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Natural Genetic Variation Affecting Calcium Homeostasis

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NATURAL GENETIC VARIATION AFFECTING CALCIUM HOMEOSTASIS

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To my husband and best friend John, who has weathered much, and supported me always.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25 dihydroxyvitamin D
25(OH)D	25 hydroxyvitamin D
2lnBF	twice the natural log of the Bayes factor
Ca	calcium
CaBPD28k	calbindin D28k
CaBPD9k	calbindin D9k
Chr	chromosome
CIM	composite interval mapping
HSS	[DNase1] hypersensitive site
IBD	identity by descent
LOD	logarithm of odds
PMCA1b	plasma membrane Ca2+ ATPase
QTL	quantitative trait locus
RI	recombinant inbred
SNP	single nucleotide polymorphism
TRPV5	transient receptor potential cation channel, subfamily V, member 5
TRPV6	transient receptor potential cation channel, subfamily V, member 6
UTR	[mRNA] untranslated region
VDR	vitamin D receptor

ABSTRACT

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Calcium (Ca) is essential for multiple functions within the body including skeletal health. The level of Ca in the serum is tightly regulated. During periods of habitual low Ca intake, the body senses a decrease in serum Ca and increases renal conversion of 25 hydroxyvitamin D (25(OH)D) to 1,25 dihydroxyvitamin D ($1,25(OH)_2D$). $1,25(OH)_2D$ acts through the vitamin D receptor (VDR) to increase intestinal Ca absorption, renal Ca reabsorption and skeletal Ca resorption. Efficient intestinal Ca absorption, especially during periods of low Ca intake, is critical for protecting bone mass. Ca absorption and its primary regulator, $1,25(OH)_2D$, are affected by both genetic and environmental factors.

However, the genetic architecture of these phenotypes has not been carefully studied in a controlled environment. Using genetically characterized mouse models in a controlled environment the studies in this dissertation characterize the natural genetic variation affecting intestinal Ca absorption, 25(OH)D, and $1,25(OH)_2D$ under normal and low dietary Ca conditions. This dietary intervention allowed for the study of gene-by-diet interactions (i.e. variability in the adaptation of these parameters to habitual low Ca intake). The relationship of Ca absorption to known regulators and cellular mediators is examined, elucidating significant effects of genetics on these relationships and

identifying gaps in our current knowledge of intestinal Ca absorption. In addition, specific genetic loci affecting intestinal Ca absorption, 1,25$(OH)_2$D, 25(OH)D, and diet-induced adaptation are identified in the mouse genome. These quantitative trait loci (QTLs) represent novel variation affecting Ca absorption and vitamin D metabolites. Identification of the causal variation underlying these QTLs will expand our knowledge of Ca homeostatic pathways. These studies serve as a foundation for identification of individual variation in Ca homeostasis and personalized dietary recommendations.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Calcium is an essential nutrient that is important for health as a signaling molecule and for maintenance of bone health throughout life. Calcium is maintained at a nearly constant concentration in the serum through a complex homeostatic mechanism that is sensitive to dietary calcium intake. The role of diet in maintaining calcium homeostasis becomes tremendously important to preserve bone mass, which serves as the largest calcium store in the body, but also as structural support for the body. For this reason, the Dietary Reference Intake for calcium is set in order to achieve optimal bone health.[(1)]

Inadequate calcium intake is common in adolescence, an age where calcium is vital for longitudinal growth and attaining peak bone mass.[(2)] This dietary inadequacy has been shown to increase with age and increase the risk for osteoporosis.[(3)] Intestinal calcium absorption is an integral part of calcium homeostasis and may also be crucial for the prevention of osteoporosis-related fractures. Calcium absorption is proposed to occur through several mechanisms.[(4)] The facilitated diffusion model of active intestinal calcium absorption responds in an adaptive manner to habitual low calcium intake. The up-regulation of calcium absorption in response to low calcium is mediated through

genomic regulation by hormonal vitamin D. Thus, dietary calcium intake and its absorption at the intestine are important regulators of a complex physiological system.

An emerging factor influencing this system is natural genetic variation. Genetics is known to affect endpoints related to calcium homeostasis such as bone mineral density and, more recently, vitamin D status.[5,6] Recent observations across racial groups suggest genetic variation controls calcium absorption efficiency directly, as well as adaptation to a low calcium diet (i.e. a gene-by-diet interaction).[7]

The focus of this thesis is to characterize and locate the natural genetic variation affecting calcium homeostasis, specifically intestinal calcium absorption and its primary regulator, vitamin D. Understanding how natural genetic variation affects calcium absorption efficiency and its regulation will help us discover new mechanisms of its regulation and functional details of the genes involved. Such research will inform how to leverage dietary interventions for maximum health benefit to the individual. This chapter contains a review of the literature focused on calcium absorption physiology and the effects of natural genetic variation.

1.2 Calcium Homeostasis

1.2.1 The Three Tissue Axis

Calcium functions as both a primary and secondary signal within cells.[8] Consequently, maintaining the concentration of this ion within the serum is of vital physiological importance. A complex interplay of environmental cues and hormonal regulation controls calcium absorption in the intestine, reabsorption in the kidney, and

resorption from the calcified matrix of the skeleton in order to maintain serum calcium concentrations within a tight range of 2.2-2.6 mM.[8] Calcium absorption at the intestine affects both calcium availability and homeostasis. As such, it is arguably the most important component of this three-tissue axis and will be discussed in detail later in this chapter. Reabsorption of calcium from the urine in the kidney occurs in both a passive fashion (primarily in the proximal tubule and thick ascending loop of Henle) and an active fashion (primarily in the distal convoluted tubule).[9] The steps and regulation of these processes have been well characterized in the kidney.[10-12] Approximately half of all serum ionized calcium is filtered by the kidney, emphasizing its importance for maintaining calcium homeostasis.[12] The skeleton serves as the body's calcium reserve, containing 99% of the body's total calcium [2]. This calcium reserve serves a dual purpose by contributing to the bone mass necessary for structural support of the body. However, homeostatic mechanisms will induce bone resorption in order to maintain serum calcium concentrations [13]. Calcium intake and absorption at the intestine must therefore be sufficient to maintain serum calcium concentration and prevent skeletal losses and fracture.

The importance of adequate calcium absorption for optimal bone health is supported by data from both clinical studies and animal models. Transgenic mice lacking a primary regulator of calcium absorption, the vitamin D receptor (VDR), have provided valuable lessons on the relationship between calcium absorption and bone health. These mice exhibit greatly decreased bone mineral density, and additional models were able to attribute this effect directly to intestinal calcium absorption.[14] Calcium absorption has been observed to increase concomitantly with bone mass accrual during puberty.[15]

Calcium absorption efficiency tends to decline with age and this malabsorption has been associated with increased hip and vertebral fracture rates.[16,17] The mechanisms and regulation of this critical process will be discussed in the following sections.

1.2.2 Mechanisms of Intestinal Calcium Absorption

Intestinal calcium absorption has been shown to be composed of a saturable and a non-saturable component.[18] This diversified system allows the intestine to efficiently maintain serum calcium homeostasis. This section will summarize the current state of calcium absorption models and discuss observations that suggest our knowledge on this topic is still incomplete.

Passive diffusion, the non-saturable component, is present throughout all sections of both the small and large intestine.[19] Passive diffusion consists of the diffusion of small molecules and ions, such as calcium, via a paracellular route between epithelial cells according to the electrochemical gradient present in the intestinal lumen.[9,20] A linear relationship of passive calcium absorption to luminal calcium load exists, accounting for absorption of 13% of luminal calcium per hour.[21] Thus, the passive component of calcium absorption predominates at normal to high dietary intake levels of calcium.[22] The paracellular route is regulated by tight junctions, which allow the intestinal epithelium to be selectively permeable.[23] Tight junctions are made up of a variety of protein families that allow them to be selective based on ion size and concentration, similar to ion channels.[24]

The saturable component of intestinal calcium absorption is a process consisting of three phases; calcium entry into the enterocyte at the brush border membrane,

facilitated diffusion of the ion across the enterocyte, and extrusion through the basolateral membrane.[22] Decades of research have characterized the kinetics and regulation of each step of this facilitated diffusion model.

Calcium entry into the enterocyte against the chemical gradient was first demonstrated using brush border membrane vesicles in rats and chicks.[25-27] Several paradigms (e.g. membrane channel or carrier) were postulated for calcium entry.[22] Peng et al. cloned the transient receptor potential cation channel, subfamily V, member 6 (TRPV6) and demonstrated its calcium transport capabilities.[28].

Once through the brush border membrane, it is necessary to buffer free ionized calcium concentrations in the cell and to preserve the slight electrochemical gradient that exists within the enterocyte.[22] A calcium binding protein was isolated from the intestine of chicks and several mammalian species.[29,30] Feher et al. found that diffusion of calcium across a dialysis chamber was accomplished only when this calcium binding protein, not bovine serum albumin, was added to the buffer.[31] Active intestinal calcium absorption was observed to correlate directly with cytosolic levels of this calcium binding protein, suggesting the intercellular trafficking step was the rate-limiting step of active calcium absorption.[22,32] The calcium binding protein, calbindin D9k, was identified as the specific protein that accomplishes facilitated diffusion in the enterocyte.[33,34] After being ferried across the enterocyte, calcium must be extruded across the basolateral membrane. Using Ussing chambers, it was demonstrated that this process is ATP dependent.[35] Later, the ATPase PMCA1b was located in chick intestine and found to be responsive to stimuli known to modulate calcium absorption.[36,37] Together, these

components make up the facilitated diffusion model of active calcium absorption. The regulation of this model will be discussed in later sections.

While the facilitated diffusion model is perhaps the most widely accepted model of saturable intestinal calcium absorption, several others have been proposed. One such method is vesicular transport, where calcium in the lumen is endocytosed, transported in lysosomes, and released as the vesicle merges with the basolateral membrane.[38] Nemere et al. found that lysosomes were filled with Ca^{45} radioisotope tracer in treated chick intestine.[38] Disruption of the lysosome's acidic pH gradient resulted in a 67% decrease in duodenal calcium transport.[39] This vesicular movement has been shown to be increased by the same regulating factor as the facilitated diffusion model (1,25dihydroxyvitamin D, to be discussed later).[40] The full mode of regulation and impact of vesicular calcium transport is unknown. Another proposed method of intestinal calcium transport is transcaltachia; the rapid, hormone-stimulated induction of calcium transport in the intestine. 1,25dihydroxyvitamin D ($1,25(OH)_2D$) hormonal treatment to the basolateral side of perfused intestinal membranes increased calcium transport within 14 minutes.[41] This non-traditional action of the hormone is proposed to work through the membrane associated, rapid response, steroid binding receptor (MARRS) and PKA signaling.[42] The study by Nemere et al. used primary cell culture from intestine-specific MARRS knockout mice to show loss of stimulated-calcium transport, but failed to show what consequence, if any, this knockout had on whole-body Ca homeostasis and bone health.[42]

Morgan et al. suggested that active calcium absorption is linked to glucose transport in the lower small intestine.[43] This active absorption is proposed to occur

through the L-type channel Cav1.3 during the presence of both glucose and sufficient calcium.[44] Morgan et al. observed that perfused rat jejunum absorbed more calcium when the perfusate contained glucose rather than mannitol.[43] Additionally, both glucose and calcium absorption were inhibited by an L-type channel inhibitor.[43] The proposed implication of these findings is that they represent a nutrient-sensing mechanism. However, there is currently no evidence that it has specificity to calcium or sensitivity to overall calcium homeostasis, as the hormonal regulation of the facilitated diffusion model does. The increase in calcium absorption efficiency in rats seen after prolactin treatment [45] has been shown to be mediated through Cav1.3 in CaCo2 cells[46]. The physiological relevance of this observation remains unclear because prolactin is regulated differently between rats and humans.[47]

1.2.3 Regulation by Dietary Calcium

Dietary calcium intake is a primary regulator of calcium homeostasis.[48] As mentioned above, the intestine plays a vital role in maintaining serum calcium concentrations. However, when dietary intake is insufficient the body must adapt each arm of the three tissue axis to maintain serum calcium within the physiologically necessary range. Habitual low dietary calcium intake induces a chain of events that activates hormonal regulation. This mode of regulation and the consequences will be discussed in the following sections.

Although it is a ready source to maintain serum calcium concentration, adaptation of bone to a low calcium diet is of particular concern. Low calcium intake increases risk of osteoporosis and fracture that accumulates across life stages.[13] For example,

Kalkwarf et al., showed in women that low calcium intake during growth limits the attainment of peak bone mass and increases fracture risk later in adulthood.[49] Calcium intake is also important in adulthood to maintain bone mass (i.e. negate bone loss) and prevent fracture.[2] Early experiments observed that a calcium deficient diet resulted in decreased trabecular bone in cats, suggesting that cancellous bone is more sensitive to calcium intake and is the more metabolically active compartment.[50] This hypothesis is supported by a time-course study in rats 70 years later.[51] However, if the deficient diet is maintained, total bone mineral density (BMD) (both trabecular and cortical bone) will be affected.[52] Calcium deficiency limited the increase in BMD during the critical pubertal growth period from 35% to 7% in mice.[53]

Low calcium intake also induces adaptation by increasing the efficiency of calcium utilization, primarily by increasing calcium absorption efficiency. Balance studies in rats conducted as early as 1938 suggested that efficiency of calcium utilization increased while feeding low calcium diets.[54] Leichsenring et al. showed that young women were more efficient at retaining calcium when maintained on a low calcium diet (300mg/d) than when supplemented (1500mg/d).[55] A population of women with a wider age range of intake also exhibited increased calcium retention efficiency on a 300mg/d compared to a 2000mg/d diet.[56] Experimental models have shown that the efficiency of calcium absorption, specifically, is increased in response to a low calcium diet.[18,57] The inverse relationship between calcium intake and calcium absorption efficiency has also been observed in multiple human studies.[58-60] This physiological response to habitual low calcium intake is mediated through vitamin D.

1.3 The Role of Vitamin D in Calcium Homeostasis

The importance of vitamin D for calcium homeostasis became apparent as disorders associated with the newly discovered vitamin overlapped with abnormalities in bone and Ca metabolism. Rickets, a disease characterized in children by soft, deformed, and under-mineralized bone, was found to be ameliorated by sun exposure or increased dietary intake of vitamin D.[61] Ostoeomalacia has a similar etiology to rickets but occurs in adulthood as a result of vitamin D deficiency.[13] As vitamin D metabolites and details of its molecular action have been revealed, vitamin D has been recognized as the primary regulator of calcium homeostasis.

1.3.1 Overview of Vitamin D Metabolism

Vitamin D is obtained from the diet [62] or synthesized *de novo* from 7-dehydrocholesterol in the skin upon exposure to sunlight [63]. Vitamin D is then hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver, most likely by the enzyme CYP2r1.[64] 25(OH)D serves as the biomarker for vitamin D status in the serum. 25(OH)D is further hydroxylated at the 1 carbon position by the enzyme 1±-hydroxylase (CYP27b1), predominantly in the kidney.[65-67] This form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is the active hormonal form, exerting influence on the kidney, bone, and especially the intestine. Serum $1,25(OH)_2D$ levels are correlated to calcium absorption efficiency in both humans and mice.[57,68-70] $1,25(OH)_2D$ binds to the vitamin D receptor (VDR) to initiate target gene transcription to maintain whole-body calcium homeostasis.[71] The renal 24-hydroxylase enzyme, CYP24, inactivates hormonal $1,25(OH)_2D$ and begins its degradation.[72]

The activation of hormonal vitamin D is influenced by calcium homeostasis and opposing hormonal signals. A low serum calcium level is sensed by the calcium sensing receptor (CaSR) in the parathyroid gland, signaling release of parathyroid hormone (PTH).[48] PTH increases renal CYP27b1 gene expression and subsequently $1,25(OH)_2D$ conversion.[73-75] The ligand-bound VDR complex then exhibits feedback action by binding the promoter of CYP27b1 and inhibiting its expression.[76] Fibroblast growth factor 23 (FGF23) produced by the skeleton also inhibits CYP27b1 expression (and therefore $1,25(OH)_2D$ activation) in response to high serum phosphate.[77] CYP24 is inhibited by PTH in order to promote availability of $1,25(OH)_2D$.[72] $1,25(OH)_2D$ operates in a feedback loop to greatly increase the gene expression of CYP24, and thereby, its own degradation.[78]

1.3.2 Genetic Disruption of Vitamin D and Calcium Metabolism in Mice

The importance of vitamin D regulation of calcium homeostasis has been illustrated by the use of transgenic mice. Transgenic mouse models disrupt a targeted gene (usually by knockout or overexpression) on an otherwise homogenous, inbred background. This method allows for detailed investigation of pathways of interest.

Three different VDR knockout (KO) mouse models have been reported and used for the study of vitamin D function.[79-81] Studies in each of these models show similar results after pups are weaned including hypocalcemia, hypophosphatemia, hyperparathyroidism, increased serum $1,25(OH)_2D$, type II vitamin D-dependent rickets, and alopecia.[79-81] Similarly, CYP27b1 knockout mice display hypocalcemia,

hyperparathyroidism, and type I vitamin D-dependent rickets.[82] These results emphasize that vitamin D action functions primarily to maintain calcium homeostasis.

These studies, and similar ones, indicate that the intestine is the primary site of $1,25(OH)_2D$ action to maintain calcium homeostasis. Intestinal calcium absorption efficiency is decreased in VDR KO mice compared to wild-type.[81] The impact of vitamin D on calcium absorption has been assessed using two different strategies. Feeding a high-calcium diet containing lactose overrides the impairment of active intestinal calcium absorption in VDR null mice.[83,84] VDR null mice fed this "rescue diet" showed normal serum calcium and phosphorus levels and a recovery from the rachitic phenotype in bone.[83] Further investigation on the effects in bone showed normal morphology and mineral apposition rate in VDR null mice fed the rescue diet.[84] These observations led the authors to conclude that both the mineral ion homeostasis and bone phenotypes of VDR KO mice are due to lack of vitamin D action on intestinal calcium absorption.[84] The second approach to examining the impact of vitamin D-mediated intestinal calcium absorption on whole-body calcium homeostasis was to restore functionality specifically to this system in a VDR KO mouse model. Xue et al. developed a transgenic mouse model with intestine-specific VDR expression in an otherwise VDR null mouse.[85] Intestinal VDR expression completely recovered the negative phenotypes of VDR KO mice, emphasizing the importance of intestinal calcium absorption.[85]

Regulation by serum $1,25(OH)_2D$ allows the intestine to be sensitive to decreases in dietary calcium intake. In wild-type animals, feeding a low calcium diet induces an increase in serum $1,25(OH)_2D$ and calcium absorption efficiency.[57] Similarly, direct

injection of 1,25$(OH)_2$D induces an increase in calcium absorption efficiency in rodents.[19,57] Adaptation of intestinal calcium absorption to habitual low dietary calcium intake occurs through 1,25$(OH)_2$D-mediated up-regulation of the facilitated diffusion model. Gene expression of TRPV6 has been shown to increase in rodents fed a low calcium diet or with 1,25$(OH)_2$D injection.[57,86,87] TRPV6 mRNA is decreased in vitamin D deficient rats and VDR KO mice.[81,86] CaBPD9k gene expression is also regulated by 1,25$(OH)_2$D genomic action. CaBPD9k is increased on a low calcium diet or after 1,25$(OH)_2$D injection [57,87,88] CaBPD9k mRNA and protein are reduced in VDR KO mice.[81]. Although typically viewed as less responsive to vitamin D, the basolateral transporter, PMCA1b, also responds to vitamin D depletion and repletion.[89] Additionally, all three components of the facilitated diffusion model were identified as vitamin D target genes in a microarray study conducted in 1,25$(OH)_2$D-injected rats.[90]

Although the vitamin D-regulated facilitated diffusion model of intestinal calcium absorption appears sound, transgenic mouse models have allowed investigators to explore the completeness of this model. Bianco et al. showed a significant decrease in calcium absorption efficiency in TRPV6 KO mice compared to WT mice fed a normal diet.[91] However, others presented that calcium absorption in the same TRPV6 KO mice was not different from WT mice fed a sufficient calcium diet.[92,93] Kutuzova et al. write that they had changed the background of the null mice developed by Bianco et al. by 3 generations of backcrossing to C57BL/6J mice.[92] Both studies showed that active calcium absorption does occur, at some level, without TRPV6, calling into question the validity of TRPV6 as a calcium transporter in the enterocyte. However, this function of TRPV6 was confirmed by a mouse model overexpressing the protein in the intestine, resulting in

hypercalcemia and extensive ectopic calcification.[94] Additionally, calbindin D9k KO mice were shown to be normocalcemic, and exhibited no difference from wild-type in calcium absorption.[93,95]

The roles of TRPV6 and CaBPD9k in low calcium adaptive response have also been investigated. Bianco et al. observed that TRPV6 KO mice had decreased calcium absorption compared to WT on both a normal or low calcium diet, suggesting that TRPV6 is necessary for intestinal calcium absorption and its adaptation to a low calcium diet.[91] Benn et al. observed that although it did not equal the response of WT mice, TRPV6 KO mice were able adapt to a low calcium diet.[93] Interestingly, $1,25(OH)_2D$ injection elicited an equal adaptive response in TRPV6 null and WT mice, suggesting that TRPV6 is not necessary for adaptive calcium homeostasis.[92,93] Lieben et al. propose that calcium homeostasis is maintained in TRPV6 knockout mice through compensation by the skeleton because TRPV6 KO mice exhibit hyperosteoidosis when fed a low calcium diet.[96]

Studies of CaBPD9k KO mice indicate that this protein is not critical for low calcium adaptive response. CaBPD9k KO mice exhibited an adaptive response equal to WT mice on both a low calcium diet and following a $1,25(OH)_2D$ injection.[93] A study by Lee et al. showed that compensatory up-regulation of calcium transport genes in the kidney and intestine may be responsible for the adaptive phenotype of CaBPD9k KO mice.[95] When both TRPV6 and CaBPD9k are knocked out in the same mouse, the adaptive response of intestinal calcium absorption to both a low calcium diet and $1,25(OH)_2D$ injection is markedly reduced.[93] However, adaptive calcium absorption is not completely eliminated in these double knockout mice.[93]

Recent studies suggest that vitamin D may also affect passive diffusion of calcium in the intestine. The proposed effect of $1,25(OH)_2D$ on passive diffusion of calcium is mediated through increasing ion permeability of tight junction, specifically through affecting the proteins claudin 2 and claudin 12.[90,97] Gene expression and protein levels of claudin 2 and claudin 12 are decreased in VDR KO mice.[97] Treatment of intestinal cells with $1,25(OH)_2D$ increases gene expression of claudins 2 and 12, resulting in increased paracellular movement of calcium.[97] Another mechanism through which vitamin D may regulate paracellular movement of calcium is by regulating cell adhesion. In a microarray study of rat intestine, $1,25(OH)_2D$ treatment suppressed gene expression of cell adhesion proteins cadherin-17 and aquaporin-8.[90]

Taken together, these observations indicate that our knowledge of intestinal calcium absorption is incomplete. There may be aspects of the facilitated diffusion model or compensatory mechanisms that are undiscovered. Alternate methods of regulation may also be possible. The paracellular movement of calcium across the intestinal epithelia appears to be more complex than simple passive diffusion along a concentration gradient. The possibility of specificity to calcium and regulation of this process by vitamin D warrant further study.

1.4 Genetic Variation and Gene-by-Diet Interactions Influence Calcium Homeostasis

1.4.1 Overview of Forward Genetic Methodologies

Heritable factors have long been recognized to affect physiology. Identification and study of the genes responsible for a trait of interest can be accomplished through two

classes of methods: forward and reverse genetics. Each method has strengths and limitations. The following section will describe paradigms and challenges of genetic studies and how they may be used to inform nutrition research.

Understanding the function of a gene can be accomplished through manipulation of the gene in a model organism and studying the outcome. This genotype to phenotype approach is known as reverse genetics.[98] Reverse genetics studies include targeted gene manipulation (e.g. knockout and transgenic mouse models) as well as examination of variation within candidate genes.[99] These studies are excellent for testing *a priori* hypotheses of the gene's function in a biological process or pathway.[100] However, this strength is also a weakness in that the study is dependent on, and limited by, existing knowledge of the biological pathway or gene.[100] It has also been suggested that another limitation of reverse genetics studies is investigator bias in the development and stringent testing of hypotheses.[100]

These limitations are overcome by forward genetic methods including positional cloning, mutagenesis screens, and linkage and association mapping.[99] Forward genetics follows observation of a phenotype to its root genotypic cause(s).[98] In contrast to reverse genetics, forward genetics is hypothesis-free.[100] This quality of forward genetic studies places the emphasis on discovery. Novel genes and regulatory pathways or novel roles of known genes can be discovered through forward genetics.[100] Linkage and association mapping methods, in particular, have come to the forefront in recent years due to successes in identifying genetic loci related to several types of cancer[101-104], Type II diabetes[105], the peripheral nervous system[98], atherosclerosis[106], and skeletal phenotypes[107] among others. This surge in forward genetics has been ushered in by a

wealth of techniques and resources. The completion of the human[108] and mouse[109] genome sequences allow identification of genes, or putative genes, within a locus. Further advances have allowed scientists to examine the polymorphisms and gene expression patterns of the entire genome.[110,111] However, the genetic basis of most physiological processes is not entirely known.

Forward genetic methods present several challenges. Extreme phenotypes may be particularly interesting, but these are monogenic, arising from extremely rare mutations in a single gene. The vast majority of physiological processes and diseases are complex, polygenic phenotypes. Therefore, the most common polymorphisms in a population may account for only small phenotypic changes, but these effects accumulate to increase risk or susceptibility in an individual. These polymorphisms can create changes in gene regulation, expression, and protein function. Additional challenges of identifying the genetic basis of a phenotype include interactions between loci (i.e. epistasis) and the modulating influence of environmental factors such as diet. Consequently, identifying and characterizing causal genetic loci in the human population is particularly difficult.

1.4.2 Mouse Models Allow Investigation of Natural Genetic Variation and Gene-by-Diet Interactions

The mouse has become the ideal model to identify the role of genetic variation in biology and disease susceptibility. Results from genetic mapping studies in mice have been shown to be translatable to humans.[112-114] Similarly, mapping results of similar traits in both mice and humans show high concordance.[115] The natural genetic variation present in the mouse species is estimated to be greater than the variation present in

humans.[99] Portions of this variation have been captured in developed populations of mice such as recombinant inbred line panels, consomic strains, and congenic strains. These mouse models offer a known genetic background with biological replicates because each line is inbred. This attribute allows the investigator to examine how an environmental stimulus or stress (e.g. diet) interacts with genetic variation to alter the phenotype of interest.[116] These gene-by-diet interactions are being studied in human populations, but do not always reach significance because of the complexity of the human environment. For example, Nettleton et al. examined the effects of whole grain intake and an overall healthy diet on previously identified diabetes risk loci, but found only a suggestive interaction of diet and genetic risk.[117,118] In contrast, mouse models under a controlled environment and intervention allow identification of gene-by-diet interactions.[116]

Gene-by-diet interactions can affect biology through several paradigms. Permissive gene-by-diet interactions are genetic effects only seen under certain dietary conditions, typically deficiency. For example, inactivating mutations in the methylenetetrahydrofolate reductase (MTHFR) enzyme reduce conversion of homocysteine to methionine.[119] Jacques et al. observed that the buildup of homocysteine in those carrying the MTHFR mutation was further exacerbated a low folate status, and suggested those individuals may have a higher folate requirement.[119] A gene-by-diet interaction may also be sensitizing, altering the functional outcome of a nutrient. Examples of this type of gene-by-diet interaction are prevalent in lipid biology.[120] Nicklas et al. reported that a low fat, low cholesterol diet had differing effects dependent on apolipoprotein E genotype; the serum lipid profile of women with

the APOE4+ genotype was improved, while women with the APOE4- genotype showed an increase in serum triglycerides and a large decrease in high-density lipoprotein.[121] The following sections will discuss genetic effects on calcium homeostasis phenotypes. The evidence for possible gene-by-diet interactions affecting calcium homeostasis will also be discussed.

1.4.3 Genetic Variation Affects Calcium and Bone Metabolism

Much focus has been paid to genetic factors affecting bone health and osteoporosis risk. Evidence collected from family and twin studies, as well as forward genetic methods in rodents and humans indicate that bone parameters are highly heritable, with heritability values ranging from 50-85%.[5] Observational twin and family studies have noted that genetics contributes to several indices of bone health including; bone size, bone mass, BMD, femur geometry, and strength.[122-125]

There are notable cases of rare, monogenic disorders that lead to extreme bone phenotypes. Candidate gene studies of affected families and knockout mouse models have identified COL1A1 and several other genes to be responsible for the bone-fragility disease osteogenesis imperfecta.[126] Interestingly, separate phenotypes were mapped to the genomic region containing the lipoprotein-related protein 5 (LRP5) gene. Linkage mapping by Johnson et al. led to identification of LRP5 mutations that were responsible for familial high bone density phenotypes.[127] Mapping conducted in families with fragile, low bone mass osteoporosis pseudoglioma also indicated LRP5 as the causative gene.[128] Linkage mapping followed by positional cloning found SOST mutations to be the cause of sclerosteosis and van Buchem disease.[129-131] These discoveries have greatly

enhanced our knowledge of bone biology, but still do not account for the large heritability of bone parameters in the general population.

The majority of variation in bone phenotypes observed in the general population is due to polygenic effects which, individually, are too small to be tracked through a single pedigree. This variation can be seen at the population level. It has been observed that racial groups (a surrogate for population structure and genetic variation) differ in bone density and strength parameters.[132,133] Similarly, bone density varies greatly across inbred mouse strains.[134] These populations display a distribution of a quantitative trait of interest, such as bone mineral density (BMD), indicating the trait is due to the contribution of many different factors, genetic and environmental.

Forward genetic approaches have been applied in diverse populations to successfully identify the more common, small-effect variation affecting bone traits. Linkage mapping in mice has identified many regions of the mouse genome linked to bone parameters.[135,136] Fewer studies, however, have been able to identify the candidate gene within these regions. Klein and colleagues identified a region of mouse chromosome 11 controlling the natural variation in peak whole body and femoral BMD between C57BL/6J and DBA/2 mice.[137] Further studies using congenic fine mapping and differential gene expression analysis indicated the gene driving changes in BMD was *Alox15*, a gene that encodes the 12/15-lipoxygenase important for peroxisome proliferator-activated receptor gamma (PPAR^3) signaling in osteoblast differentiation.[138] A cross between C57BL/6J mice and CAST/EiJ mice identified significant linkage between distal chromosome 1 and BMD which was attributable to variation in the chemokine receptor *Darc*.[139] Farber et al. investigated the genetic basis of femur

morphology in mice and found that variation in cadherin 11 (*Cdh11*) significantly affected femur width.[140]

Recently, genome-wide association studies (GWAS) have been used to identify genetic polymorphisms associated with bone traits in humans.[107] GWAS are hampered by the extremely large sample size they require, hidden structure in the chosen population (which increases the chance for false positives), and the inability to fully account for environmental differences.[141] The results of available bone-related GWAS and mouse linkage studies were overlaid in 2010, showing that 26 out of 28 loci identified in human GWAS were also identified in mice.[115] Thus, there is high concordance between the two species in both the genomes and susceptibility to complex diseases (e.g. osteoporosis), allowing genetic studies in mice to inform the biology of humans.[141,142]

Another important factor to calcium homeostasis, vitamin D status, has been shown to be heritable and altered by genetic factors. Serum 25(OH)D has been shown to vary across racial groups, mostly owing to differences in skin tone (i.e. Whites>Hispanics>Blacks).[143] However, heritability of 25(OH)D was shown to differ between similar Hispanic American populations, suggesting genetic factors beyond skin color influence vitamin D status.[144] Heritability estimates for serum 25(OH)D range from 23-80%.[144-149] The genetic potential of an individual becomes most important when environmental factors, such as sunlight exposure, are at a minimum. Some of the largest heritability estimates of 25(OH)D were measured in winter, when the environmental effect of sunlight does not play a large role.[146,147] Serum $1,25(OH)_2D$ has also been shown to be heritable, though not to the extent of 25(OH)D. Heritability of $1,25(OH)_2D$ has been reported to be 16-20% in Hispanic Americans, 48% in African

Americans, and 30% in European populations.[144,149] There are few studies examining the genetic determinants of serum 25(OH)D. Such studies of serum $1,25(OH)_2D$ are extremely limited, possibly due to the myriad of physiological and environment also influencing it.

Recent associations between vitamin D and multiple health outcomes have led to increased interest in defining the genetic architecture of vitamin D biology.[150] Engelman et al. examined the relationship of serum 25(OH)D and $1,25(OH)_2D$ to polymorphisms in three vitamin D candidate genes; vitamin D binding protein (GC), CYP27B1, and VDR. Only GC showed significant association, with one SNP affecting both 25(OH)D and $1,25(OH)_2D$ levels, and another SNP associated with 25(OH)D only.[144] Another candidate gene study by Bu et al. tested for relationships between 25(OH)D status and SNPs in nine candidate genes known to affect vitamin D; alkaline phosphatase (ALPL), CYP24A1, CYP27A1, CYP27B1, CYP2R1, CYP3A4, GC, VDR, and PTH.[151] The investigators identified and replicated associations between CYP2R1 and GC polymorphisms that were associated with serum 25(OH)D levels in two Caucasian populations.[151]

Forward genetics methods have been applied to understanding genetic variability on vitamin D metabolites in two clinical studies. Wjst et al. sought to identify genetic regions linked to either serum 25(OH)D or $1,25(OH)_2D$ in German and Swedish families with asthma.[149] This study found that serum 25(OH)D levels were more highly heritable than $1,25(OH)_2D$, 80% and 30%, respectively.[149] Five loci were significantly linked to serum 25(OH)D in this population, but no previously known vitamin D-related genes were near these markers.[149] The largest and most comprehensive study as of yet is a

GWAS of serum 25(OH)D in over 4000 individuals of European descent.[150] Ahn et al. identified single nucleotide polymorphism in only one gene, GC, to be significantly associated with serum 25(OH)D.[150] Three other genes contained variation putatively associated with vitamin D status; 7-dehydrocholesterol reductase (DHCR7), acyl-Coenzyme A dehydrogenase (ACADSB), and CYP2R1.[150]

These studies reaffirm relationships seen in candidate gene studies, but also indicate that unknown genetic factors affecting vitamin D biology do exist. Surprisingly, the use of animal models to unravel these questions is largely absent. Natural variation was noted in the susceptibility of different dog breeds to rickets in some of the earliest studies of vitamin D physiology.[152] More recently, a screen of 18 inbred mouse lines identified significant variation in serum 25(OH)D.[153] Despite animal models being used extensively to characterize vitamin D biology and the genetics of bone health (as discussed earlier), they have not been leveraged to identify the genetic factors influencing vitamin D metabolites.

There are indications that calcium absorption is affected by genetic variation, but the factors influencing it have not been well studied. A wide amount of variation has been observed in human populations. Heaney et al. observed a range of 0.05-0.65 in true fractional calcium absorption in four combined populations of postmenopausal, non-osteoporotic women, even after adjustment for dietary calcium intake.[154] Wolf et al. observed a range of 0.17-0.58 in true fractional calcium absorption.[155] Several environmental factors are commonly associated with intestinal calcium absorption efficiency, including age, estrogen, calcium intake, and serum $1,25(OH)_2D$.[70,155,156] Still other dietary components have been indicated to influence intestinal calcium absorption

efficiency such as fat, fiber, and protein.[155,157,158] However, these environmental factors and others explain 25%, at most, of the variation seen in calcium absorption.[155,157] It could be hypothesized that genetics makes up the remaining variation. This idea is supported by several pieces of evidence.

Similar to BMD, calcium absorption has been observed to vary by race.[60,159-161] Abrams et al. observed significantly higher fractional calcium absorption in black than white pre and postmenarchal girls.[159] Several balance and kinetic studies in black and white adolescent girls conducted by Dr. Connie Weaver's research group have confirmed this observation under controlled dietary conditions.[60,160,161] Chinese adolescent girls were also observed to have higher calcium absorption efficiency across a range of controlled calcium intakes.[59]

It has been postulated that polymorphisms in the VDR, specifically, may account for the variation seen in intestinal calcium absorption. However, these candidate gene studies have been largely inconclusive and dependent on the haplotype or population studied. Several studies have found a positive association between VDR polymorphisms and calcium absorption [162-164], while others found no association at all [155,165-167]. These discrepancies may be due to uncontrolled environmental factors, such as dietary calcium intake.

1.4.4 Do Gene-by-diet interactions Influence Calcium Homeostasis?

As discussed above, calcium homeostasis is sensitive to dietary calcium intake which prompts a complex network of physiological and hormonal signals. Thus, gene-by-diet interactions may likely influence calcium homeostasis. Evidence for gene-by-diet

interactions is particularly evident in skeletal health. Observed differences in heritabilities of bone mass between monozygotic and dizygotic twins increase with age, suggesting bone is affected by interaction of genetics and accumulated environmental exposures (e.g. diet).[123] Specific examples of gene-by-diet interactions affecting bone have been shown.

Farrow et al. identified a permissive gene-by-diet interaction between mutations in FGF23 and iron status.[168] Mutations in a proteolytic cleavage site of FGF23 result in inappropriate maintenance of intact, active serum FGF23 and the disease autosomal dominant hypophosphatemic rickets (ADHR).[169] Low iron status increases gene expression of FGF23. Individuals carrying the mutant allele cannot compensate for this increase because the mutation inhibits the degradation of FGF23. [168] Thus, intact FGF23 is increased during periods of low iron status, causing the hypophosphatemia and osteomalacia that characterize ADHR. [168] A sensitizing interaction between PPARG and dietary fat has also been observed to affect skeletal health. Ackert-Bicknell et al. identified PPARG as a candidate gene modifying bone mineral density between C3H/HeJ and C57BL/6J mice.[112] The effect of this genetic variation on BMD was found to be modulated by dietary fat intake. [112] The authors were also able to identify variation in PPARG that influenced BMD in the Framingham Offspring Cohort. [112] In this population, certain SNPs showed significant interactions with dietary fat intake; a high fat diet was detrimental to BMD in one genotypic class and beneficial to BMD in the other genotypic class.[112] These studies emphasize the impact of natural genetic variation and the important influence of the dietary environment on an individual level.

Evidence suggests that gene-by-diet interactions influence calcium absorption as well. The adaptive increase in intestinal calcium absorption caused by habitual low dietary calcium intake is different among racial groups. Data from Black and White adolescent girls indicate that Black girls display the predicted adaptive increase in calcium absorption with decreasing dietary calcium levels, but White girls do not.[161] Similarly, Chinese-American girls have higher calcium absorption efficiencies than white girls.[59]. The disparity between these racial groups is greater at low calcium intakes, supporting the hypothesis that a gene-by-diet (calcium) interaction influencing intestinal calcium absorption efficiency exists.[59]

The genomic sources of this gene-by-diet interaction are not currently known. Candidate gene studies have attempted to relate VDR polymorphisms with calcium absorption efficiency, but do not always factor in the effect of dietary calcium.[165] Dawson-Hughes et al. examined calcium absorption efficiency in postmenopausal women genotyped for the BSM1 VDR polymorphism.[170] Results of that experiment showed calcium absorption efficiency was similar between genotypic classes on a high calcium diet, but differed significantly on a low calcium diet.[170] Specifically, the BB genotype showed decreased adaptation to a low calcium diet than the bb genotype.[170] Wishart et al. found no associations between the BSM1, APA1 and Taq1 polymorphisms and calcium absorption efficiency until the population was corrected for dietary calcium intake, serum $1,25(OH)_2D$, and body weight.[162] Thus, the effect of VDR genotype on intestinal calcium absorption efficiency is dependent on the dietary and physiological environment.

Characterization of the gene-by-diet interaction affecting adaptation of calcium absorption to a low calcium diet is needed. However, candidate genes studies such as the ones described above are unlikely to reveal the genetic root of gene-by-diet interactions because they are limited to what is known about genomic control of the trait of interest, and by how completely environmental exposure can be ascertained. Controlled, discovery-focused research is needed to identify what genetic factors allow some individuals a genetic advantage to protect their bone while on a low calcium diet while others are susceptible to this dietary deficiency.

1.5 Gaps Remain in Our Understanding of Calcium Homeostasis and Its Regulation

Considerable research has gone into the molecular mechanisms involved in intestinal calcium absorption. Careful characterization in animal models has led to the development of the facilitated diffusion model of active intestinal calcium absorption. This model has been shown to be regulated by $1,25(OH)_2D$ in response to low dietary calcium intake. However, the $1,25(OH)_2D$-mediated facilitated diffusion model does not solely and completely explain intestinal calcium absorption. For instance, considerable calcium absorption capacity remains in knockout mouse models of two facilitated diffusion components, TRPV6 and CaBPD9k. Calcium absorption in these mice was still able to respond to $1,25(OH)_2D$. Additionally, the strong relationship between intestinal calcium absorption and $1,25(OH)_2D$ seen in cell and wild-type animal models is not seen in human populations. These observations suggest that there are still details of calcium absorption to be discovered. Particularly, novel mechanisms of regulation by vitamin D remain to be elucidated.

Observations in racial groups indicate that natural genetic variation may influence calcium absorption efficiency. Although genetic approaches have been used to study related phenotypes such as bone mineral density and vitamin D status, the effect of genetics of calcium absorption has not been widely studied. Candidate gene approaches have explained little, and are limited to current gene function and pathway knowledge. No discovery-focused forward genetic methods have been used to identify genetic variation affecting intestinal calcium absorption. The adaptation of calcium absorption to habitual low dietary calcium intake appears to have a genetic component as well, but this has not been investigated in depth. Again, candidate gene studies have had limited success explaining this gene-by-diet interaction. These studies were conducted in human populations with little to no control over the environment and other genetic factors. Forward genetic studies are needed to identify the genetic sources of this gene-by-diet interaction. Animal models are best suited to this purpose because of their known genetic background and the ability of the investigator to control and manipulate the dietary environment.

The goal of this thesis is to address several of these gaps in our knowledge of calcium homeostasis. Inbred mouse lines were chosen as a research model in order to control environment and genetic background. The first research goal was to characterize the natural genetic variation present in intestinal calcium absorption and vitamin D metabolites in the laboratory mouse population. The adaptive response of these parameters to a low calcium diet was also assessed. The second goal was to use forward genetics to locate genetic regions controlling calcium absorption and vitamin D

metabolites. Gene-by-diet interaction loci were also mapped to identify genetic loci affecting the response to a low calcium diet.

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CHAPTER 2. GENE-BY-DIET INTERACTIONS INFLUENCE CALCIUM ABSORPTION AND BONE DENSITY IN MICE

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Supplemental material is included with this chapter

Keywords

calcium; intestinal absorption; genetics; diet; bone

2.1 Abstract

Dietary calcium (Ca) intake is needed to attain peak bone mineral density (BMD). Habitual low Ca intake increases intestinal Ca absorption efficiency to protect bone mass but the mechanism controlling, and the impact of genetics on, this adaptive response is not clear. We fed 11 genetically diverse inbred mouse lines a normal (0.5%) or low (0.25%) Ca diet from 4-12 wks of age (n=8 per diet per line) and studied the independent and interacting effects of diet and genetics on Ca and bone metabolism. Significant genetic variation was observed in all bone, renal, and intestinal phenotypes measured including Ca absorption. Also, adaptation of Ca absorption and bone parameters to low dietary Ca was significantly different among the lines. Ca absorption was positively correlated to femur BMD ($r=0.17$, $p=0.02$), and distal femur BV/TV ($r=0.34$, $p<0.0001$). While Ca absorption was correlated to 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) ($r=0.35$, $P<0.0001$), the adaptation of Ca absorption to low Ca intake did not correlate to diet-induced adaptation of $1,25(OH)_2D$ across the 11 lines. Several intestinal proteins have been proposed to mediate Ca absorption; claudins 2 and 12, voltage gated Ca channel v1.3 (Cav1.3), plasma membrane Ca ATPase 1b (PMCA1b), transient receptor potential vanilloid member 6 (TRPV6) and calbindin D_{9k} (CaBPD9k). Only the mRNA levels for TRPV6, CaBPD9k, and PMCA1b were related to Ca absorption ($r= 0.42$, 0.43, and 0.21, respectively). However, a significant amount of the variation in Ca absorption is not explained by the current model and suggests that novel mechanisms remain to be determined. These observations lay the groundwork for discovery-focused initiatives to identify novel genetic factors controlling gene-by-diet interactions affecting Ca/bone metabolism.

2.2 Introduction

Whole body calcium (Ca) homeostasis is maintained by the coordination of a three-tissue axis of intestine, kidney, and bone[1] and this coordination is crucial for developing peak bone mass and minimizing adult bone loss[2,3]. In humans, fractional Ca absorption is positively correlated to bone mass [4-6] while low Ca absorption efficiency is associated with increased fracture risk [7] and can reduce the efficacy of Ca treatment in the prevention of osteoporosis [4]. Despite these relationships, we still lack a clear understanding for how Ca absorption occurs.

Ca absorption follows both a saturable, transcellular pathway and a passive, paracellular pathway that is directly proportional to dietary Ca intake.[8] Active intestinal Ca absorption is especially crucial to the development of optimal bone density when dietary Ca intake is low. During these periods, renal conversion of serum 25 hydroxyvitamin D (25OHD) to the active hormone 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) is increased. Molecular events regulated by binding of $1,25(OH)_2D$ to the vitamin D receptor (VDR) subsequently increase Ca absorption efficiency as well as reduce urinary Ca loss.[9-11] Deletion of intestinal VDR in mice reduces active intestinal Ca absorption efficiency by 70% and this is directly responsible for the hypocalcemia and osteomalacia seen in VDR knockout mice.[11]

The most studied mechanism for vitamin D-regulated intestinal Ca absorption is the facilitated diffusion model.[8] In this model, $1,25(OH)_2D$ increases the expression of target genes whose protein products mediate Ca entry into the enterocyte through the apical membrane channel transient receptor potential vanilloid member 6 (TRPV6), movement through the cell by binding to calbindin D_{9k} (CaBPD9k), and extrusion from

the cell through the ATP dependent-plasma membrane Ca pump, PMCA1b.[8] While close relationships exist between components of the facilitated diffusion model in CaCo-2 cells[12] and C57BL/6J mice[13,14] recent studies in knockout mice indicate that the facilitated diffusion model may not be accurate [15].

In human studies, the efficiency of intestinal Ca absorption varies from 7-75%.[16-18] The variation in intestinal Ca absorption efficiency in humans is likely due to the influence of multiple physiologic (e.g. growth, pregnancy, lactation, aging), environmental variables (e.g. dietary Ca intake, vitamin D status), and genetic factors. Twin studies, genetic mapping studies in mice, and GWAS in humans reveal that several aspects of whole-body Ca and bone homeostasis are influenced by genetics, e.g. multiple bone endpoints[19,20] (human findings reviewed in[21]), serum 25OHD [22]. However, less information is available for the impact of genetics on the efficiency of Ca absorption and the adaptive upregulation of Ca absorption to low dietary Ca intake. Two groups have reported that the efficiency of intestinal Ca absorption is higher in C3H/HeJ mice compared to C57BL/6J mice, suggesting genetic background may influence this trait.[23,24] In addition, racial differences in the ability of adolescent girls to increase Ca absorption efficiency during periods of low dietary Ca intake indicate that this adaptive response also has a genetic component.[25,26]

To determine the genetic contribution to the efficiency of Ca absorption, we examined 11 inbred lines of mice fed defined diets containing either high or low Ca content from weaning to 12 wks of age. Using this population, we have identified genetic variation in intestinal Ca absorption efficiency as well as a number of other parameters relevant to whole body Ca homeostasis. There were independent genetic

effects controlling the adaptive response of Ca absorption and other parameters to low dietary Ca intake. Collectively, our findings suggest that Ca absorption physiology is more complex than suggested by the facilitated diffusion model and that novel genetic factors affecting Ca absorption as well as diet-induced adaptation of Ca/bone metabolism have yet to be identified.

2.3 Materials and Methods

2.3.1 Experimental Design

Four week old, male mice from 11 common laboratory inbred strains were obtained from The Jackson Labs (Bar Harbor, ME): 129S1/SV1mJ (129S), A/J, AKR/J (AKR), C3H/HeJ (C3H), C57BL/6J (B6), CAST/EiJ (CAST), CBA/J (CBA), DBA/2J (DBA), PWK/PhJ (PWK), SWR/J (SWR), and WSB/EiJ (WSB). This panel of lines was chosen to encompass three mouse subspecies (*Mus musculus domesticus, M.m.musculus,* and *M.m. castaneus*), to include classical inbred strains as well as more genetically divergent wild-derived inbred lines, and to represent parental strains of available genetic mapping resources.[27] To minimize differences in availability and breeding efficiency among the lines, lines were shipped in groups of 4-8 mice within each of two shipment periods. At arrival, an equal number of mice from each line were randomly assigned to either a 0.5% Ca (adequate) or 0.25% Ca (low) diet (AIN93G base with 200 IU vitamin D_3/kg diet, Research Diets, New Brunswick, NJ) (n=8/diet/line). Dietary Ca levels were chosen to maintain Ca homeostasis (0.5% Ca) or elicit an adaptive response in serum $1,25(OH)_2D$ (0.25% Ca). Mice were maintained in rooms with UV blocking filters over

lights (Pegasus Lighting, Beaver Falls, PA) and a 12 h light/dark cycle; they were given food and water *ad libitum*. At 12 wks of age mice were fasted overnight, anesthetized with a cocktail of ketamine and xylazine, and Ca absorption was measured by Ca^{45} appearance in the serum 10 min after an oral gavage test as previously described.[10] Blood was drawn and serum was prepared for the analysis of intestinal Ca absorption by liquid scintillation and $1,25(OH)_2D$ levels by radioimmunoassay as previously described.[11,28] Duodenum and kidney were prepared for mRNA analysis. Data from a pilot study characterizing the response of B6, DBA, and PWK mice to dietary Ca restriction were included in the final analysis of available phenotypes (total sample size for these lines=16-24/diet). All animal experiments were approved by the Purdue University Animal Care and Use Committee.

2.3.2 Intestinal Calcium Absorption

Anesthetized mice were given an oral gavage of a solution containing 0.1 mM $CaCl_2$, 125 mM NaCl, 17 mM Tris, and 1.8 g/L fructose, enriched with 20 µCi $^{45}CaCl_2$/ml (Perkin Elmer, Waltham, MA) (15 µL/g body weight). Ten minutes later, 200 µL of blood was drawn from the superficial temporal vein using the Goldenrod Lancet (Medipoint, Inc., Mineola, NY). Serum was isolated by centrifuging samples for 10 min. at 500 x g. Each serum sample was bleached by dilution (1:10) in a 1:4 solution of 3N potassium hydroxide and 30% hydrogen peroxide for 30 min, after which pH was neutralized with 3N hydrochloric acid. Samples were then counted by liquid scintillation for 1 min.

2.3.3 Gene Expression

Mucosal scrapings were obtained from the first 2 cm of the duodenum. The left kidney was minced and approximately 50 µg saved. These samples were mixed and immediately frozen in TriReagent. Total mRNA was isolated and reverse transcribed into cDNA[28]. Real-time PCR was conducted on samples using the MyiQ RT-PCR system containing SYBR green (Bio-Rad, Hercules, CA). mRNA levels were normalized to the expression of ribosomal protein, large, P0 (RPLP0). Primers used for qPCR have been described elsewhere: RPLP0[28], CaBPD9k[9], and TRPV6[9], PMCA1b[29], $Ca_v1.3$[30], claudin 2 (CLDN2)[31], claudin 12 (CLDN12)[32], TRPV5[9], CaBPD28k[11].

2.3.4 Bone Phenotyping

Hindlimbs were removed, skin was removed, and the remaining tissue was fixed in 10% neutral buffered formalin for 2 weeks after which the tissue was stored in 70% ethanol. After all muscle was removed bones were transferred back to 70% ethanol. Formalin-fixed femora were scanned using a PIXImus densitometer (Lunar; GE-Healthcare, Madison, WI) to yield bone mineral content (BMC, g) and bone mineral density (BMD, g/cm^2) or by microcomputed tomography (µCt 40, Scanco Medical, AG, Bassersdorf, Switzerland) at the midshaft and distal metaphysis.[33] Samples were scanned for µCt while immersed in 70% ethanol. Images were obtained using a cubic voxel size of 16 µm, X-ray tube potential of 55 kVp, an X ray intensity of 145 µA, and 300 ms integration time.

The region of interest (ROI) for cortical bone was at 50% of the length of the bone (midshaft) where 15 slices (0.24 mm) were scanned and reconstructed. Cortical

bone volume fraction (Ct.Ar/Tt.Ar, %) and cortical thickness (Ct. Th, mm) were calculated. For trabecular bone, the ROI was set at 1 mm from the growth plate, 94 slices were scanned (1.5 mm) and 56 slices (1 mm) were reconstructed starting from the first slice containing no evidence of growth plate or primary spongiosa. Reference contours to delineate trabecular bone region were drawn manually a few voxels away from the endocortical surface, the shape of the reference contours were automatically adapted to the bone surface approximately every 10 slices. Images were segmented using Gaussian filtration (Sigma = 0.8, support = 1). Parameters calculated for trabecular bone were bone volume/total volume (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular spacing (Tb.Sp, mm), and trabecular thickness (Tb.Th, mm). All images were reconstructed using the Scanco software and measures obtained were based on 3D model-independent algorithms. A single global threshold value was set manually for each ROI. The reproducibility of this method for mouse femur is reported elsewhere.[33,34]

2.3.5 Statistical Analysis

Data points with a z score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. Adherence to a normal distribution was determined by Anderson-Darling tests. Data not normally distributed were transformed as follows: Ca absorption (log 10); VDR ($y^{0.5}$); TRPV6 and 1,25$(OH)_2$D ($y^{0.25}$); duodenal and renal CaBPD9k, PMCA1b, CLDN2, CLDN12, Cav1.3, Tb.Sp, Tb.Th, BV/TV, TRPV5, and CaBPD28k (natural log). Adherence to a normal distribution was confirmed after transformation. Each phenotype was assessed for the presence of main effects (line, diet) and a line-by-diet interaction using ANCOVA with body weight (BW) and femur

length (FL) as body size covariates.[35] When a significant F statistic was detected, specific *a priori* post-hoc comparisons were made using a permutation-based t-test procedure (Supplemental Table 2.3). Relationships between phenotypes were determined after, significant, independent confounding effects of BW and/or FL were removed by linear regression [35]. Phenotypes affected by BW and FL were: BMD, BMC, Tb.N, Tb.Sp, and renal CaBPD9k; while Ca absorption, TRPV6, duodenal CaBPD9k, PMCA1b, VDR, CLDN2, CLDN12, $1,25(OH)_2D$, Tb.Th, Ct.Th, and Ct.Ar/Tt.Ar were affected by BW only and BV/TV was affected by FL only. The resulting residual values were used in Pearson's correlations, full model linear regression, and principal components analysis (PCA). The number of factors extracted in PCA was based on the Kaiser criterion (eigenvalue >1) and scree plot examination.[36] Factors were next rotated using the orthogonal Varimax rotation. Factor loadings >0.4 or <-0.4 were used for interpretation of each principal component.

For several phenotypes, a unique adaptation parameter reflecting the response to low dietary Ca intake was generated for each mouse on the 0.25% Ca diet. This was calculated as the percent difference between the phenotype value for an individual (i) fed the 0.25% Ca diet (x) and the line (j) mean for the phenotype value from the 0.5% Ca diet (y), standardized to the line mean for the phenotype value from the 0.5% Ca diet and multiplied by 100, i.e. $\left[\left(x_{ij} - \bar{y}_j\right)/\bar{y}_j\right]$*100. The adaptation parameter was normally distributed and not affected by body-size covariates for any phenotype. Summary statistics of adaptation parameters are given in Supplemental Table 2.4. The effect of genetic variation on the adaptation parameter was tested in a one-way ANOVA. The impact of low dietary Ca intake on phenotypes within individual lines was determined

using a one-sample t-test (H_0=0). All analyses were conducted using SAS Enterprise 4.2 (SAS Institute, Inc., Cary, NC) and significance determined at $p<0.05$.

Our primary research goal was to determine the effect of genetics on Ca absorption and so the study was powered based on variance estimates from B6 mice for this phenotype (n=8, 50% difference between dietary groups, SD = 30% of mean, α = 0.05, power = 0.872). Using this sample size we had sufficient power to detect significant differences in mRNA endpoints (100% difference, SD = 50%, power = 0.96) and distal femur μCT parameters (30% difference, SD = 20%, power = 0.797) but femur midshaft, BMD, and BMC had reduced power 0.461). Our linear associations with n=123 have the power (0.8) to see a significant correlation of r = 0.25 ($p<0.05$).

2.4 Results

2.4.1 Ca Absorption and Its Adaptation to Low Dietary Ca Intake

Unadjusted values and least squares means from ANCOVA are provided for all parameters in Supplemental Tables 2.1 and 2.2, respectively. Intestinal Ca absorption was significantly affected by genetic background (line) regardless of the level of dietary Ca fed (Figure 2.1A, $p<0.0001$) with CBA, A/J, and WSB lines having the highest absorption efficiency on the 0.5% Ca diet and 129S, CAST, and DBA lines having the lowest.

As expected, Ca absorption efficiency was significantly increased by low dietary Ca intake in the B6 reference line (+82%, $p<0.0001$). However, the adaptation of Ca absorption to low dietary Ca stress varied significantly among the inbred lines (line-by-

diet interaction, p=0.009). Low Ca intake significantly increased Ca absorption only in the B6 and 129S lines (Figure 2.1A). Consistent with this observation, analysis of the Ca absorption adaptation parameter shows that the B6 and 129S lines were significantly up-regulated ($p<0.05$) while a trend towards increased Ca absorption was also seen for CAST, DBA, C3H, and SWR ($p<0.1$, Figure 2.1B). Lines with no diet-induced increase in Ca absorption were: A/J, AKR, CBA, PWK, and WSB.

2.4.2 Bone Parameters, and Their Adaptation to Low Dietary Ca Intake

BMD was affected by significant line and diet main effects, as well as a significant line-by-diet interaction (p=0.038). Variability in BMD across the population for each diet can be seen in Figure 2.2A. Similar levels of variation were also seen for Ct.Ar/Tt.Ar, distal femur BV/TV, Tb.Th, Tb.N, and Tb.Sp (Supplemental Table 2.3). However, the impact of line on bone loss due to dietary Ca restriction was different among BMD, Ct.Ar/Tt.Ar, and BV/TV (line effect p=0.005, p=0.8, $p<0.0001$, respectively; Figure 2.2B-D). Only PWK mice were resistant to diet-induced bone loss in all three measures. The adaptive response of distal femur BV/TV to low Ca diets was most heterogeneous with significant loss of BV/TV observed in 129S, AKR, and DBA lines, no change in A/J, C3H, CAST, CBA, PWK, and WSB lines, and an increase in B6 (p=0.04) and SWR lines (p=0.1) (Figure 2.2D).

2.4.3 Ca Absorption Efficiency Is Correlated to Bone Mass

Ca absorption was positively correlated to BMD (r=0.17, p=0.02, Table 1). This effect was due to the beneficial impact of Ca absorption on trabecular bone (BV/TV,

Figure 2.3, Tb.Th, Table 1). In contrast, while Ct.Ar/Tt.Ar correlated to Ca absorption in mice on the 0.5% Ca diet (r = 0.25, p for trend = 0.052), the relationship was not significant for mice fed the 0.25% Ca diet or for the combined population.

2.4.4 Regulation of Intestinal Ca Absorption by $1,25(OH)_2D$

Using *in situ* ligated loops, we previously showed that Ca absorption efficiency is significantly correlated to serum $1,25(OH)_2D$ in B6 mice ($r = 0.92$[14]). We confirmed this relationship in B6 mice using the oral gavage test (r=0.65, p=0.0006) (Figure 2.4, black symbols). In the full panel of 11 inbred lines the relationship was still significant, but weakened (r=0.35, p<0.0001, Figures 2.4 and 2.5) due to the high degree of diversity in the relationship between adaptation of Ca absorption and adaptation of serum $1,25(OH)_2D$ to low Ca intake among the lines. Figure 2.6 shows that only two lines had a proportional, diet-induced increase in Ca absorption and serum $1,25(OH)_2D$ (B6, C3H). In contrast, other lines were hyper-responders (i.e. the diet-induced increase in Ca absorption was high relative to the diet-induced increase in serum $1,25(OH)_2D$ in 129S and CAST), or hypo-responders (i.e. a blunted response in Ca absorption in relation to a large increase in serum $1,25(OH)_2D$ in PWK and SWR). Two other lines increased Ca absorption with little to no corresponding increase in serum $1,25(OH)_2D$ (i.e. vitamin D-independent in WSB, DBA), while three lines did not increase either Ca absorption or serum $1,25(OH)_2D$ on a low Ca diet (i.e. non-responders were CBA, A/J, AKR) (Figure 2.5, Supplemental Table 2.4).

2.4.5 Correlations between Duodenal mRNA Levels and Ca Absorption

Several proteins have been proposed to contribute to basal and vitamin D-regulated intestinal Ca absorption, i.e. VDR, TRPV6, CaBPD9k, PMCA1b, CLDN2, CLDN12, and Cav1.3.[8] Line effects influenced the duodenal mRNA level for each of these genes (Supplemental Table 2.3). However, only TRPV6 and CaBPD9k mRNA levels were significantly increased by dietary Ca restriction. Consistent with their proposed roles in the facilitated diffusion model[8], TRPV6, CaBPD9k, and PMCA1b mRNA were each significantly, positively correlated with Ca absorption efficiency (Figure 2.7A-C). Additionally, each gene target was positively correlated to serum $1,25(OH)_2D$ (Table 2) and the three mRNA levels were closely correlated with one another (Table 2, Figure 2.8). When all three mRNAs were included in a multiple linear regression model for Ca absorption, only CaBPD9k mRNA level remained significant, indicating that these three factors are not independent determinants of Ca absorption. Duodenal CLDN2, CLDN12, and Cav1.3 mRNA levels were not significantly correlated to Ca absorption or to serum $1,25(OH)_2$ D levels (data not shown). Although VDR mRNA correlated with TRPV6, CaBPD9k, and PMCA1b mRNA, it did not correlate with Ca absorption (Table 2). Diet-induced changes in CaBPD9k mRNA, but not the other mRNAs, were correlated to the adaptation of Ca absorption to low Ca intake ($r=0.38$, $p<0.0001$, Figure 2.9). However, while VDR mRNA levels did not correlate to the adaptive increase in Ca absorption using mean values from the full panel of 11 inbred line ($n = 11$, $r = 0.44$, $p = 0.17$), a significant correlation was observed when the biological outlier line, A/J, was removed ($n = 10$, $r = 0.81$, $p<0.01$, data not shown).

2.4.6 Renal mRNA Levels

Renal levels of TRPV5, CaBPD28k, and CaBPD9k mRNA were significantly affected by genetic background (Supplemental Tables 2.1 and 2.3) while only CaBPD28k and CaBPD9k were significantly influenced by diet and no significant line-by-diet interaction was detected for any of the three renal mRNAs. CaBPD28k and CaBPD9k mRNA were significantly, positively correlated with serum $1,25(OH)_2D$ ($r = 0.27$ and 0.33, respectively, $p<0.001$) but TRPV5 mRNA was not (Supplemental Table 2.5). TRPV5 mRNA was negatively correlated with intestinal Ca absorption ($r = -0.23$, $p=0.007$) while TRPV5, CaBPD28k, CaBPD9k mRNA levels were negatively correlated with several bone parameters (e.g. $r = -0.25, -0.43, -0.33$ with Ct.Th., respectively, $p \leq 0.002$, Supplemental Table 2.5).

2.4.7 Principal Components Analysis (PCA)

Two significant principal components (PC1, PC2) were extracted and they account for 30.8% and 25.9% of the total variance, respectively (Supplemental Table 2.6). PC1 contained intestinal Ca absorption, duodenal mRNA levels, and renal CaBPD9k mRNA. PC2 contained factors from each arm of the 3-tissue axis; Ct.Ar/Tt.Ar, BV/TV, Ca absorption, and renal mRNA levels.

2.5 Discussion

Identification of genetic diversity in inbred mouse lines has been the foundation for quantitative trait loci (QTL) mapping, candidate gene, and genome wide association studies to identify molecular determinants of phenotypes like BMD.[20] However, few

studies have examined the effects of genetics on tissues controlling Ca metabolism other than bone, nor have interactions between genetics and diet been accounted for, resulting in inconsistencies in the association between dietary Ca intake and bone health.[37-41] Our study addresses this knowledge gap with a special focus on intestinal Ca absorption. We demonstrate that although feeding Ca restricted diets to growing mice initiates a physiological adaptation to protect bone, the robustness of this adaptive response is dependent on genetic background (Figure 2.1 and 2.2, Supplemental Table 2.4). For most of the phenotypes we examined, there was no correlation between the basal genetic effect and adaptation of a phenotype to low Ca intake, demonstrating that these genetic effects are distinct.

Bone is the most abundant store of Ca in the body and is influenced by regulatory mechanisms occurring at the bone, intestine, and kidney. Our data show that while significant gene-by-diet interactions control the adaptation of bone mass and intestinal Ca absorption to low dietary Ca intake, no interaction influenced the renal levels of transcripts related to Ca reabsorption. This suggests that while genetic variation affecting renal Ca handling may contribute to bone health, it may be less critical for the adaptive response to Ca restriction in growing mice. We expected that renal TRPV5, CaBPD28k, and CaBPD9k levels, as surrogate markers of renal Ca reabsorption, would be tightly correlated to each other and positively associated with both serum $1,25(OH)_2D$ and bone parameters (i.e. improved Ca retention = improved bone).[9,42] However, while CaBPD9k and D28k mRNA levels were positively associated with serum $1,25(OH)_2D$ levels ($r = 0.33, 0.27$, respectively, $p<0.001$), TRPV5 mRNA was not. The lack of correlation between renal TRPV5 and serum $1,25(OH)_2D$ is consistent with previous reports showing

modest changes in renal TRPV5 mRNA levels between wild-type and VDR knockout mice[10,13] and following injection with pharmacologic levels of $1,25(OH)_2D$[9]. Previous studies report that urinary Ca excretion is higher in mice with TRPV5 gene deletion or low renal CaBPD9k and D28k levels[42,43] suggesting a positive role for these proteins in limiting bone loss and maintaining bone density. In contrast, we found that the three renal mRNA levels were individually, and in a principal components analysis, negatively associated with various bone endpoints. We hypothesize that this is an indirect effect that reflects a reduced need for renal Ca reabsorption when intestinal Ca absorption is high. This idea is supported in part by a negative association between renal TRPV5 mRNA and Ca absorption.

The primary goals for our study were to evaluate the genetic influences on intestinal Ca absorption as well as to use our genetically diverse population to learn more about mechanisms for Ca absorption and the contribution of Ca absorption to development of peak bone mass. Two previous studies showed that Ca absorption was greater in 8-12 week old female C3H than B6 mice fed a Ca-sufficient diet (0.4% or 1.2% Ca).[23,24] We have extended these observations to 11 inbred strains and our data reveal a large amount of variation in Ca absorption efficiency in mice fed an adequate or low Ca diet, as well as in the ability of mice to adapt to low dietary Ca intake (Figure 2.1).

Armbrecht et al.[24] previously showed that the maximal response of Ca absorption to $1,25(OH)_2D$ injection was not different between C3H and B6 mice. In contrast, our study examined a nutritionally and physiologically relevant condition – restriction of dietary Ca by 50%, i.e. similar to the relationship between Ca intake and Ca requirements seen in adult women in the U.S.[44] Previously, we reported that B6 mice

follow the traditional model of adaptation to low dietary Ca intake[9]; increases in serum $1,25(OH)_2D$ induce active intestinal Ca absorption that protect mice from bone loss through a VDR dependent mechanism[8,9,13]. However, while we see variation in the Ca absorption response to low dietary Ca intake across the 11 lines, this variation was not strongly associated with diet-induced increases in serum $1,25(OH)_2D$ levels (Figure 2.6). Subpopulations were identified in the panel of 11 lines that reflected "normal" and "hyper" adapters, vitamin D-independent adapters, vitamin D-resistant adapters, and non-adapters. The lack of a strong relationship between adaptation of Ca absorption and diet-induced changes in serum $1,25(OH)_2D$ were not due to obvious line-specific differences in duodenal VDR mRNA level (Supplemental Tables 2.2 and 2.4). We previously reported that growth hormone or IGF-1 contributes to the residual Ca absorption efficiency that exists in growing VDR knockout mice.[13] However, we measured Ca absorption in mice that were past their rapid growth phase and our data was adjusted for body size to minimize growth-related effects on our phenotypes. Taken together, these observations indicate the existence of a vitamin D-independent, enhancing effect of low Ca intake on Ca absorption but the mechanism for this is not clear.

In B6 mice there is a close relationship between serum $1,25(OH)_2D$ levels and Ca absorption efficiency (Figure 2.3, $r = 0.65$; $r = 0.9$[14]). However, when the genetic diversity available in our 11 line panel is considered, the relationship of Ca absorption to serum $1,25(OH)_2D$ is more similar to that reported in humans ($r = 0.23$-0.35)[45-47](Figure 2.4). Known environmental factors such as diet, age, and circulating hormones account for, at most, one quarter of the variation in true fractional Ca absorption seen in human populations.[45,48] Our data suggest that the remainder of the variation in Ca absorption is

due to genetic factors and gene-by-diet interactions. Consistent with this concept, adolescent black girls have higher Ca absorption compared to white girls[49] and this may contribute to the higher bone deposition seen in black girls[50]. Also, serum $1,25(OH)_2D$ is a significant predictor of Ca absorption in black but not white women, suggesting an impact of genetics on this relationship.[16] Here we found that adaptive increases in Ca absorption were not strongly correlated to diet-induced changes in serum $1,25(OH)_2D$. This suggests that resistance or hyper-responsiveness to the action of $1,25(OH)_2D$ may reflect defects in the VDR-dependent regulatory system.[12,14] However, there were also no obvious polymorphisms in the VDR gene that segregate with the responses of the mouse lines studied here. Identification of the genetic factors controlling Ca absorption has been difficult because studies on polymorphisms in candidate genes have been limited and inconsistent.[8] In addition, the environmental and genetic complexity of free-living human populations makes identifying gene-by-diet interactions difficult, especially for a hard to measure physiologic trait like Ca absorption. In contrast, our study in genetically well-characterized mouse models raised in a controlled environment provides a strong foundation for future gene mapping studies to identify the genetic variants that control intestinal Ca absorption efficiency as well as its adaptation to low dietary Ca intake.[27]

Our study has also allowed us to examine three models proposed to describe intestinal Ca absorption[8], i.e. the facilitated diffusion model, passive diffusion through the tight junction proteins CLDN2 and CLDN12 [51], and transcellular Ca transport through the voltage gated Ca channel $Ca_v1.3$ [52]. Although CLDN2, CLDN12, and Cav1.3 mRNA levels were each detected in duodenum and each was significantly

affected by line, none of them were influenced by diet nor were they significantly associated with Ca absorption or serum $1,25(OH)_2D$. This observation indicates they play a minimal role in Ca absorption under our experimental conditions (i.e. a low Ca load in our absorption test designed to reveal transcellular, not paracellular, Ca transport).

In the facilitated diffusion model[53], TRPV6, CaBPD9k, and PMCA1b work in coordination to mediate Ca absorption, e.g. in B6 mice, TRPV6 and CaBPD9k levels are elevated by increased serum $1,25(OH)_2D$ levels and they are associated with increased Ca absorption efficiency[9]. However, the role of these proteins in Ca absorption has been questioned due to lack of a dramatic phenotype in TRPV6 and CaBPD9k knockout mice.[15] Our data indicate that TRPV6, CaBPD9k, and PMCA1b likely perform as a single functional unit; multiple linear regression and PCA indicated that TRPV6, CaBPD9k, and PMCA1b mRNA were not independent predictors of Ca absorption. However, the correlations of these messages with Ca absorption, while significant, are weak (r values ≤ 0.43 lead to $r^2 \leq 0.18$ or less), indicating that only a small portion of the variability in Ca absorption is dependent upon the facilitated diffusion model (Table 2.2). In addition, only the adaptation of CaBPD9k mRNA to low Ca intake was significantly correlated to low dietary Ca-induced adaptation of Ca absorption. This observation supports our hypothesis that CaBPD9k expression is a response to the elevated intracellular Ca levels that accompany Ca absorption, but it does not strongly support an exclusive role for the facilitated diffusion model as the mediator of Ca absorption.[28]

In conclusion, we have shown that genetic variation and gene-by-diet interactions affect not only active intestinal Ca absorption, but also its relationship to bone. These interactions are partially accounted for by variation in the traditional cellular mediators

(i.e. TRPV6, CaBPD9k, PMCA1b mRNA) and the hormonal regulator (i.e. $1,25(OH)_2D$) of Ca absorption. However, the characterization done here, on 11 inbred lines of mice in a carefully controlled environment, indicates that there are aspects of Ca homeostasis that remain to be discovered. Future studies using mouse genetic mapping populations, such as recombinant inbred line panels, are needed to map genetic loci responsible for our observation.[27] Further characterization of the gene-by-diet interactions identified here will provide insight into their impact on fracture risk and will provide scientific support for defining dietary requirements for individuals or genetically distinct subgroups.

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Table 2.1 Correlation of Bone Density and Morphometry Parameters to Ca Absorption

Phenotype	*r* (95% CL)	p	n
BMD	0.172 (0.025-0.312)	p=0.02	n=176
BMC	0.048 (-0.104-0.198)	p=0.54	n=168
Ct.Ar/Tt.Ar	0.085 (-0.086-0.251)	p=0.33	n=134
Ct.Th	0.102 (-0.066-0.265)	p=0.24	n=138
BV/TV	0.338 (0.178-0.481)	p<0.0001	n=133
Tb.Th	0.277 (0.134-0.438)	p=0.0004	n=140
Tb.Sp	-0.106 (-0.269-0.063)	p=0.22	n=137
Tb.N	0.055 (-0.115-0.221)	p=0.53	n=135

Pearson's correlation coefficients are given: *r* (upper, lower 95% confidence limits). Body size corrected residuals were used for analysis.

Table 2.2 Pearson's Correlation Coefficients of Ca Absorption Regulators

	Ca Absorption	TRPV6	CaBPD9k	PMCA1b	$1,25(OH)_2D$
TRPV6	0.42 (0.26, 0.55) p<0.0001, n=127				
CaBPD9k	0.43 (0.27, 0.55) p<0.0001, n=153	0.74 (0.65, 0.80) p<0.0001, n=140			
PMCA1b	0.21 (0.04, 0.37) p=0.02, n=129	0.53, (0.40, 0.64) p<0.0001, n=142	0.47, (0.33, 0.59) p<0.0001, n=141		
$1,25(OH)_2D$	0.35 (0.21, 0.48) p<0.0001, n=166	0.44, (0.30, 0.57) p<0.0001, n=139	0.39, (0.27, 0.51) p<0.0001, n=189	0.19, (0.02, 0.34) p=0.03, n=140	
VDR	-0.02 (-0.19, 0.15) p=0.82, n=132	0.45, (0.31, 0.54) p<0.0001, n=147	0.23, (0.06, 0.37) p=0.01, n=145	0.53, (0.40, 0.63) p<0.0001, n=148	0.09, (-0.07, 0.25) p=0.29, n=146

Pearson's correlation coefficients are given: *r* (upper, lower 95% confidence limits), p-value, n. Body size corrected residuals were used for analysis.

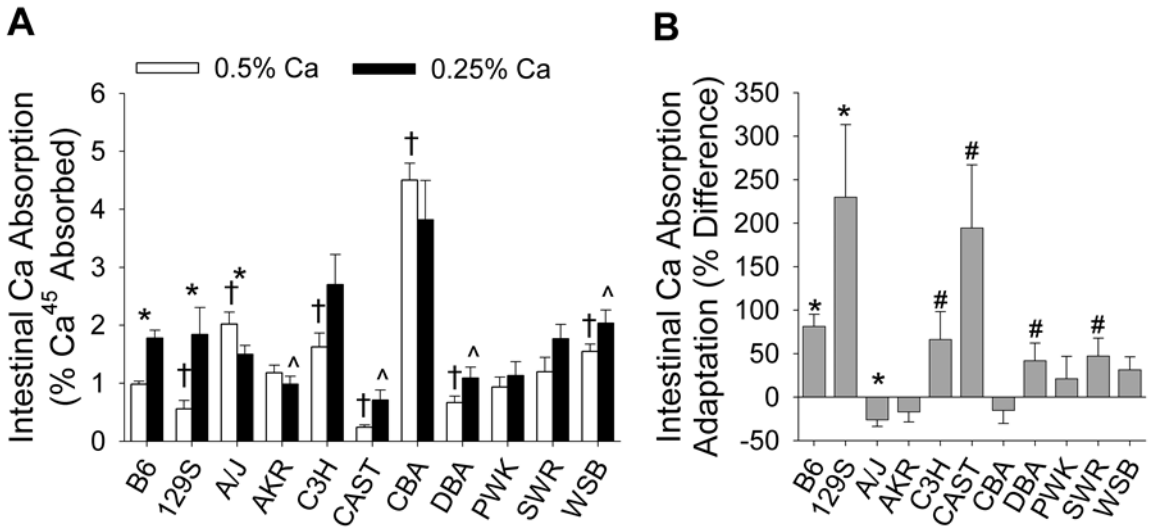

Figure 2.1 Ca absorption and adaptation of Ca absorption to low Ca intake is variable among 11 inbred mouse lines. Bars reflect the mean ±SEM (n=4-14 per diet for each line). (A) Unadjusted Ca absorption values, * dietary groups within a line differ significantly ($p<0.05$); line mean differs significantly relative to the B6 reference line ($p<0.05$), † for the 0.5% Ca group, ^ for the 0.25% Ca group. (B) Adaptation of Ca absorption to low dietary Ca intake; adaptation significantly differs from 0 (*, $p<0.05$; #, $p<0.1$).

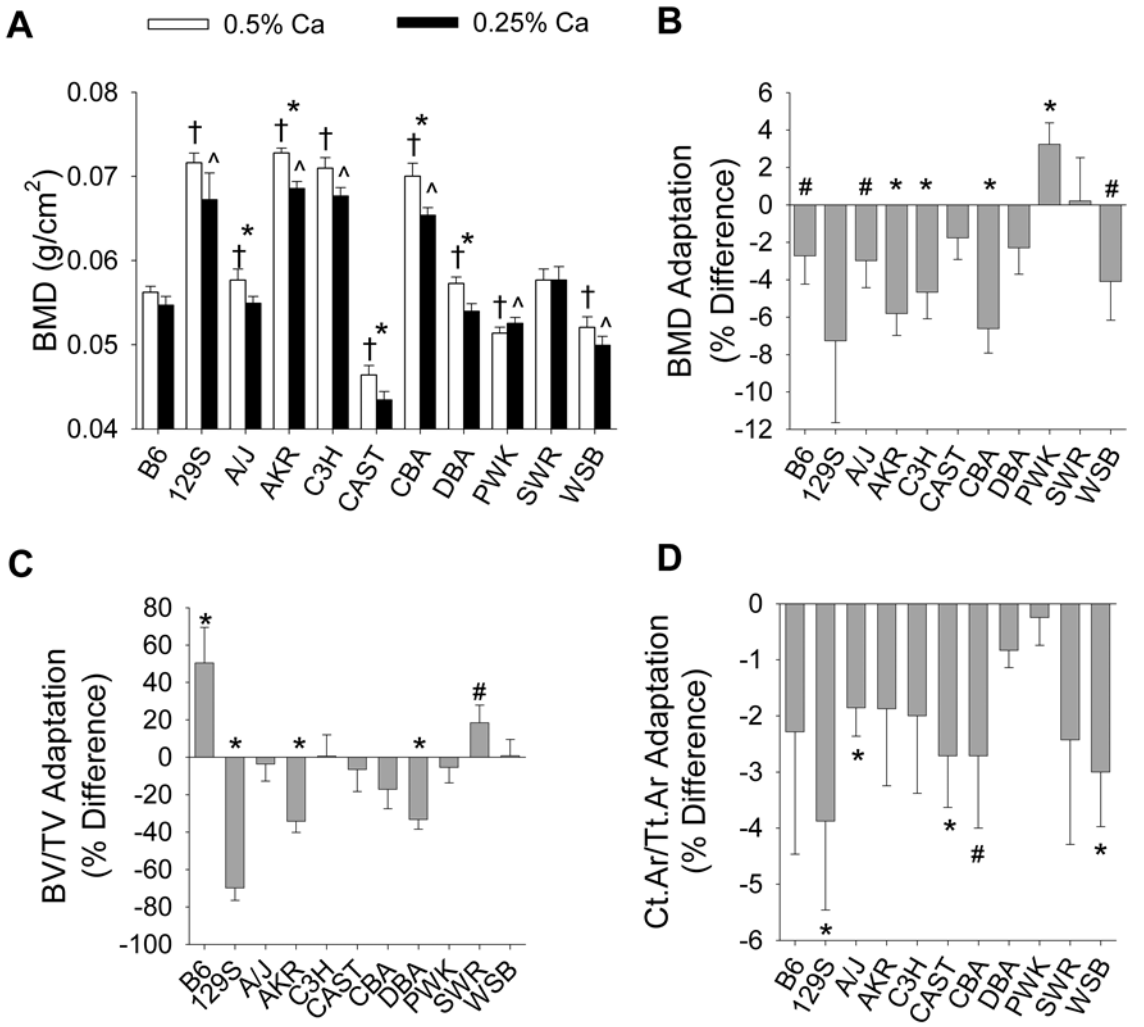

Figure 2.2 Bone parameters and adaptation of bone parameters to low Ca diets are variable among 11 inbred mouse lines. Bars reflect the mean ±SEM (n=7-20 per diet for each line). (A) Unadjusted BMD values, * dietary groups within a line differ significantly ($p<0.05$); line mean differs significantly relative to the B6 reference line ($p<0.05$), † for the 0.5% Ca group, ^ for the 0.25% Ca group. (B-D) Adaptation to low dietary Ca intake for (B) BMD, (C) BV/TV, and (D) Ct.Ar/Tt.Ar; adaptation significantly differs from 0 (*, $p<0.05$; #, $p<0.1$).

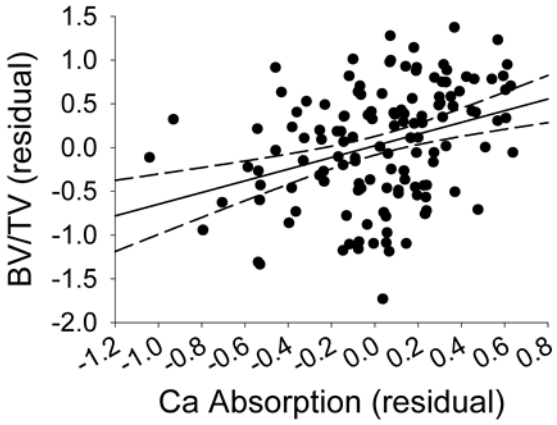

Figure 2.3 Distal femur BV/TV is significantly, positively correlated with intestinal Ca absorption. Pearson's correlation was calculated using individual residual values from mice representing all 11 inbred lines from both diet groups with data points present for both phenotypes. Solid line = regression ($r=0.34$, $p<0.05$), dotted line = 95% confidence interval, n=133.

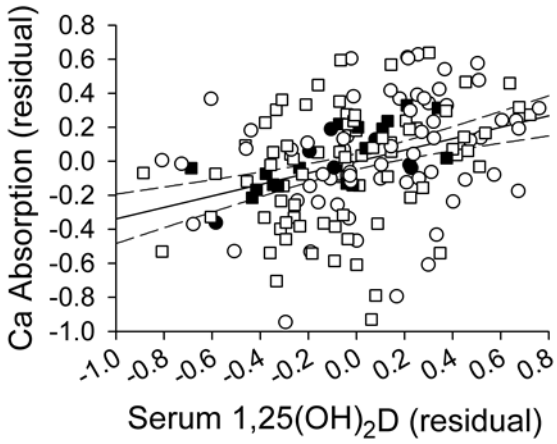

Figure 2.4 Relationship between serum $1,25(OH)_2D$ and intestinal Ca absorption across the 11 inbred lines (r=0.35, p<0.001, all symbols). Values for the B6 reference line alone are shown as filled symbols (r=0.65, p<0.01). 0.25% Ca diet (circles), 0.5% Ca diet (squares). Body size-corrected residual values are plotted, dotted line = 95% confidence interval, n=166.

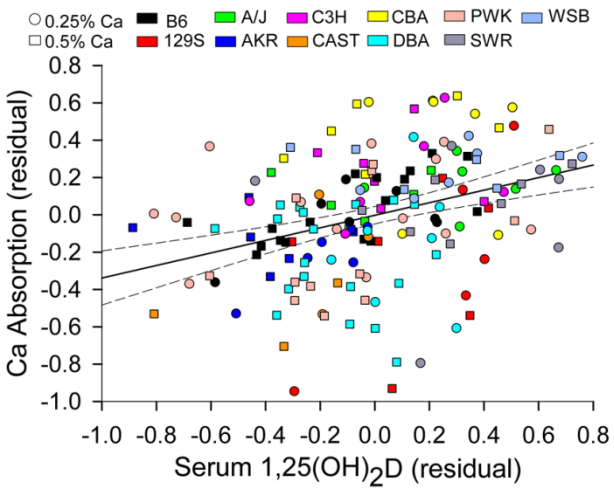

Figure 2.5 Genetic diversity affects the relationship between $1,25(OH)_2D$ and Ca absorption. . The relationship between serum $1,25(OH)_2D$ and intestinal Ca absorption across the 11 inbred lines (r=0.35, p<0.001, n=166, dotted line = 95% confidence interval) is weaker than that observed for the B6 line alone (r=0.65, p<0.01, n=24). 0.25% Ca diet (circles), 0.5% Ca diet (squares). Body-size corrected residuals are plotted.

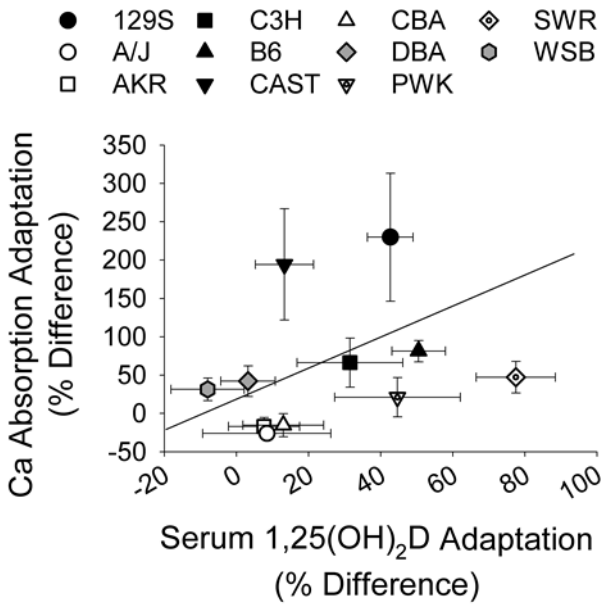

Figure 2.6 Correlation between adaptation of serum $1,25(OH)_2D$ and intestinal Ca absorption to low Ca diets in the 11 inbred lines. An adaptation parameter was calculated for each mouse on the 0.25% diet and the line mean values (± SEM) were plotted.

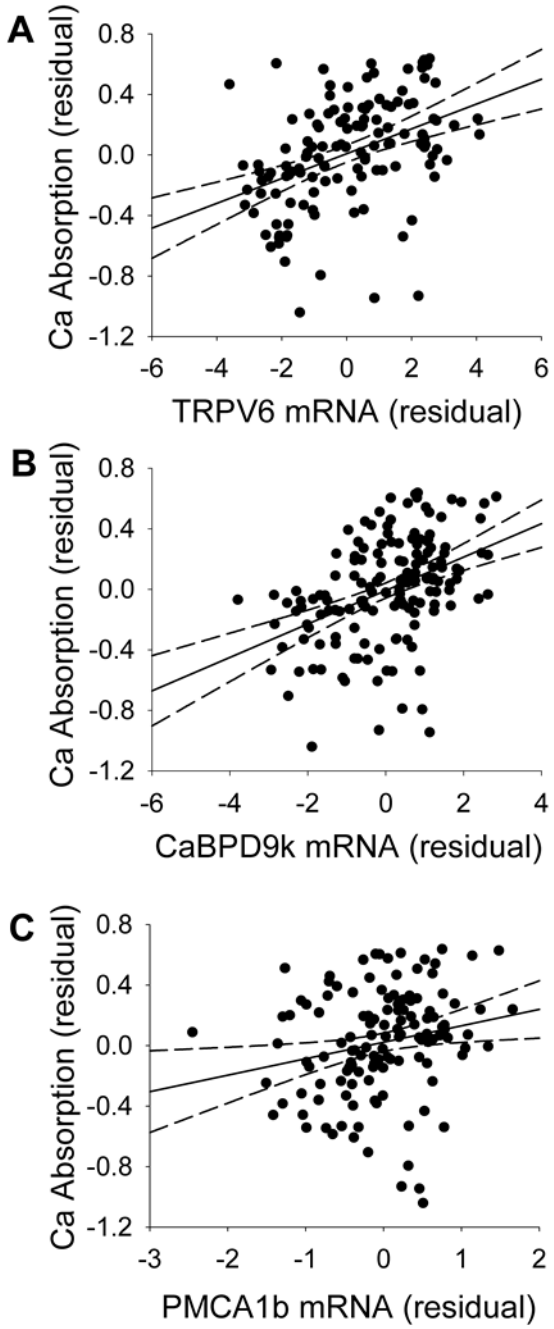

Figure 2.7 Correlation between mRNA levels for the key members in the facilitated diffusion model and Ca absorption. (A) TRPV6 mRNA ($r=0.42$, $p<0.001$, $n=127$), (B) CaBPD9k mRNA ($r=0.43$, $p<0.001$, $n=153$), and (C) PMCA1b mRNA ($r=0.21$, $p<0.05$, $n=129$). Body size-corrected residuals with 95% confidence intervals are shown.

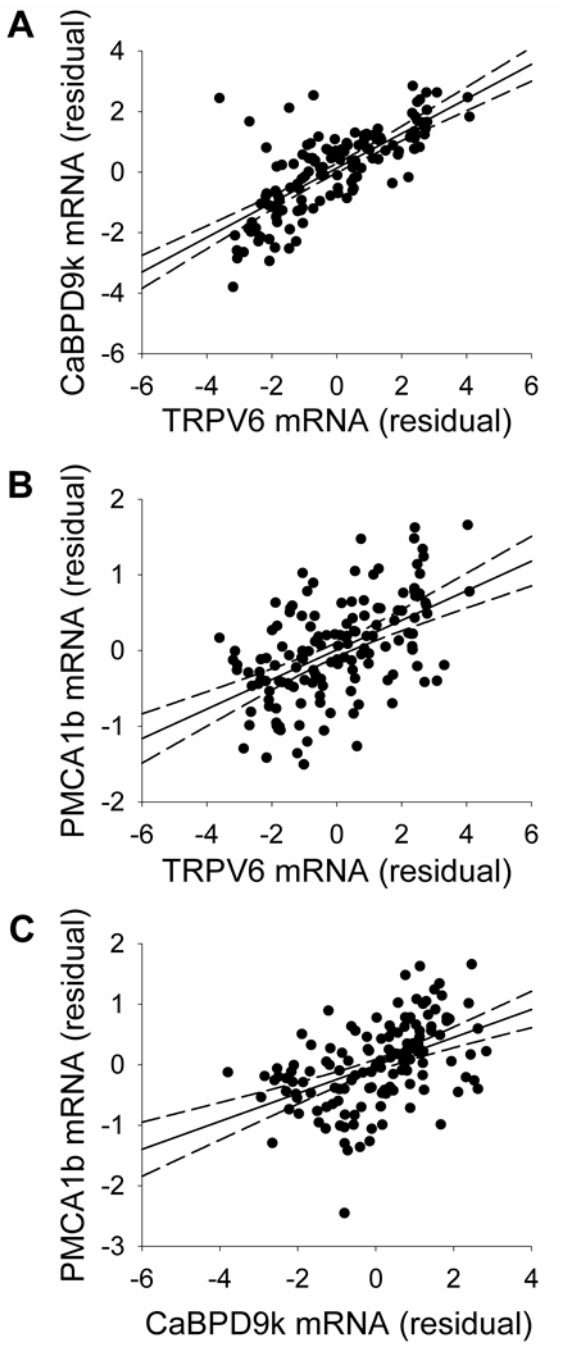

Figure 2.8 Key members in the facilitated diffusion model are significantly interrelated at the mRNA level. (A) TRPV6 to CaBPD9k ($r=0.74$, $p<0.0001$, $n=140$), (B) TRPV6 to PMCA1b ($r=0.53$, $p<0.0001$, $n=142$), and (C) CaBPD9k to PMCA1b ($r=0.47$, $p<0.0001$, $n=141$). Data are shown as body size-corrected residuals with 95% confidence intervals.

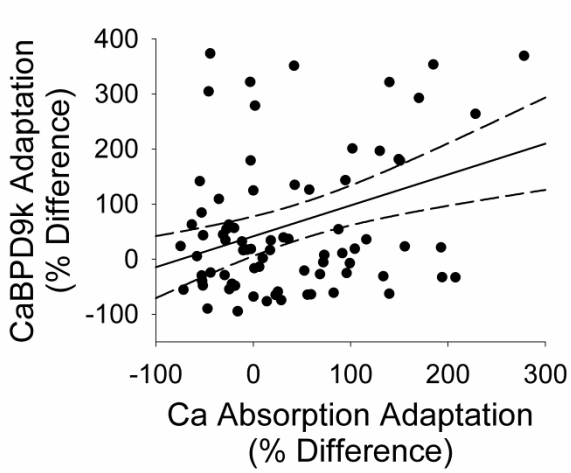

Figure 2.9 Adaptation of CaBPD9k to a low Ca diet is significantly, positively correlated with adaptation of Ca absorption to a low Ca diet. An adaptation parameter was calculated for each mouse on the 0.25% diet. Pearson's correlation was calculated using individual values from mice representing all 11 inbred lines with data points present for both phenotypes ($r=0.38$, $p<0.001$, $n=79$), dotted line = 95% confidence interval.

CHAPTER 3. NATURAL GENETIC VARIATION AFFECTING THE RESPONSE OF SERUM 1,25$(OH)_2$D TO DIETARY CALCIUM RESTRICTION IN MICE

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Keywords

Vitamin D; gene by diet interaction; genetics; QTL; dietary calcium

3.1 Abstract

Vitamin D regulates calcium (Ca) homeostasis and is necessary for proper bone health. Low dietary Ca intake increases the renal conversion of 25 hydroxyvitamin D (25(OH)D) to the hormone 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), a regulator of gene expression through the nuclear vitamin D receptor (VDR). While studies have identified genetic variation controlling serum 25(OH)D levels, it is unclear whether genetics control serum $1,25(OH)_2D$ levels or their adaptation to low Ca diets. To test this, male mice from 11 inbred lines (Study 1) and 51 BXD recombinant inbred lines (Study 2) were placed on controlled diets containing 200 IU vitamin D and either 0.5% (the rodent Ca requirement) or 0.25% Ca from 4-12 wks of age (n=8/line/diet). Significant variation was identified in Study 1 in serum 25(OH)D, serum $1,25(OH)_2D$, renal hydroxylases controlling vitamin D metabolism, and adaptation of $1,25(OH)_2D$ to low dietary Ca. In Study 2, narrow sense heritability (h^2) of 25(OH)D and $1,25(OH)_2D$ on the 0.5% Ca diet was 0.40 and 0.66 , respectively. The adaptive response of $1,25(OH)_2D$ to the low Ca dietary environment ranged from -6% to 297% across BXD lines (h^2=0.59). Quantitative trait loci associated with serum $1,25(OH)_2D$ did not overlap those linked to diet-induced adaptation, indicating the genes controlling the physiologic response to low dietary Ca are distinct from those controlling baseline levels. Our results indicate serum vitamin D metabolite levels are controlled by multiple genetic factors that, in some cases, interact with the dietary environment.

3.2 Introduction

Vitamin D deficiency is characterized by rickets and osteomalacia, highlighting its important role in calcium (Ca) and bone homeostasis.[1] Additionally, vitamin D has been proposed to have unique, beneficial effects in several other diseases including cancer, heart disease, and autoimmune disease.[1] The regulation of vitamin D synthesis, metabolism, and action are complex processes. Vitamin D is acquired from the diet or by *de novo* synthesis in the skin, after which it is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D) and released into the serum. Circulating 25(OH)D levels are used to assess vitamin D status. 25(OH)D is the biologically inactive precursor to the hormonal metabolite, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$). $1,25(OH)_2D$ functions through the vitamin D receptor (VDR) to maintain serum Ca homeostasis by regulating gene expression in the intestine, kidney and bone.[2] This method of regulation is sensitive to the dietary Ca environment. Habitually low dietary Ca intake stimulates an adaptive increase in the conversion of 25OHD to $1,25(OH)_2D$ in the kidney by the enzyme CYP27b1.[2-4]

Serum vitamin D metabolite levels are influenced by genetic and environmental factors.[5,6] The heritability of serum 25(OH)D in human populations ranges from 23-80%.[6-11] However, only a few genetic factors accounting for a small amount of variance (1-14%) in serum 25(OH)D levels have been identified.[5,12] Combined, environmental factors and selected genotypes have explained just 54% of total variance in serum 25(OH)D levels.[12] Together, these data suggest novel genetic factors influencing vitamin D status remain to be identified.

Heritability of $1,25(OH)_2D$ has been estimated at 16-48% in human populations.[13,14] Studies examining the effect of genetic modifiers on serum $1,25(OH)_2D$ level are limited. What data are available are inconclusive because this metabolite is particularly sensitive to environmental (e.g. dietary Ca) and physiological cues (e.g. low serum Ca). Research suggests that natural genetic variation can interact with the dietary Ca environment to influence $1,25(OH)_2D$-mediated Ca homeostasis.[15-17] However, gene-by-diet interactions affecting serum $1,25(OH)_2D$ levels have not been carefully studied. Understanding the influence of natural genetic variation, the dietary environment, and their interaction on serum $1,25(OH)_2D$ may elucidate new biology in the vitamin D metabolic pathway. In turn, this knowledge may clarify the hormone's roles in other systems.

We have characterized the natural genetic variation in serum 25(OH)D and $1,25(OH)_2D$ present in a diverse population of inbred mice. Using animal models allowed us to control genetic background as well as environmental conditions, thus overcoming the highly variable nature of $1,25(OH)_2D$ present in free-living populations. Additionally, we have used a physiologically relevant habitual dietary Ca restriction in order to study the effect of natural genetic variation on the adaptation of serum $1,25(OH)_2D$ to this stress and have conducted quantitative trait locus (QTL) mapping to identify genetic regions controlling serum vitamin D metabolite levels and the adaptation of $1,25(OH)_2D$ to low Ca intake. This is the first linkage mapping study to identify loci controlling serum $1,25(OH)_2D$ or its adaptation to a low Ca diet under controlled environmental conditions.

3.3 Materials and Methods

3.3.1 Animal Models

Study 1 was a characterization of serum vitamin D metabolites in a diverse population of 11 inbred mouse lines; 129S1/SV1mJ (129S), A/J, AKR/J (AKR), C3H/HeJ (C3H), C57BL/6J (B6), CAST/EiJ (CAST), CBA/J (CBA), DBA/2J (DBA), PWK/PhJ (PWK), SWR/J (SWR), and WSB/EiJ (WSB). Intestinal Ca absorption and bone related phenotypes for this population have been reported previously.[18] Study 2 was a forward genetic linkage mapping study to identify QTL influencing serum vitamin D metabolites using the BXD recombinant inbred (RI) panel. In the BXD RI panel, each line is a unique recombination of the B6 and DBA parental genomes, but because each BXD line is inbred, each individual within a line is genetically identical.[19] This aspect allows for biological replicates to test an environmental intervention such as diet.[19]

3.3.2 Experimental Design

Using the study design reported previously[18], male mice from the 11 inbred lines (Study 1) and 51 BXD RI lines (Study 2) were obtained at 4 wks of age (The Jackson Labs, Bar Harbor, ME). At arrival, an equal number of mice from each line were randomly assigned to either a 0.5% Ca (adequate) or 0.25% Ca (low) diet (AIN93G base with 200 IU vitamin D3/kg diet, Research Diets, New Brunswick, NJ) (n=8/diet/line). Dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca) or elicit an adaptive response to low Ca intake in serum $1,25(OH)_2D$ (0.25% Ca). Mice were maintained in an UV free environment (12h light/dark cycle) and given food and

water ad libitum. At 12 wks of age mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. Mice were euthanized by exsanguination and the left kidney was removed. Serum was separated by centrifugation at 700 rcf for 10 min at room temperature. Serum vitamin D metabolites 25(OH)D and $1,25(OH)_2D$ were measured using commercially available radioimmunoassays (IDS, Plc., Scottsdale, AZ) according to the manufacturer's directions. Renal tissue was harvested and mRNA isolated as described previously.[18] Renal CYP24 and CYP27b1 mRNA was measured by real-time PCR as previously described.[20] All animal experiments were approved by the Purdue University Animal Care and Use Committee.

3.3.3 Statistical Analysis

Statistical methods for Study 1 can be found in Replogle et al.[18] Study 1 was transformed as follows; $1,25(OH)_2D$ ($y^{0.25}$), CYP24 and CYP27b1 (natural log) and adherence to a normal distribution confirmed using the Anderson-Darling test for normality following transformation. ANCOVA was used in Study 1 to test for the main effects of genetic background (i.e. line) and diet as well as a line-by-diet interaction while controlling for the effect of body weight (BW) and femur length (FL) as covariates. Select, *a priori* post-hoc comparisons were made using Fisher's LSD. Relationships between phenotypes were done with Pearson's correlation tests using covariate corrected residuals. 25(OH)D, $1,25(OH)_2D$, and CYP27b1 were corrected for BW while CYP24 was corrected for FL using simple linear regression as described elsewhere.[18] In Study 2 the covariate effect of BW was removed by linear regression and residuals were used for linkage mapping.[21] Narrow-sense heritability was calculated using the r^2 of a one-way

ANOVA (main effect=line) for each diet/phenotype population. An additional parameter reflecting adaptation of $1,25(OH)_2D$ to the low Ca diet was calculated as the percent difference between the raw phenotype value for an individual (i) fed the 0.25% Ca diet (x) and the raw line (j) mean for the phenotype value from the 0.5% Ca diet (y), standardized to the line mean for the phenotype value from the 0.5% Ca diet, i.e. $\left[\left(x_{ij} - \bar{y}_j\right)/\bar{y}_j\right]$*100. Statistics were conducted using SAS Enterprise Guide 4.2 (SAS Institute Inc., Cary, NC).

3.3.4 QTL Mapping

Marker information and BXD genotypes were downloaded from GeneNetwork (http://www.genenetwork.org/genotypes/BXD.geno) and the genetic location of each marker was updated using the Mouse Map Converter tool at the Jackson Labs Center for Genome Dynamics (http://cgd.jax.org/mousemapconverter/).[22] Markers with duplicate genetic locations or perfectly correlated genotypes in our panel of BXD lines were removed. The final genetic map for the 51 lines contained 1558 markers (the list is available on request).

Composite interval mapping (CIM) was conducted using Windows QTL Cartographer v2.5_011 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) with RI line means (n=51). Forward selection identified 5 significant background markers. CIM was carried out using a Haldane map function, 2 cM walking speed, and a 10 cM window. Each diet (0.5% or 0.25% Ca) group and the adaptation to low dietary Ca ($1,25(OH)_2D$ only) were mapped separately. Permutations (n=500) were used to determine significance for each analysis.[23] A Bayesian QTL mapping method using individual animal values was also used to validate CIM findings. This method, as described in

Zhang et al., accurately detects multiple QTL in a study with a large number of marker effects (*p*) and small sample size (*n*).[24] Bayes factors were used as a measure of significance for the presence of a QTL. Specifically, twice the natural log of the Bayes factor (2lnBF) was used as a test statistic because it scales similarly to an LOD score.[25,26] The strength of the 2lnBF test statistic was assessed according to the rubric defined by Kass and Raftery; "0-2 not worth a bare mention, 2-6 positive, 6-10 strong, >10 very strong".[25] Each diet population was mapped separately as well as included together in a full model to test the genetic main effect. The use of these two QTL mapping methods allowed us to test for QTLs within the traditional framework, but still leverage our study design which contained biological replicates. Results that were replicated among the analyses lent more confidence to the outcome and were prioritized for further study.

3.3.5 Bioinformatic Characterization

The QTL candidate region was defined using 1-LOD support intervals which approximate 95% confidence intervals.[27,28] QTL candidate regions were populated with genome features including protein-coding genes, non-coding RNA genes, gene fragments, and unclassified genes from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/).[29] Genome-wide DNase1 hypersensitive site (HSS) data from the mouse ENCODE project was used to identify potential regulatory regions (i.e. open chromatin) within QTL regions.[30] DNase1 HSS peaks in 10 adult tissues (fat pad, genital fat pad, heart, kidney, large intestine, liver, lung, skeletal muscle, spleen, and brain) were merged using the UCSC Genome Browser.

Gene locations were identified as the region from the first to last exon plus 5000 bp region upstream of the gene coordinates for exon 1 (i.e. the proximal promoter region). Genomic regions that were identical by decent (IBD) between B6 and DBA were identified using the Mouse Phylogeny Viewer (http://msub.csbio.unc.edu/).[31] Regions that were 100% IBD were eliminated from further consideration. Genes that were less than 100% IBD and the remaining non-IBD regions were used to query for polymorphisms between B6 and DBA using the Mouse Phenome Database (MPD, http://phenome.jax.org/). MPD annotations were used to categorize polymorphisms by gene attribute; intronic, mRNA un-translated region (5' and 3' UTR), promoter region (5000 bp upstream), and exon-associated (i.e. synonymous and non-synonymous codons, stop codons, splice sites, or frameshift mutations). HSS data was then overlaid with the polymorphisms present in the QTL region. Reference amino acids and variants resulting from non-synonymous polymorphisms were categorized based on charge, polarity, size, and special cases using the classification set out by Zhang et al with modifications.[32] Amino acids were classified into only two categories for size: small; C, P, G, A, S, T, N, D, Q, E, I, L, M, and V; and large; R, H, K, F, W, and Y. Three special cases are considered; C, P, and G. An amino acid substitution was considered deleterious if the reference and variant alleles were in a different category for any classification.

3.4 Results

3.4.1 Variation Related to Vitamin D Metabolites and Metabolic Enzymes Was Seen across the Diverse 11 Inbred Line Population

Serum 25(OH)D was significantly affected by genetic background in the 11 inbred line population (line effect $p<0.0001$, Figure 3.1A) but there was no diet effect nor line-by-diet interaction observed. Significant line and diet main effects were found for serum 1,25$(OH)_2$D ($p<0.0001$, Figure 3.1B) while a trend for a gene-by-diet interaction was found ($p=0.09$). A significant line effect influenced the diet-mediated change in serum 1,25$(OH)_2$D ($p<0.0001$, Figure 3.1C). However, the adaptive increase in serum 1,25$(OH)_2$D was significant for only five lines; B6, 129S, PWK, and SWR ($p<0.05$), C3H ($p<0.1$). CYP27b1 mRNA levels in the kidney were significantly affected by the main effects of line ($p<0.0001$) and diet ($p=0.0005$), but no line-by-diet interaction was seen ($p=0.4$)(Figure 3.2A). Renal CYP24 mRNA levels showed a significant influence of line ($p<0.0001$)(Figure 3.2C) while no diet effect ($p=0.6$) or line-by-diet interaction ($p=0.12$) was observed. Serum 1,25$(OH)_2$D levels did not significantly correlate to either renal CYP27b1 ($r=0.08$, $p=0.3$) or CYP24 ($r= -0.03$, $p=0.7$) mRNA levels (Figure 3.2B,D).

3.4.2 Genetic Loci Controlling Serum 25(OH)D

A significant line effect influencing serum 25(OH)D was seen in the BXD RI panel ($p<0.0001$). The main effect of diet was not significant ($p=0.2$), but a significant line-by-diet interaction ($p<0.0001$) was observed for serum 25(OH)D (Figure 3.3A for Z

scores). The narrow sense heritability (h^2) of 25(OH)D was 0.40 and 0.45 in the 0.5% and 0.25% Ca diet groups, respectively. When significant QTL mapping results from all analyses were considered together, 13 QTLs were identified (Figure 3.4A, Table 3.1). A detailed summary of the test statistics for each analysis can be found in Table 3.2.

Two 25(OH)D QTLs were selected for in-depth bioinformatics analysis: *25D2* (chr 1, 95.4 cM) and *25D4* (chr 6, 28.2 cM). Table 3.3 lists the characteristics of the 1-LOD support interval for each of these QTL. The number of genome features remaining after IBD filtering was 14 and 50 for *25D2* and *25D4*, respectively. Further classification of functional polymorphisms at each locus is given in Table 3.4. Genes contained within the 1-LOD support interval are listed in Table 3.5.

3.4.3 Genetic Loci Controlling Serum 1,25$(OH)_2$D and Its Adaptation to Low Ca Intake

Serum 1,25$(OH)_2$D was significantly affected by line and diet main effects ($p<0.0001$) as well as a line-by-diet interaction ($p=0.0002$) in the BXD RI panel (see Z-scores in Figure 3.3B). Serum 1,25$(OH)_2$D had a narrow-sense heritability (h^2) of 0.66 and 0.65 in the 0.5% and 0.25% Ca diet groups, respectively. The adaptation of serum 1,25$(OH)_2$D to low Ca intake was significantly affected by line ($p<0.0001$, Figure 3.3C), and had a heritability of 0.59.

Twelve QTLs for serum 1,25$(OH)_2$D were significant in at least one mapping method and one population (Table 3.1, Figure 3.4B). Two of these loci were significant for the adaptation of 1,25$(OH)_2$D to a low Ca diet: *125D10* (chr 10, 61.7 cM) and *125D16* (chr 18, 25.0 cM) (Table 3.1, Figure 3.4B, see Table 3.6 for detailed test statistics for each QTL). Five loci were selected for in-depth, bioinformatic investigation

based on concordance between analyses and association with the adaptation parameter: *125D7*, *125D10*, *125D15*, *125D16*, and *125D17*. 1-LOD candidate regions of these 5 QTLs were characterized by the number of genome features remaining after IBD filtering, polymorphisms, and HSS characteristics (Table 3.3). The number of genome features remaining in *125D7*, *125D10*, *125D15*, *125D16*, and *125D17* after IBD filtering was 9, 11, 18, 36, and 17, respectively. Further classification of functional polymorphisms at each locus is given in Table 3.4. Genes contained within the 1-LOD support interval are listed in Table 3.7.

3.5 Discussion

Although vitamin D has attracted attention as a novel contributor to many health outcomes, strong associations between vitamin D status and health outcomes are often difficult because of the complex milieu of factors influencing vitamin D metabolite levels in a free living population. The genetic factors influencing the status marker, 25(OH)D, and the active hormone, $1,25(OH)_2D$, are poorly understood. We have used genetically variable mouse models to show that serum 25(OH)D and $1,25(OH)_2D$ levels are significantly affected by genetic background in a diverse population of mice. Variation in serum $1,25(OH)_2D$ was not explained by variation in the enzymes that control its activation and degradation, CYP24 and CYP27b1. The heritability of 25(OH)D in human populations is widely variable (23-80%) due to the effects of environmental factors, mainly UV exposure.[6,7,13,14,33,34] Heritability of 25(OH)D in our BXD RI panel fell within this range at 40-45%. Serum $1,25(OH)_2D$ has also been shown to be heritable in several human populations; 16-20% in Hispanic Americans, 48% in African

Americans, and 30% in European populations.[13,14] Heritability of serum $1,25(OH)_2D$ in our BXD RI panel was slightly higher, 65-66%, likely due to the ability of our experimental model to distill the effect of genetics by tightly controlling environmental factors. Additionally, our experimental model allowed us to test for a gene-by-diet interaction. Individuals within each BXD RI line serve as biological replicates, allowing us to test how natural genetic variation affects the adaptation of $1,25(OH)_2D$ to habitual low Ca intake. We found that the low dietary Ca adaptation of $1,25(OH)_2D$ was significantly affected by genetic background in both the 11 inbred mouse lines and the BXD RI panel (h^2= 59%). This forward genetic study represents the first characterization of the effect of natural genetic variation on serum $1,25(OH)_2D$ and its adaptation to habitual low Ca intake under controlled conditions.

Our data demonstrate that the genetic control of serum vitamin D metabolites is multi-focal and complex. Both 25(OH)D and $1,25(OH)_2D$ mapped to multiple, separate locations in the BXD RI panel (13 and 12 loci, respectively). The majority of genetic loci identified are unique to each metabolite and indicate independent controls on the serum levels of each metabolite. This observation is consistent with the traditional viewpoint that $1,25(OH)_2D$ is highly sensitive to physiological cues and subject to multiple points of regulation (e.g. habitual low dietary Ca intake [35]) while 25(OH)D is not. In addition, the adaptive response of $1,25(OH)_2D$ to low Ca intake also mapped to unique loci, suggesting that genetic controls on the adaptive capability of an individual are independent of the factors controlling baseline levels.

The loci affecting 25(OH)D in this BXD RI panel do not contain the genes whose natural variants have been associated with vitamin D status in humans in either candidate

gene studies, (i.e. the vitamin D binding protein (GC) and the 25-hydroxylase, CYP2R1 [13,36]) or genome wide association studies (GWAS) (i.e. GC, 7-dehydrocholesterol reductase (DHCR7), acyl-Coenzyme A dehydrogenase (ACADSB), and CYP2R1.[37] Both DHCR7 and ACADSB are involved in cholesterol metabolism and *de novo* synthesis of vitamin D from cholesterol upon exposure to UV radiation.[37,38] In our study, all vitamin D was supplied in the diet and mice were shielded from UV radiation. Thus, we would not expect natural genetic variation in cholesterol metabolism genes (e.g. DHCR7 and ACADSB) to influence serum 25(OH)D in this experiment. In addition, the BXD RI panel represents just 20% of the total variation known to exist in the mouse genome.[39] Thus, although the BXD lines in this study did not harbor variants similar to those found in published human studies, other mouse crosses might. None-the-less, our linkage mapping in BXD lines has identified new loci not yet associated with vitamin D status by other methods.

One example of the unique variation we identified in the BXD RI panel is the chr 6 QTL *25D4* (chr 6, 28 cM) After examining genes within the *25D4* candidate regions for Gene Ontology (GO) terms relating to kidney filtration, lipid absorption, lipid trafficking, vesicular transport, and vitamin metabolism, pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8 (PLEKHA8 or FAPP2, 54 Mb) was identified. Expression of this gene is highly enriched in the intestine and kidney [40] and PLEKHA8 has been indicated in lipid synthesis, apical membrane trafficking in polarized kidney epithelial cells, and structural confirmation of apical tubule carriers.[41-43] These functions may impact recycling of the apical membrane proteins megalin and cubilin. Megalin and cubilin are cell surface receptors which bind

and internalize GC-bound vitamin D from the urine in the kidney.[44] We hypothesize that variation in PLEKHA8 disturbs the proper localization and internalization of the ligand-bound megalin/cubilin complex, thus altering the circulating concentration of 25(OH)D by influencing urinary 25(OH)D levels. In the BXD panel PLEKHA8 contains no non-synonymous coding polymorphisms, but does contain 5 polymorphisms within DNase1 HSS. This variation suggests that gene expression or regulation of PLEKHA8, rather than protein function, may be altered in the BXD RI panel. Future studies are needed to identify whether renal PLEKHA8 mRNA or urinary 25(OH)D levels differ across the BXD panel.

Few studies have examined the genetic architecture of serum $1,25(OH)_2D$ in a comprehensive manner. Engelman et al. found polymorphisms in GC to be associated with $1,25(OH)_2D$ levels in Hispanic and African Americans [13] while Wjst et al. found no significant loci controlling serum $1,25(OH)_2D$ in a linkage mapping analysis of a population of German families.[14]. These findings illustrate that, despite a moderate level of heritability, genetic determinants of $1,25(OH)_2D$ are difficult to identify in a mixed, free-living population. However, the BXD RI panel is ideal for investigating a physiologically complex phenotype such as serum $1,25(OH)_2D$.[19] This mouse model allows us to have complete lifetime control of environmental exposure, an impossible task in human studies. In addition, we were able to leverage a major strength of the BXD RI panel, biological replicates, in order to test for gene-by-diet interactions affecting the adaptation of serum $1,25(OH)_2D$ to habitual low Ca intake.[19] Our QTL characterization highlights a gene known to mediate $1,25(OH)_2D$ metabolism, as well as identifies novel variation that may affect $1,25(OH)_2D$ pathways.

125D15 (chr 15, 51 cM), does not contain any obvious new candidates, but the VDR gene (chr 15, 97.8Mb) is located < 1Mb from the 1-LOD region. VDR is necessary for mediating the negative feedback regulation of its ligand, $1,25(OH)_2D$. Ligand-bound VDR regulates gene expression of several genes that control vitamin D metabolism (i.e. CYP27b1, CYP24, and PTH).[45-48] No predicted deleterious, non-synonymous amino acid substitutions or other coding region polymorphisms were found in the VDR between B6 and DBA. However, polymorphisms were found in potential regulatory regions controlling transcription of the VDR gene. Previously, Zella et al. used osteoblasts to identify seven enhancer regions within and around the VDR gene [49]: 5 within the VDR gene (S1, S2, S4, and S5 are intronic, S3 spans exon 2), 1 at the proximal promoter (PP), and 1 approximately 7 kb upstream of the VDR transcription start site (U1).[49] Using ENCODE data, 20 DNase1 HSS regions were found within the enhancer regions identified by Zella et al.[49] Ten polymorphisms were found in these HSS: 3 within the S1 region, 1 within the S3 region, 4 within the S5 region, 1 within the PP region, and 1 within the U1 region. These polymorphisms may affect VDR gene expression by altering binding of transcription factors to regulatory sequences within the enhancer regions. Previous data from our group shows that duodenal VDR mRNA level does not differ between B6 and DBA mice [18]. However, lack of differential duodenal VDR expression does not eliminate the possibility of variability of VDR gene expression in other vitamin D target tissues. The global transcription factor profile, and therefore gene expression profile, varies by tissue.[50] Each VDR enhancer region identified by Zella et al. in osteoblasts was shown to be targeted by specific transcription factors.[49] However, our group has evaluated the VDR enhancer regions in colon epithelial cells and found only 3

to be active; S3, PP, and U1 (Fleet lab, unpublished data). Variation in the BXD RI panel may influence the complex and tissue specific gene expression of VDR and, downstream, serum $1,25(OH)_2D$ levels.

Other QTLs linked to serum $1,25(OH)_2D$ in this study represent novel variation. For example, *125D7* (chr 9, 18 cM) was highly significant across all of our analyses. Within the *125D7* candidate region is the gene encoding the transcription factor E26 avian leukemia oncogene 1 (Ets1). Ets1 has the potential to impact the $1,25(OH)_2D$ negative feedback mechanisms mediated through $1,25(OH)_2D$-target genes CYP24 and FGF23, whose protein products inactivate and inhibit synthesis of $1,25(OH)_2D$, respectively.[51-53] Previous studies from our group and others have identified Ets1 as a cofactor necessary for maximal $1,25(OH)_2D$-induced, transcription of the CYP24 gene.[54,55] Additionally, conserved Ets1 binding sites in the FGF23 proximal promoter suggest that Ets1 may upregulate FGF23 gene expression.[56] Thus, similar to the classic feedback regulation of $1,25(OH)_2D$, Ets1 is induced by $1,25(OH)_2D$ at the gene transcription level and then acts indirectly to down-regulate $1,25(OH)_2D$ serum levels. Others have shown that $1,25(OH)_2D$ treatment increases gene expression Ets1 in rat osteogenic sarcoma cells.[56] The Ets1 gene is highly polymorphic the BXD RI panel. It contains 120 polymorphisms that fall within DNase1 HSS located either upstream or within introns. Future studies will be necessary to determine whether these variants influence Ets1 gene regulation.

125D17 (chr 18, 49 cM) was highly significant in all analyses of serum $1,25(OH)_2D$ concentration. Examination of GO terms within the *125D17* candidate region identified RAB27b (member RAS oncogene family) as an interesting candidate.

In the BXD RI panel Rab27b contains 6 intronic HSS polymorphisms and two 3' mRNA untranslated region polymorphisms which may influence its regulation and gene expression. Rab27b is involved in the fusion of secretory granules with the plasma membrane of secretory cell types.[57] In addition it is important for apical membrane trafficking in polarized epithelial cells.[57] Parathyroid hormone (PTH), a major regulator of $1,25(OH)_2D$ serum levels, is released into the circulation via fusion of secretory granules with the apical plasma membrane of the parathyroid gland.[58] Also, localization of proteins to the apical membrane of parathyroid cells is important for signal transduction as well as for trafficking and fusion of secretory granules.[59] In addition, apical membrane trafficking is also necessary for delivery of GC-vitamin D transport proteins (i.e. megalin and cubilin) to the apical plasma membrane of the kidney. Unfortunately, expression of Rab27b in the kidney is low and it has not been evaluated in the parathyroid gland. As a result, the role of Rab27b in PTH release or renal vitamin D metabolite handling is unknown.

In summary, using a mouse genetics approach we have identified considerable genetic diversity controlling serum vitamin D metabolite levels. Our study is the first to examine the genetic architecture of serum $1,25(OH)_2D$ in a controlled, genetically diverse animal model. Our data show that genetic variation controlling serum $1,25(OH)_2D$ concentrations is multifocal and complex, independent from the variation controlling serum 25(OH)D levels, and independent from the variation controlling adaptation of $1,25(OH)_2D$ to a low Ca diet. Although we have not been able to definitively identify the causal variants affecting serum vitamin D metabolite levels, we have identified several plausible candidate genes for future study. The genetic loci identified in this study

serve as a starting point to identify novel pathway members or gene functions that will expand our knowledge of vitamin D physiology and pave the way for personalized health recommendations to better manage vitamin D status.

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Table 3.1 Significant QTLs Identified for Serum Vitamin D Metabolites

Chr	Point Estimate (cM)	Point Estimate (Mb)	Parental Influence
25(OH)D			
1	65.3	155.0	B6
1	**95.4**	**190**	**B6**
3	34	75.9	B6
6	**28.2**	**59.1**	**B6**
6	66.2	135.3	B6
7	28.8	51.8	B6
8	63.2	119.1	B6
10	45.1	87.1	B6
11	11.8	19.4	B6
13	14.4	36.0	B6
15	39.4	82.9	B6
17	46.1	75.3	B6
X	4.4	9.1	B6
$1,25(OH)_2D$			
1	16.8	38.5	B6
2	13.2	19.0	DBA
3	33.7	74.5	B6
7	45.6	87.4	B6
9	**18.1**	**33.6**	**B6**
9	55.8	103.8	DBA
10	4.7	12.7	B6
10	**61.7**	**114.6**	**DBA**
13	53.8	102.4	B6
15	**51.3**	**96.0**	**DBA**
18	**25.0**	**47.2**	**DBA**
18	**48.8**	**73.4**	**B6**

QTLs in bold are prioritized for candidate region characterization

Table 3.2 Test Statistics for 25(OH)D QTLs Across Analyses

Chr	Peak (cM)	Analyses Represented					
		0.5% Ca		0.25% Ca		Full[1]	
		C[2]	B[3]	C	B	C	B
1	65.3		10				
1	**95.4**	**4**	**72**	**5**		**8**	**23**
3	34		4		5		10
6	**28.2**	**5**	**7**				**7**
6	66.2		2				6
7	28.8				4		
8	63.2				4		
10	45.1				3		4
11	11.8						12
13	14.4			6	15		11
15	39.4			4			5
17	46.1				7		
X	4.4					4	

[1] "Full" denotes the full combined diet population

[2] CIM (C) results are given in LOD, significance determined at 3.1 LOD for adaptation, 3.8 LOD for all other analyses. LOD >2 is considered putative

[3] Bayesian (B) results are given in 2lnBF, significance determined at 2lnBF>6, putative 2lnBF>2

Table 3.3 Characteristics of Vitamin D Metabolite QTL Candidate Regions

QTL ID	Chr	1-LOD Candidate Region (Mb)	Total Polymorph.[1]	Total Genome Features[2]	Protein-Coding Genes	Functional Polymorph.[3]	Dnase1 HSS Peaks	Polymorph. in Dnase1 HSS	Polymorph. in Dnase1 HSS associated with genes
25(OH)D									
25D2	1	188.9-190.7	3971	14	7	8	546	215	100
25D4	6	54.6-68.5	6690	50	32	5	968	328	183
1,25(OH)$_2$D									
125D7	9	32.3-34.4	18628	9	6	1	496	750	287
125D10	10	111.7-114.9	3902	11	8	1	204	110	16
125D15	15	95.6-97.1	4123	18	8	5	642	345	202
125D16	18	45.2-48.9	8915	36	19	7	826	323	142
125D17	18	69.2-73.5	9298	17	7	9	613	258	172

[1] Polymorphisms

[2] Genome features include; protein-coding genes, non-coding RNA genes, gene fragments, and unclassified genes

[3] The Functional Polymorphism category contains non-synonymous, splice site, frameshift, and stop site polymorphisms

Table 3.4 Classification of Polymorphisms in Vitamin D Metabolite QTLs

QTL ID	Chr	1-LOD Candidate Region (Mb)	5kb Promoter[1]	Intronic	Splice Site Acceptor	Exon-associated Polymorphisms: Synonymous Codon	Non-Synonymous Codon	Gain of Stop Codon	Frameshift	5' UTR	3' UTR
25(OH)D											
25D2	1	188.9-190.7	160	1056	0	15	7	1	0	3	38
25D4	6	54.6-68.5	310	3120	0	8	5	0	0	6	36
1,25(OH)$_2$D											
125D7	9	32.3-34.4	365	2508	0	10	1	0	0	4	67
125D10	10	111.7-114.9	96	2293	0	2	1	0	0	0	27
125D15	15	95.6-97.1	277	1616	1	8	5	0	0	6	72
125D16	18	45.2-48.9	410	3309	0	13	7	0	0	0	20
125D17	18	69.2-73.5	188	3849	0	6	7	1	1	10	20

[1]Numbers indicate total number of polymorphisms per category in each QTL candidate region

Table 3.5 Candidate Genes Remaining for 25(OH)D QTLs after IBD Filtering

25D2		*25D4*				
n-Tn3	Gm24092	Fkbp14*	**Crhr2***	Gm3793*	Vmn1r13	Gm20158
Kctd3*[1]	**Cenpf***	Plekha8*	Gm25963	Vmn1r4	Mmrn1	9630021D06Rik[#]
Kcnk2*	**Ptpn14***	Gm15863	Inmt*	Vmn1r5	A730020E08Rik*	Gm25205
2900042K21Rik[#]	Smyd2*	2410066E13Rik*	**Fam188b***	Vmn1r6[#]	Ccser1*	**Atoh1***
Gm3837	Prox1*	2610209C05Rik*	Gm25458	Vmn1r7[#]	B230204D01Rik[#]	C530040J15Rik
A430027H14Rik	Gm17566*	Znrf2*	Aqp1	Vmn1r8	A730075L09Rik[#]	2610300M13Rik[#]
Gm2149	Gm23153	Nod1*	Ghrhr*	Vmn1r9[#]	Gm24645	Mad2l1[#]
		Gm24230*	6430584L05Rik*	Vmn1r10	Gm22212[#]	Smarcad1*
		Ggct*	Adcyap1r1*	Vmn1r11	9330118I20Rik	Vmn1r32[#]
		Gars*	Nt5c3*	Vmn1r12[#]	Grid2*	Fkbp9

Genes are listed in ascending order of 5' top strand start site bp location (given by the Mouse Genome Informatics Database)

[1]Genes containing at least one functional polymorphism are in bold

*indicates the presence of a polymorphism within a DNase1 HSS within the gene, #indicates a gene with no polymorphisms

Table 3.6 Test Statistics for Significant 1,25$(OH)_2$D QTLs Across Analyses

Chr	Peak (cM)	Analyses Represented						
		0.5% Ca		0.25% Ca		Full[1]		Adaptation[2]
		C[3]	B[4]	C	B	C	B	C
1	17	2	3	2	2		72	
2	13						6	
3	34	5	72					
7	46			2	72		72	
9	**18**	**13**	**72**	**11**	**72**	**16**	**72**	
9	56						8	
10	5				13		72	
10	**62**							**4**
13	54	5	72			2		
15	**51**		**6**	**8**	**72**	**10**	**72**	
18	**25**							**4**
18	**49**	**6**	**72**	**6**	**13**	**9**	**72**	

[1]"Full" denotes the full combined diet population

[2] "Adaptation" denotes the calculated adaptation to a low Ca diet

[3] CIM results are given in LOD, significance determined at 3.1 LOD for adaptation, 3.8 LOD for all other analyses

[4] Bayesian results are given in 2lnBF, significance determined at 2lnBF>6

Table 3.7 Candidate Genes Remaining for Five 1,25$(OH)_2$D QTLs after IBD Filtering

125D7	*125D10*	*125D15*	*125D16*		*125D17*
Kcnj5*	Gm20758*	**Dbx2**	Kcnn2	Gm3734#	Tcf4*
Kcnj1*	Krr1	Gm6961*	A330093E20Rik*	Cdo1*	6030446J10Rik*
Fli1*[1]	Glipr1*	A130051J06Rik*	Gm26262	Atg12	Gm24845*
Gm22060	Glipr1l1	Ano6*	Trim36*	Ap3s1	4732423E21Rik*
Ets1*	Glipr1l2	Gm17546*	1700018A14Rik	A430019L02Rik	9630026C02Rik*
Gm3331*	Glipr1l3	Gm25070*	**Pggt1b***	**4833403I15Rik***	**Gm20343***
Gm21540*	**Caps2**	3110045A19Rik*	Gm23914	Gm23610	Gm22508*
Gm25439	Kcnc2	D030018L15Rik	Ccdc112*	Arl14epl	Ccdc68*
7630403G23Rik*	Trhde*	2610037D02Rik#	n-Tt10	**Commd10***	1700061H18Rik*
	4930473D10Rik#	4833422M21Rik*	4930415P13Rik*	Gm22791	4930448D08Rik#
	Gm15723#	E330033B04Rik#	Gm4107*	Hspe1-rs1*	Rab27b*
		Arid2*	Gm24617#	Gm25036*	**Poli***
		Gm25397	Mospd4#	**Sema6a***	**Mbd2***
		Scaf11*	Gm24076	9130209A04Rik	C230075M21Rik*
		Slc38a1*	Fem1c*	Gm5095*	**Dcc***
		Gm22045*	Ticam2*	Gm4146*	5730478J17Rik*
		Slc38a2*	Tmed7*	G630055G22Rik	Gm19825
		Slc38a4*	Eif1a*	Gm5506#	

Genes are listed in ascending order of start site bp location (given by the Mouse Genome Informatics Database)

[1]Genes containing at least one functional polymorphism are in bold

*indicates the presence of a polymorphism within a DNase1 HSS within the gene, #indicates a gene with no polymorphisms

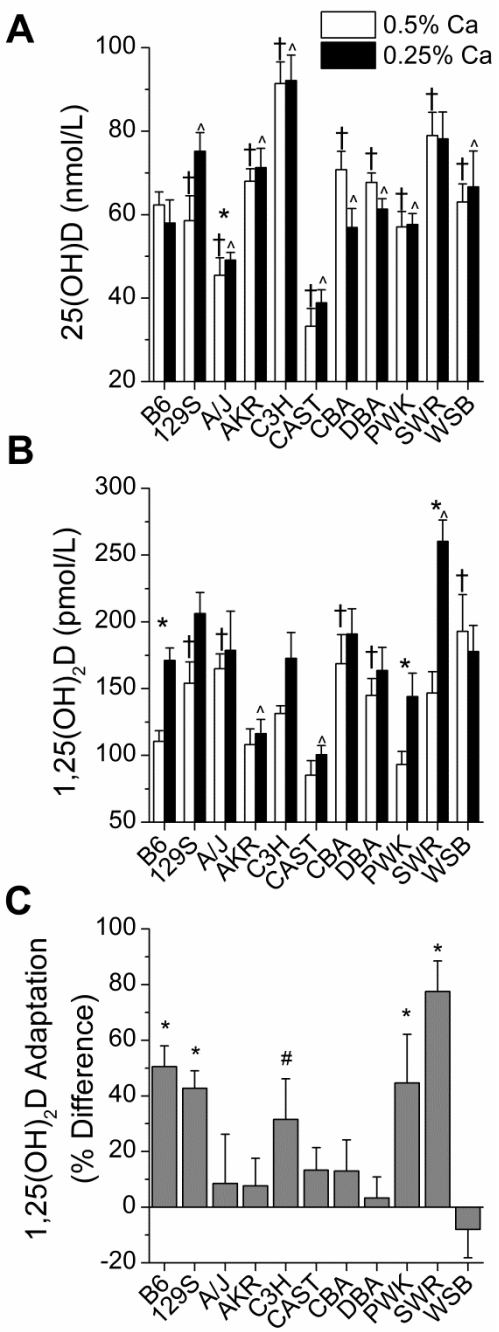

Figure 3.1 Serum vitamin D metabolites and the low dietary Ca adaptation of $1,25(OH)_2D$ are variable in a genetically diverse population of 11 inbred mouse lines. Bars reflect the mean ±SEM (n=6-20 per diet per line). (A) Unadjusted serum 25(OH)D values, *dietary groups within a line differ significantly; line mean differs significantly relative to the B6 reference line, † for the 0.5% Ca group, ^ for the 0.25% Ca group ($p<0.05$) (B) Unadjusted serum $1,25(OH)_2D$ values, *dietary groups within a line differ significantly; line mean differs significantly relative to the B6 reference line, † for the 0.5% Ca group, ^ for the 0.25% Ca group ($p<0.05$) (C) Adaptation of $1,25(OH)_2D$ to low dietary Ca intake; adaptation significantly differs from 0 (*, $p<0.05$; #, $p<0.1$).

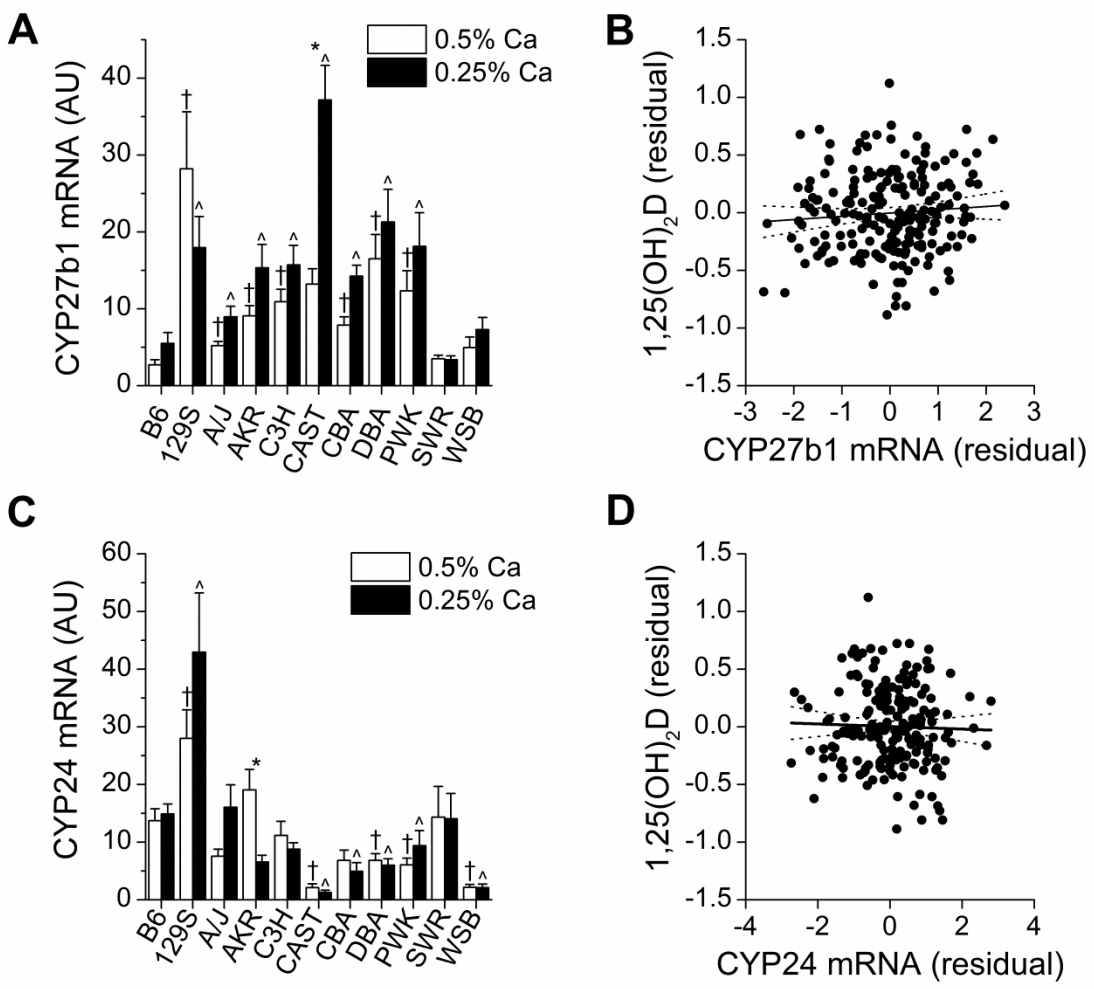

Figure 3.2 CYP27b1 and CYP24 enzymes are variable in a diverse population, but do not correlate to serum $1,25(OH)_2D$. (A&C) CYP27b1 and CYP24 mRNA levels (arbitrary unit means ±SEM), *dietary groups within a line differ significantly; line mean differs significantly relative to the B6 reference line, † for the 0.5% Ca group, ^ for the 0.25% Ca group ($p<0.05$, n=6-20 per diet per line) (B&D) Correlation of CYP27b1 and CYP24 to serum $1,25(OH)_2D$. Solid line = regression line, 95% confidence interval = dotted line. n=204 and 202, respectively.

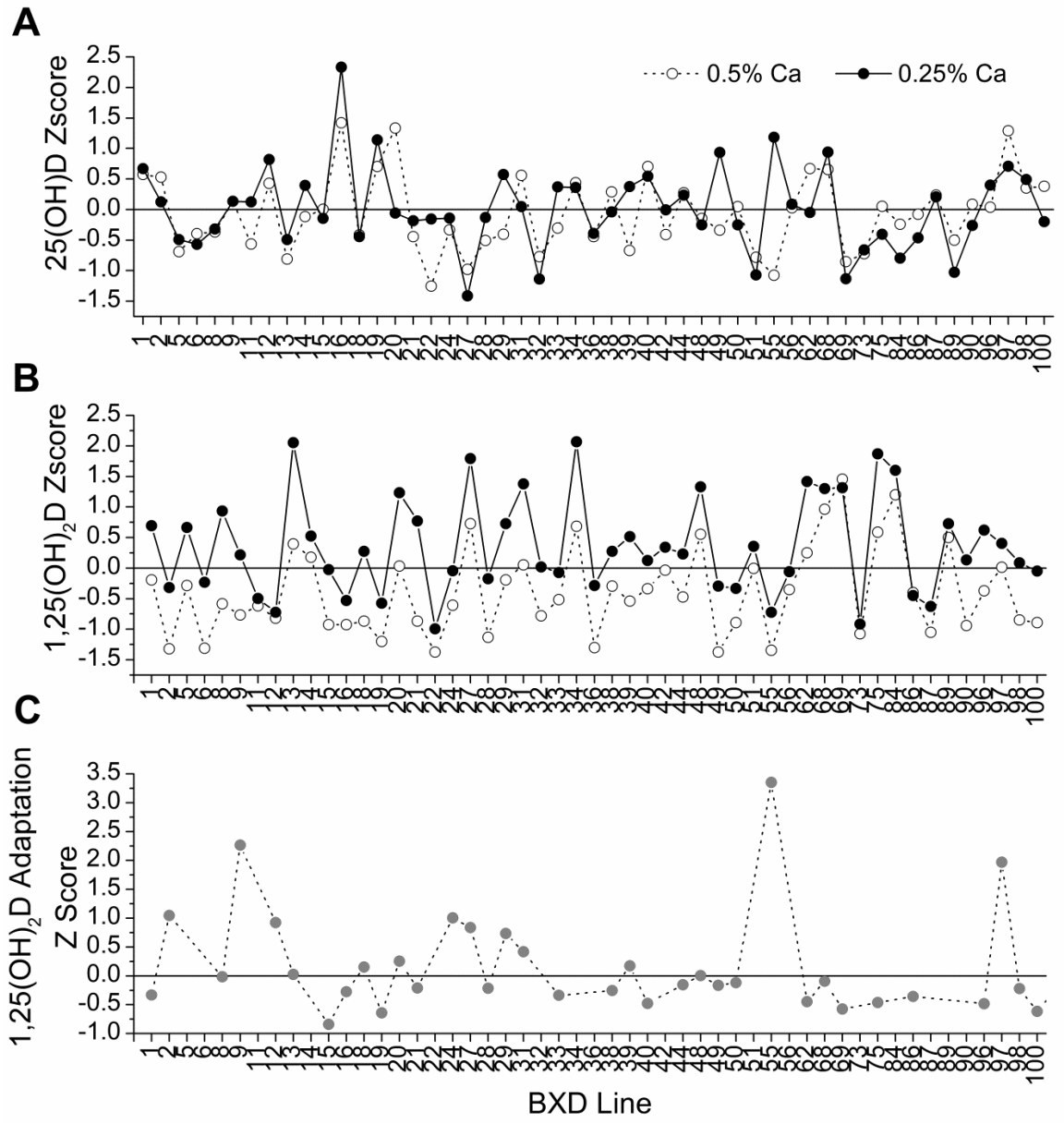

Figure 3.3 Serum vitamin D metabolites and the low dietary Ca adaptation of $1,25(OH)_2D$ are genetically variable in the BXD RI panel. Z-scores were calculated for each BXD line mean on each diet population in reference to the combined population mean for (A) serum 25(OH)D, (B) $1,25(OH)_2D$, and (C) low dietary Ca adaptation of $1,25(OH)_2D$.

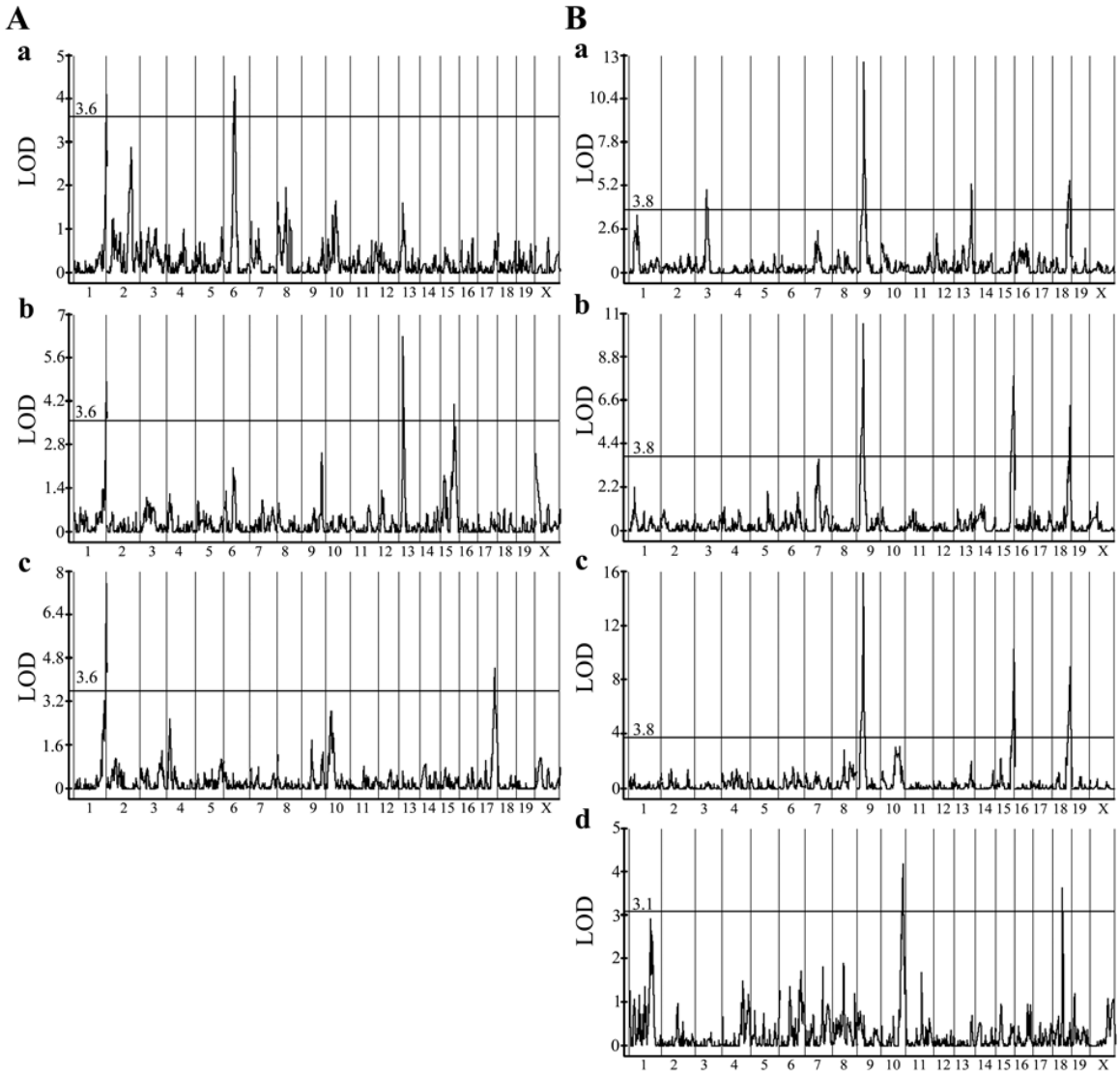

Figure 3.4 Composite Interval Mapping (CIM) identified multiple QTLs for 25(OH)D and 1,25$(OH)_2$D. (A) 25(OH)D CIM results for (a) 0.5% Ca, (b) 0.25% Ca, and (c) Full combined diet population. (B) 1,25$(OH)_2$D CIM results for (a) 0.5% Ca, (b) 0.25% Ca, and (c) Full combined diet population, and (d) Adaptation to a low Ca diet. Significance was determined separately for each data set by permutation (n=500), LOD cutoff shown as solid horizontal line.

CHAPTER 4. NATURAL GENETIC VARIATION AFFECTING INTESTINAL CALCIUM ABSORPTION AND ITS RESPONSE TO DIETARY CALCIUM RESTRICTION IN MICE

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Keywords

Calcium absorption; gene by diet interaction; genetics; QTL; dietary calcium

4.1 Abstract

Intestinal calcium (Ca) is a vital component of the three-tissue axis (i.e. intestine, kidney, and bone) that maintains serum Ca homeostasis, particularly in times of low dietary Ca intake. Natural genetic variation has been indicated to play a role in Ca absorption efficiency and its adaptation to a low Ca diet, but the genetic factors controlling this gene-by-diet interaction are not known. We measured Ca absorption in male mice from 51 BXD recombinant inbred (RI) lines that were fed a normal (0.5%) or low (0.25%) Ca diet from 4-12 wks of age (n=8 per diet per line). We used these data to map quantitative trait loci (QTLs) controlling Ca absorption efficiency as well as its adaptation to a low Ca diet. We found that Ca absorption is genetically variable in the BXD RI panel with narrow sense heritability (h^2) of 0.37 and 0.4 in the 0.5% and 0.25% Ca groups, respectively. Ca absorption efficiency mapped to 12 QTLs in the mouse genome. Diet-induced adaptation of Ca absorption ranged from -36% to 124% (h^2=0.36). The three QTLs linked to diet-induced adaptation of Ca absorption did not overlap the twelve QTLs linked to Ca absorption efficiency on one or more diets. Thus, genetic control of diet-induced adaptation of Ca absorption is independent of baseline efficiency. No known Ca absorption genes are found in these QTLs. These results point to novel genetic variation that will further our knowledge of Ca absorption physiology.

4.2 Introduction

Intestinal calcium (Ca) absorption is critical for maintaining whole-body Ca homeostasis and protecting bone mass.[1] Ca absorption is positively correlated to bone density in mice and humans.[2-4] Higher Ca absorption efficiency during growth leads to higher bone deposition and decreased Ca absorption efficiency in adulthood is associated with increased fracture risk.[5-7] Regulation of Ca absorption is sensitive to both genetic and environmental (e.g. diet) influences, but the genetic factors affecting Ca absorption, as well as its response to diet, are not well defined.

Ca absorption occurs through both a passive, paracellular component and an active, saturable transcellular pathway that is regulated in response to dietary Ca intake. During periods of habitual low dietary Ca intake serum Ca concentration drops, signaling release of parathyroid hormone (PTH) which upregulates the conversion of serum serum 25 hydroxyvitamin D (25OHD) to the active hormone 1,25 dihydroxyvitamin D ($1,25(OH)_2D$). Genomic action of $1,25(OH)_2D$, through binding to the vitamin D receptor (VDR), increases gene expression of the factors that mediate the facilitated diffusion model of intestinal Ca absorption.[8] This model consists of Ca transport into the enterocyte by apical membrane channel transient receptor potential vanilloid member 6 (TRPV6), movement of Ca across the cell bound to calbindin D9k (CaBPD9k), and extrusion across the basolateral membrane by the ATP dependent-plasma membrane Ca pump, PMCA1b.[8] However, recent knockout models of TRPV6 and CaBPD9k indicate that the facilitated diffusion model is not the only mechanisms by which Ca absorption adapts to a low Ca diet.[9]

Wide variation in Ca absorption efficiency (7-75%) has been observed in human populations.[10-12] A large portion of this variation is likely due to natural genetic variation because environmental factors account for, at most, 25% of the variability.[13,14] Ca absorption efficiency has also been shown to differ between racial groups, a surrogate of genetic background.[6,15] Similarly, racial differences exist in the adaptive response of Ca absorption to low dietary Ca intake and relationship to serum $1,25(OH)_2D$.[10,15,16] Our group has previously characterized the significant genetic diversity of Ca absorption and its response to a low Ca diet present in a diverse population of mice.[4] We now build on these observations using the BXD RI panel to map specific genomic regions (i.e. quantitative trait loci, QTLs) controlling intestinal Ca absorption efficiency and its adaptation to a low Ca diet. This is the first study to map QTLs linked to Ca absorption under controlled and physiologically relevant conditions.

4.3 Materials and Methods

4.3.1 Animal Models

The BXD recombinant inbred (RI) panel was used in this forward genetic linkage mapping study to identify QTL influencing intestinal Ca absorption efficiency. In the BXD RI panel, each line is a unique recombination of the C57BL/6J (B6) and DBA/2J (DBA) parental genomes, but because each BXD line is inbred, each individual within a line is genetically identical.[17] This advantage allows for biological replicates to test an environmental intervention such as diet.[17]

4.3.2 Experimental Design

Using the study design reported previously[4], male mice from the 51 BXD RI lines available from The Jackson Labs (Bar Harbor, ME) were obtained at 4 wks of age. At arrival, an equal number of mice from each line were randomly assigned to either a 0.5% Ca (adequate) or 0.25% Ca (low) diet (AIN93G base with 200 IU vitamin D3/kg diet, Research Diets, New Brunswick, NJ) (n=8/diet/line). Dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca) or elicit an adaptive response to low Ca intake in serum $1,25(OH)_2D$ (0.25% Ca). Mice were maintained in a UV free environment (12 h light/dark cycle) and given food and water ad libitum. At 12 wks of age mice were fasted overnight, anesthetized with an intraperitoneal injection of ketamine and xylazine, and Ca absorption measured by Ca^{45} radioisotope appearance in the serum 10 min after an oral gavage test, as previously described.[4,18] All animal experiments were approved by the Purdue University Animal Care and Use Committee.

4.3.3 Statistical Analysis

Data points with a z score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. Ca absorption was normalized using a natural log transformation and adherence to a normal distribution confirmed using the Anderson-Darling test following transformation. A significant covariate effect of femur length was determined by Pearson's correlation and removed by simple linear regression. Residuals were used for ANOVA and linkage mapping.[19] ANOVA was used to test for the presence of significant main effects of genetic background (i.e. line) and diet as well as a line-by-diet interaction. An additional parameter reflecting adaptation of Ca absorption

to the low Ca diet was calculated as the percent difference between the raw phenotype value for an individual fed the 0.25% Ca diet and the raw line mean for the phenotype value from the 0.5% Ca diet, standardized to the line mean for the phenotype value from the 0.5% Ca diet, i.e.$\left[\left(0.25\%\ x_{ij} - 0.5\%\ \bar{x}_j\right)/0.5\%\ \bar{x}_j\right]$*100. ANOVA was used to determine the effect of genetic background on this parameter. Narrow-sense heritability (h^2) was calculated using the r^2 of a one-way ANOVA (main effect=line) for each diet/phenotype population. Statistics were conducted using SAS Enterprise Guide 4.2 (SAS Institute Inc., Cary, NC).

4.3.4 QTL Mapping

Marker information and BXD genotypes were downloaded from GeneNetwork (http://www.genenetwork.org/genotypes/BXD.geno) and the genetic location of each marker was updated using the Mouse Map Converter tool at the Jackson Labs Center for Genome Dynamics (http://cgd.jax.org/mousemapconverter/).[20] Markers with duplicate genetic locations or perfectly correlated genotypes in our panel of BXD lines were removed. The final genetic map for the 51 lines contained 1558 markers (the list is available on request).

Composite interval mapping (CIM) was conducted using Windows QTL Cartographer v2.5_011 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) with RI line means (n=51). Forward selection identified 5 significant background markers. CIM was carried out using a Haldane map function, 2 cM walking speed, and a 10 cM window. Each diet (0.5% or 0.25% Ca) group and the adaptation to low dietary Ca ($1,25(OH)_2D$ only) were mapped separately. Permutations (n=500) were used to determine

significance for each analysis.[21] A Bayesian QTL mapping method using individual animal values was also used to validate CIM findings. This method, as described in Zhang et al., accurately detects multiple QTL in a study with a large number of marker effects (p) and small sample size (n).[22] Bayes factors were used as a measure of significance for the presence of a QTL. Specifically, twice the natural log of the Bayes factor (2lnBF) was used as a test statistic because it scales similarly to an LOD score.[23,24] The strength of the 2lnBF test statistic was assessed according to the rubric defined by Kass and Raftery; "0-2 not worth a bare mention, 2-6 positive, 6-10 strong, >10 very strong".[23] Each diet population was mapped separately as well as included together in a full model to test the genetic main effect. The use of these two QTL mapping methods allowed us to test for QTLs within the traditional framework, but still leverage our study design which contained biological replicates. Results that were replicated among the analyses lent more confidence to the outcome and were prioritized for further study.

4.3.5 Bioinformatic Characterization

The QTL candidate region was defined using 1-LOD support intervals which approximate 95% confidence intervals.[25,26] QTL candidate regions were populated with genome features including protein-coding genes, non-coding RNA genes, gene fragments, and unclassified genes from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/).[27] Genome-wide DNase1 hypersensitive site (HSS) data from the mouse ENCODE project was used to identify potential regulatory regions (i.e. open chromatin) within QTL regions.[28] DNase1 HSS peaks in 10 adult tissues (fat

pad, genital fat pad, heart, kidney, large intestine, liver, lung, skeletal muscle, spleen, and brain) were merged using the UCSC Genome Browser.

Gene locations were identified as the region from the first to last exon plus 5000 bp region upstream of the gene coordinates for exon 1 (i.e. the proximal promoter region). Genomic regions that were identical by decent (IBD) between B6 and DBA were identified using the Mouse Phylogeny Viewer (http://msub.csbio.unc.edu/).[29] Regions that were 100% IBD were eliminated from further consideration. Genes that were less than 100% IBD and the remaining non-IBD regions were used to query for polymorphisms between B6 and DBA using the Mouse Phenome Database (MPD, http://phenome.jax.org/). MPD annotations were used to categorize polymorphisms by gene attribute; intronic, mRNA un-translated region (5' and 3' UTR), promoter region (5000 bp upstream), and exon-associated (i.e. synonymous and non-synonymous codons, stop codons, splice sites, or frameshift mutations). HSS data was then overlaid with the polymorphisms present in the QTL region. Reference amino acids and variants resulting from non-synonymous polymorphisms were categorized based on charge, polarity, size, and special cases using the classification set out by Zhang et al with modifications.[30] Amino acids were classified into only two categories for size: small; C, P, G, A, S, T, N, D, Q, E, I, L, M, and V; and large; R, H, K, F, W, and Y. Three special cases are considered; C, P, and G. An amino acid substitution was considered deleterious if the reference and variant alleles were in a different category for any classification.

4.4 Results

4.4.1 Ca Absorption Is Genetically Variable and Affected by a Gene-by-Diet Interaction in the BXD RI Panel

Ca absorption was significantly affected by the main effect of diet ($p<0.0001$, ANOVA) in the BXD RI panel. Ca absorption was genetically variable as shown by a significant main effect of BXD line ($p<0.0001$, ANOVA). The portion of variability in Ca absorption accounted for by additive genetic effects (i.e. narrow-sense heritability, h^2) was 0.37 and 0.4 in the 0.5% and 0.25% Ca groups, respectively. A significant gene-by-diet interaction affecting Ca absorption was observed in the BXD RI panel ($p=0.0008$). This gene-by-diet interaction was investigated further by analyzing the calculated adaptation parameter. Line means of the diet-induced adaptation of Ca absorption ranged from -36% to 124% across the BXD RI panel. Low Ca adaptation of Ca absorption was significantly affected by genetic background (line main effect $p<0.0001$, ANOVA). Heritability of the adaptation of Ca absorption to a low Ca diet was estimated at 0.36.

4.4.2 Ca Absorption and Its Adaptation to a Low Ca Diet Map to Multiple, Independent Genetic Loci

Linkage mapping identified twelve QTLs that were significantly associated with Ca absorption efficiency in at least one mapping analysis conducted (Figure 4.1A-C, Table 4.1). The diet-induced adaptation parameter mapped to three separate loci on chr 3, 8, and 15 (Figure 4.1D, Table 4.1). Detailed test statistics for QTL analyses can be found in Table 4.2. Five QTLs were chosen for in-depth bioinformatic analysis based on

concordance among analyses and association with the adaptation parameter; *CaAbs2 (chr 4, 47cM), CaAbs5 (chr 7, 30 cM), CaAbs12 (chr3, 30 cM), CaAbs13 (chr 8, 24 cM), CaAbs14 (chr 15, 32 cM)*. The characteristics of the 1-LOD support interval for each of these QTLs are listed in Table 4.3. After IBD filtering the number of protein coding genes remaining in each region was 26, 220, 49, 67, and 57, respectively. Further classification of functional polymorphisms at each locus is given in Table 4.4. Genes contained within the 1-LOD support interval are listed in Table 4.5.

4.5 Discussion

Whole-body Ca homeostasis is maintained by absorption of dietary Ca in the intestine, reabsorption of urinary Ca by the kidney, and resorption of Ca from bone.[8] Sufficient dietary Ca intake is recommended for maintenance of bone health. However, not every study sees a clear relationship between Ca intake and bone mineral density (BMD).[31-33] Variability in intestinal Ca absorption efficiency provides a functional link between Ca intake and BMD. Intestinal Ca absorption has been shown to be the critical element in maintaining Ca homeostasis and rescuing the low bone mass seen in VDR knockout mice.[1,18] Low Ca absorption efficiency was shown to increase the fracture risk of postmenopausal women with low dietary Ca intake.[7] This study highlights the importance of the physiological adaptive increase in Ca absorption in response to a low Ca diet in bone health. Our group has previously shown that Ca absorption efficiency is significantly, positively correlated with femoral aBMD and trabecular bone volume fraction in a genetically diverse population of mice fed adequate or low Ca diets.[4] Identifying the genetic factors that affect intestinal Ca absorption efficiency and its

adaptive response to a low Ca diet is necessary for understanding the relationship between Ca intake and bone health. We have conducted the first study to estimate the genetic component of intestinal Ca absorption and map it to linked loci in the mouse genome.

Our study demonstrates that intestinal Ca absorption and its adaptation to a habitual low Ca diet are influenced by both dietary environment and natural genetic variation in the BXD RI panel. The portion of the variability in Ca absorption that is due to genetic background (i.e. narrow sense heritability, h^2) was 37% and 40% in the 0.5% and 0.25% Ca diet groups, respectively. These heritability values are similar to those for other Ca homeostasis traits measured in the BXD RI panel.(See Ch. 3 and [34]) Heritability of Ca absorption has not been estimated in human populations, but several lines of evidence suggest that genetics is a significant contributor to variability in intestinal Ca absorption efficiency in humans. First, Ca absorption efficiency is observed to be widely variable in clinical studies. Heaney et al. observed a range of 0.05-0.65 in true fractional calcium absorption (TFCA), even after adjustment for dietary calcium intake in four combined populations of postmenopausal, non-osteoporotic women.[35] Wolf et al. observed a range of 0.17-0.58 in TFCA.[13] Environmental variables (e.g. dietary components, smoking, estrogen status) account for, at most, 25% of this variation[13,14], suggesting that genetic and gene-by-environment interactions account for the remainder. Additionally, Ca absorption efficiency has been observed to vary by racial background, which can be used as a surrogate for genetic background. Specifically, fractional Ca absorption has been shown to be higher in black than white adolescent

girls.[6,16,36,37] Chinese adolescent girls were also observed to have higher Ca absorption efficiency across a range of controlled calcium intakes.[15]

The adaptation of intestinal Ca absorption to a low Ca diet was also influenced by genetics in our study, as evidenced by a significant line-by-diet interaction influencing Ca absorption in the full BXD RIL panel. Additionally, the calculated adaptation parameter was significantly variable across BXD lines and had a heritability of 36%. Variation in the response of Ca absorption to a habitual low Ca intake has also been observed between racial groups. Ca absorption assessed by balance over a range of Ca intakes indicated black adolescent girls increase Ca absorption efficiency on a low Ca diet, but white girls do not.[16] In a separate study, Asian adolescents also exhibited an increase in Ca absorption efficiency on low Ca diets.[15] The genetic sources of this gene-by-diet interaction are unknown, but could greatly increase our ability to target individual dietary interventions. Our study is the first forward genetic study to map the genetic variation controlling Ca absorption and its adaptation to a low Ca diet.

Our results indicate that genetic control of Ca absorption is multifocal and complex. Additionally, QTLs linked to diet-induced adaptation of Ca absorption did not overlap with other Ca absorption QTLs, indicating that regulation of the adaptive response is independent from regulation of baseline Ca absorption efficiency. Identifying the causative variation driving these QTLs is a long and complex process that presents several challenges.[38] It is helpful to first examine candidate genes that have previously been associated with the trait of interest, but because this is the first forward genetic mapping study of Ca absorption, no previous candidate loci data is available for

comparison. However, current models of intestinal Ca absorption provide possible *a priori* candidate genes.

Facilitated diffusion is the most widely accepted model of intestinal Ca absorption and consists of transport of Ca across the apical membrane by TRPV6, escort across the enterocyte by CaBPD9k, and extrusion across the basolateral membrane by PMCA1b.[8] The facilitated diffusion model is up-regulated by the PTH-vitamin D endocrine axis in response to a low Ca diet. Each of these genes is a target of $1,25(OH)_2D$ genomic action, mediated through the vitamin D receptor (VDR). Additionally, diffusion of Ca between enterocytes has been suggested to be regulated by tight junction proteins CLDN2 and CLDN12.[39] Others have suggested that Ca absorption is linked to glucose absorption by the L-type Ca channel Cav1.3 [40]

However, these genes are not located in, or near, the QTLs identified in this study. This finding could be due to the fact that the BXD population represents only a small portion (~20%) of the variance observed in the mouse genome.[41] Thus, the BXD RI panel does not necessarily harbor genetic variation that alters known candidate gene function or regulation. Instead, the variation inherent in the BXD RI panel that is driving Ca absorption QTLs represents novel factors or functions influencing Ca absorption.

Recent findings support the hypothesis that novel regulation of intestinal Ca absorption and its adaptation to a low Ca diet remains to be found. One such line of evidence is that current models do not explain the variation in Ca absorption observed in a diverse population mice (including the BXD parent lines).[4] For example, in a study of 11 inbred mouse lines, mRNA levels of facilitated diffusion model components were significantly related to each other and Ca absorption, but explained, at most, only 18% of

the variation in Ca absorption present in that population.[4] CLDN2, CLDN12, and Cav1.3 were not significantly related to Ca absorption efficiency in that diverse mouse population.[4] Similarly, Ca absorption efficiencies of TRPV6, CaBPD9k, and TRPV6/CaBPD9k double knockout mice were indistinguishable from wild-type mice on a high Ca diet.[9] From these observations, genes proposed to mediate intestinal Ca absorption efficiency explain a limited amount, if any, of the variation observed in this trait.

These studies also illustrate that the vitamin D endocrine axis does not fully explain the adaptation of intestinal Ca absorption to a low Ca diet. Replogle et al. showed that subpopulations existed within the diverse 11 inbred line panel, including vitamin D-independent adaptors (i.e. lines that are able to increase Ca absorption efficiency without a corresponding increase in serum $1,25(OH)_2D$) and hyper-responders (i.e. those lines exhibiting a larger diet-induced increase in Ca absorption relative to the diet-induced increase in vitamin D), indicating multiple facets exist in the regulation of Ca absorption.[4] When fed a low Ca diet, TRPV6 and TRPV6/CaBPD9k double knockout mice exhibited diminished, but not abolished, Ca absorption.[9] In the present study only one Ca absorption QTL, *CaAbs12* (chr 3, 30cM), coincided with a QTL our group had previously linked to serum $1,25(OH)_2D$ levels (*125D3*, chr 3, 34cM) in the same BXD population (see Ch. 3). This co-localization of genetic association between the two traits indicates a potential shared upstream regulator of $1,25(OH)_2D$ serum level and intestinal Ca absorption adaptation. However, the other fourteen QTLs linked to Ca absorption in this study do not track with those identified for vitamin D. Taken together, these observations indicate that genetic variation in intestinal Ca absorption is not fully

dependent on variation in its accepted primary regulator, $1,25(OH)_2D$, or the facilitated diffusion model.

Although *a priori* candidate genes and serum $1,25(OH)_2D$ do not explain the majority of QTLs identified in this study, examination of genetic mapping of related biological functions can shed light on systems biology affecting Ca absorption. Such is the case for *CaAbs2* (chr 4, 47.35 cM), a QTL highly significant in 5 out of 6 analyses for intestinal Ca absorption efficiency. Several bone-related traits map to this region of chr 4 as well. For example, femur cross-sectional area and cortical thickness mapped nearby (chr 4, 46.99 cM) in a heterogenous population derived from BALB/cJ, C3H/HeJ, B6, and DBA mice.[42] Another study linked multiple vertebral trabecular bone traits (bone volume, bone volume fraction, trabecular thickness, and trabecular number) to this region of mouse chr 4 (45.76 cM) in an F2 cross between B6 and C3H/HeJ mice.[43] These results indicate that natural genetic variation which alters Ca absorption efficiency has downstream effects on bone health. Additionally, a QTL for circulating thyroxine hormone was identified in this genomic region (chr 4, 46.99 cM) in the same heterogeneous mouse population as Volkman et al.[44] Treatment with thyroid hormones has been shown to have a synergistic effect on $1,25(OH)_2D$-stimulated intestinal Ca absorption in cultured embryonic chick small intestine.[45] Similarly, hyperthyroid hormone status has been shown to increase intestinal Ca transport in rats.[46] These observations suggest that genetic variation affecting thyroid hormone is an upstream mediator of Ca absorption.

Identifying causative variation driving novel Ca absorption QTLs presents enormous challenges. Bioinformatic analysis of QTL candidate regions in this study

indicated several functional candidates, but these were not supported by polymorphism data. Thus, we are not able to definitively identify candidate genes driving the Ca absorption QTLs in this study. Functional filtering of candidate genes included a wide variety of Gene Ontology (GO) terms, summarized by the following categories; ion homeostasis and transport, intestinal and renal integrity, intracellular trafficking, circulating factors, and regulation of gene expression. This range of functions was chosen to encompass primary functions of genes involved in Ca homeostasis (i.e. TRPV6, CaBPD9k, and VDR) and general cellular ion homeostasis. The addition of intracellular trafficking terms were included after a linkage mapping study of iron status implicated the gene Mon1a, as a novel factor influencing mineral homeostasis through intracellular trafficking.[47] Several genes were highlighted by this functional filtering including: Zdhhc13, a magnesium transporter with palmitoyl acyltransferase activity[48]; Rap2B, a Ras-like GTPase involved in the stimulation of phospholipase C-epsilon[49]; potassium channels, Kcnab1 and Kcnk9; and Fat1, a protein in the cadherin superfamily that is involved in cell-cell junctions of podocytes in the renal glomerulus[50]. However, candidate genes from GO-term filtering contain no polymorphisms that would affect protein sequence or function (i.e. non-synonymous codon, splice site, frameshift, or stop codon changes). Furthermore, with the exception of Kcnab1, the GO-term candidate genes did harbor polymorphisms within DNase1 HSS, suggesting that proximal regulation of mRNA expression of these genes is not variable in the BXD panel. However, this does not preclude the possibility that these QTLs represent distal regulatory elements of genes located outside of the support interval.[51] Further study is

required to elucidate how genetic variation in the BXD RI panel affects Ca absorption regulatory pathways.

In summary, this is the first study to examine the genetic architecture of intestinal Ca absorption efficiency. Using a genetically and environmentally controlled mouse model, we demonstrate that Ca absorption is significantly affected by genetic background. In addition, Ca absorption is influenced significantly by a gene-by-diet interaction in the BXD RI panel. The degree to which Ca absorption adapts in response to a low Ca diet is affected by genetic background, and independent from the variation controlling baseline efficiency. Our study demonstrates that there is still much unknown about genetic regulation of Ca absorption and its adaptation to a low Ca diet. The QTLs found in the BXD RI panel represent novel variation affecting Ca absorption, but we are unable to definitively identify the causal variants at this time. The QTLs identified in this study are an important first step in identifying novel factors or pathways influencing intestinal Ca absorption, and will serve as a foundation for identifying individuals who are most at-risk to the detrimental effects of habitual low Ca intake.

4.6 Acknowledgements

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Table 4.1 Significant QTLs Identified for Intestinal Ca Absorption

Chr	Point Estimate (cM)	Point Estimate (Mb)	Parental Influence
3	19.43	40.36	B6
3	**29.55**	**61.17**	**B6**
4	**47.35**	**103.10**	**DBA**
5	40.50	76.57	B6
5	77.12	139.20	DBA
6	48.01	103.49	DBA
7	**30.25**	**46.50**	**B6**
8	**23.69**	**38.00**	**B6**
8	31.43	64.09	DBA
8	66.02	120.90	DBA
9	43.70	79.17	DBA
10	64.60	115.57	B6
12	6.90	13.86	DBA
15	**32.31**	**71.81**	**B6**
17	49.98	79.56	DBA

QTLs in bold are prioritized for candidate region characterization

Table 4.2 Test Statistics for Significant Intestinal Ca Absorption QTLs Across Analyses

Chr	Peak (cM)	Analyses Represented						
		0.5% Ca		0.25% Ca		Full[1]		Adaptation[2]
		C[3]	B[4]	C	B	C	B	C
3	19				2		72	
3	30							6
4	47	8	72		21	6	12	
5	41				4		7	
5	77					4		
6	48			2		6		
7	30	4			2		72	
8	24							5
8	31	6	17					
8	66				3		11	
9	44			4				
10	65	4	7				2	
12	7	2		4				
15	32							5
17	50				23			

[1]"Full" denotes the full combined diet population

[2] "Adaptation" denotes the calculated adaptation to a low Ca diet

[3] CIM results are given in LOD, significance determined at 3.7 (0.5% Ca), 3.4 (0.25% Ca), 2.5 (adaptation), and 3.8 (full) LOD.

[4] Bayesian results are given in 2lnBF, significance determined at 2lnBF>6

Table 4.3 Characteristics of Intestinal Ca Absorption QTL Candidate Regions

QTL ID	Chr	1-LOD Candidate Region (Mb)	Total Polymorph.[1]	Total Genome Features[2]	Protein-Coding Genes	Functional Polymorph.[3]	Dnase1 HSS Peaks	Polymorph. in Dnase1 HSS	Polymorph. in Dnase1 HSS associated with genes
CaAbs2	4	100.6-104.7	5744	46	26	11	271	152	78
CaAbs5	7	41.1-49.0	20333	275	220	0	177	3	2
CaAbs12	3	58.9-66.6	12608	79	49	8	761	260	116
CaAbs13	8	36.1-47.7	31268	117	67	104	2406	2499	1677
CaAbs14	15	69.1-75.9	17052	91	57	10	1017	362	362

[1] Polymorphisms

[2] Genome features include; protein-coding genes, non-coding RNA genes, gene fragments, and unclassified genes

[3] The Functional Polymorphism category contains non-synonymous, splice site, frameshift, and stop site polymorphisms

Table 4.4 Classification of Polymorphisms in Ca Absorption QTLs

					Exon-associated Polymorphisms			
QTL ID	Chr	1-LOD Candidate Region (Mb)	5kb Promoter[1]	Intronic	Synonymous Codon	Non-Synonymous Codon	5' UTR	3' UTR
CaAbs2	4	100.6-104.7	79	208	0	0	0	0
CaAbs5	7	41.1-49.0	3	26	0	0	0	1
CaAbs12	3	58.9-66.6	306	2683	17	8	9	24
CaAbs13	8	36.1-47.7	2248	25855	129	104	42	201
CaAbs14	15	69.1-75.9	507	3785	31	10	21	36

[1]Numbers indicate total number of polymorphisms per category in each QTL candidate region

Table 4.5 Candidate Genes Remaining for Ca Absorption QTLs After IBD Filtering

CaAbs2	*CaAbs5*		*CaAbs12*			
Pde4b*	Mrgpra6#	Mrgprb13#	Serp1	4930449A18Rik	**Gpr149***	**Vmn2r6**
4921539E11Rik*	Mrgpra9	Mrgprb8#	Eif2a	Gm16527	Gm22433	Vmn2r7
4930456L15Rik*	Mrgpra1	Mrgprb1	6720482G16Rik	Gm8298#	Mme*	Kcnab1*
Oma1*	Gm25326#	Mrgprx2	Selt	C130079G13Rik	Gm24946	A330015K06Rik*
4931409D07Rik#	Mrgpra2a#	Mrgprb2#	Fam194a	Aadacl2	Plch1	A730090N16Rik*
Gm12718*	Mrgpra3	Mrgprb3#	AU022133	Aadac	Gm23500#	Ssr3*
Gm23064*	Gm22427#	Zdhhc13	Siah2	Sucnr1	Gm24227	4931440P22Rik*
Gm12720#	Mrgpra4#	Csrp3	4930593A02Rik	Mbnl1	E130311K13Rik	Gm22279
Gm25877#	Mrgprx1	E2f8*	A930028O11Rik	Gm19816#	Slc33a1*	Tiparp*
Dab1*	2700078K13Rik#	Gm2788*	Fam188b2	P2ry1	Gm20031#	Lekr1
2900034C19Rik*	Mrgprb5#	Mrgpra2b	Clrn1	B430305J03Rik	Gm26850#	Ccnl1
	Mrgprb4	Ptpn5	Gm22491	Rap2b#	Gmps	**Veph1***
			Gm10071	Gm22162*	Vmn2r1	Gm26442
			Med12l	9330121J05Rik#	Gm22165	Ptx3
			Gm19372#	Arhgef26*	Vmn2r4	
			Gm5538	**Dhx36***	Vmn2r5	

Genes are listed in ascending order of 5' top strand start site bp location (given by Mouse Genome Informatics Database)

[1]Genes containing at least one functional polymorphism are in bold

*indicates the presence of a polymorphism within a DNase1 HSS within the gene, # indicates a gene with no polymorphisms

Table 4.5 is continued on the next page.

Table 4.5 (continued) Candidate Genes Remaining for Ca Absorption QTLs After IBD Filtering

CaAbs13					*CaAbs14*	
D8Ertd82e*	9330187G09Rik	Gm26584#	AY512931#	Ufsp2#	4930573C08Rik	3100002H20Rik*
3010031K01Rik*	Gm23128	**Slc7a2***	Mtnr1a#	Ankrd37#	4930504C09Rik*	Peg13*
4933430A20Rik*	Tusc3*	**Pdgfrl***	Gm6329#	Lrp2bp#	n-Tm3#	Gm3150*
Lonrf1*	6430500C12Rik	**Mtus1***	F11#	Snx25	Gm24232	Chrac1*
Gm26150*	Gm6213*	B430010I23Rik*	Klkb1#	Gm24684#	Gm23433	Ago2*
6430573F11Rik*	**Msr1***	Gm16192*	Cyp4v3#	Gm23030#	Gm25330	Ptk2*
Dlc1*	Gm10682	Gm16193*	Fam149a	4933411K20Rik#	Gm24058	8030476L19Rik*
9530004P13Rik*	**Fgf20***	**Fgl1***	Tlr3#	Gm15634#	Gm23987	1700085D07Rik*
9430047G12Rik*	**Micu3***	Gm16348*	Gm19634#	Slc25a4#	Gm19782	Mir151
Gm19274*	**Zdhhc2***	**Pcm1***	Sorbs2	Gm23812#	**Fam135b***	Gm24787
G630064G18Rik*	Cnot7*	Asah1*	Gm23604#	Gm24669#	Gm22519#	C130079B09Rik*
AI429214*	Vps37a*	Frg1#	Gm16351#	Helt#	**Col22a1***	**Gm10362***
Gm10683	Mtmr7*	2810404M03Rik	D330022K07Rik	Acsl1	Gm16308#	Dennd3*
1700016D18Rik*	Gm2085*	Zfp353#	Gm16352#	Mlf1ip#	Gm19794	Slc45a4*
Gm25126	**Adam24***	Gm23531#	Gm25309#	Ccdc111	Gm23217	1700010B13Rik*
Sgcz*	**Adam25**	Gm9908#	Pdlim3	Casp3#	Gm24390*	Gpr20*
n-Tl14#	**Adam20**	Fat1	Ccdc110#	Gm16675#	A930009L07Rik	Ptp4a3*
Mir383	**Adam39**	Gm2366#	1700029J07Rik#	Irf2#	Kcnk9	Gm22106*
					Trappc9*	**Mroh5***
						Gm6569*

Genes are listed in ascending order of 5' top strand start site bp location (given by Mouse Genome Informatics Database)
[1]Genes containing at least one functional polymorphism are in bold
*indicates the presence of a polymorphism within a DNase1 HSS within the gene, # indicates a gene with no polymorphisms

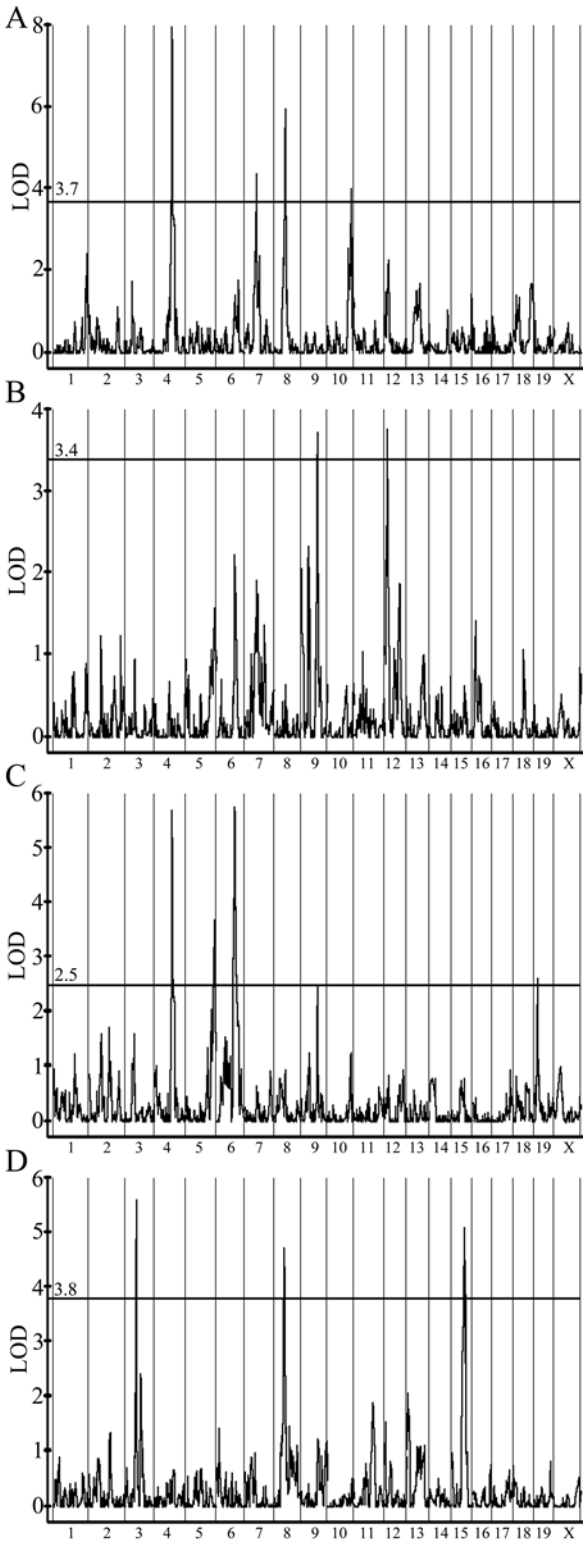

Figure 4.1 Composite Interval Mapping (CIM) identified multiple QTLs affecting intestinal Ca absorption. (A) 0.5% Ca, (B) 0.25% Ca, and (C) full combined diet population, and (D) adaptation to low Ca diet. Significance was determined separately for each data set by permutation (n=500), LOD cutoff shown as solid horizontal line.

CHAPTER 5. FUTURE DIRECTIONS

5.1 Research Summary

Calcium (Ca) is a vital nutrient that is necessary for cell signaling and structural integrity of the skeleton. Sufficient dietary Ca is recommended for maintenance of proper bone health. Intestinal Ca absorption mediates the availability of dietary Ca for these purposes and is affected by environmental (e.g. dietary Ca) and genetic factors. This dissertation has focused on characterizing the natural genetic variation affecting intestinal Ca absorption efficiency and its primary regulator, serum $1,25(OH)_2D$ under normal and low dietary Ca conditions.

The influence of genetics on Ca absorption has been suggested by observations between racial groups in clinical studies.[1,2] Serum 25(OH)D has been mapped in genome-wide association studies which pointed to regulation by genes known to function in vitamin D biology.[3] Serum $1,25(OH)_2D$, although heritable, has not been significantly linked to genetic loci in the limited studies available. These studies are limited by the fact that they cannot separate complex phenotypes such as Ca absorption and $1,25(OH)_2D$ from the myriad physiological and environmental factors that are present in free-living human populations. We have overcome this limitation by using genetically well-defined mouse models in a controlled environment.

We have clearly shown that natural genetic variation influences intestinal Ca absorption and vitamin D metabolites in these controlled animal models. Intestinal Ca absorption, serum 25(OH)D, and serum 1,25$(OH)_2$D were significantly variable across a diverse population of 11 inbred mouse lines (Ch. 2&3). Interestingly, Ca absorption efficiency was significantly correlated to femoral trabecular bone volume in the 11 inbred mouse line population (Ch. 2). These same parameters were also significantly affected by genetics in the BXD RI panel (Ch. 3&4). The heritability of serum vitamin D metabolites compared well with estimates in human populations. The study in Chapter 4 is the first to estimate the heritability of Ca absorption, and shows it is moderately heritable (h^2 = 0.37-0.4).

Another goal of this dissertation was to examine if natural genetic variation influenced the adaptation of Ca homeostasis to the common dietary insufficiency of low Ca intake. We observed that gene-by-diet interactions affect both 1,25$(OH)_2$D and Ca absorption in the 11 inbred line population as well as the BXD RI panel. This result concurs with observations between racial groups.[4,5] Adaptation to a low Ca diet of both 1,25$(OH)_2$D and Ca absorption was significantly affected by genetic background in both mouse populations studied. This is the first study to characterize the effect of genetic on 1,25$(OH)_2$D and Ca absorption using the relevant physiological condition of habitual low Ca intake.

The study of 11 inbred mouse lines in Chapter 2 highlights that gaps remain in our understanding of intestinal Ca absorption and its regulation. Regulation of intestinal Ca absorption is proposed to occur through low dietary Ca triggering an increase in serum 1,25$(OH)_2$D, which initiates genomic events that lead to increased active intestinal

Ca absorption. This model holds true in B6 mice (the standard reference inbred line) and 129S mice, but not in the other 9 lines studied. In the whole population, variation in facilitated diffusion component mRNA levels explained only a small portion of variation in Ca absorption. Additionally, adaptation of Ca absorption was not fully explained by adaptation in $1,25(OH)_2D$.(Ch. 2) These results, along with data from TRPV6 and CaBPD9k knockout mice[6], point to the existence of novel compensatory mechanisms of Ca absorption regulation.

The forward genetics studies in Ch. 3&4 address these gaps in our knowledge and highlight novel variation affecting serum $1,25(OH)_2D$ and intestinal Ca absorption. Linkage mapping results indicate that genetic control of each phenotype is multifocal and complex. The vitamin D receptor emerged as a candidate gene for one QTL affecting $1,25(OH)_2D$. Additionally, genetic regulation of each phenotype is, for the most part, independent. For example, genetic regulation of serum $1,25(OH)_2D$ was independent from that regulating serum 25(OH)D. Genetic loci linked to Ca absorption did not overlap those linked to $1,25(OH)_2D$, with one exception, indicating that genetic regulation of these two traits is independent. In both Ca absorption and $1,25(OH)_2D$, genetic regulation of the diet-induced adaptation was independent from that controlling baseline trait levels.

These studies illustrate that natural genetic variation is a significant contributor to Ca absorption physiology. We have mapped this genetic variation to specific loci in the mouse genome. All but one QTL identified in these studies represent novel genetic factors affecting Ca absorption or vitamin D metabolites. Although some promising candidates have emerged through bioinformatic analysis, further study is needed to

identify the causal variants affecting our traits of interests. Identification of the novel factors influencing Ca absorption and vitamin D metabolites will further our knowledge of these systems and inform personalized dietary recommendations in the future.

5.2 Future Directions

The studies in this dissertation have identified QTLs which affect intestinal Ca absorption and serum vitamin D metabolites. However, the causal variants driving these loci could not be definitively determined. Therefore, future studies should focus on determining and characterizing the causative genes and variants. This question could be addressed by physically narrowing the QTL region by fine-mapping in additional mouse crosses such as advanced intercross or congenic mouse lines. However, I believe further analysis of the BXD RI panel by transcriptomics and transcription factor analysis will yield functional information for candidate gene identification.

1. Identify Genetic Loci Linked to Differential Gene Expression in the BXD Panel

The majority of QTLs identified in Ch. 3&4 pointed to differences in gene regulation, rather than protein function. Consequently, transcript levels of the candidate gene should differ across the BXD panel. However, a large number of transcripts will be variable in this population but have no relationship to our phenotypes of interest, Ca absorption or serum vitamin D metabolites. It is therefore necessary to link transcript variation to genetic variation, and these results can be compared to the Ca absorption and serum vitamin D metabolite QTLs identified in this dissertation. This goal can be accomplished by using a method called expression QTL (eQTL) mapping.

eQTL mapping uses mRNA transcript levels in a target tissue as linkage mapping phenotypes. Specific, individual transcripts can be used but this requires prior knowledge of the gene affected. The majority of QTLs identified for Ca absorption and vitamin D metabolites represent novel variation, so the target transcripts are not known. Instead, I would recommend an "omic" approach that consists of measuring the transcriptome of a target tissue by microarray and mapping these results to the mouse genome. eQTL mapping results can then be compared with Ca absorption and vitamin D metabolite results in two ways. eQTLs that overlap with previous identified Ca homeostasis QTLs indicate that the target transcript may be influencing the Ca homeostatic trait of interest. Transcript values can also be correlated to the trait of interest (e.g. Ca absorption) across the BXD panel to confirm a direct relationship. Differential gene expression has been used successfully to identify candidate genes in bone-related QTLs.[7,8]

eQTL mapping has advantages that will move the identification of causal genes in the BXD RI panel forward. Primarily, eQTL mapping allows for identification of both *cis* and *trans* genetic loci affecting a given transcript level. *Cis* regulation of a gene occurs at the gene's location in the genome and may include alterations of the proximal promoter or intergenic enhancer elements. *Trans* regulation occurs at a secondary site, far away (possibly even on different chromosomes) from the target gene's coding sequence. Examples of *trans* regulation include distal regulatory elements and transcription factors. This method can also be used to assess the gene-by-diet interaction observed in Ca absorption and $1,25(OH)_2D$ by mapping the fold change of transcripts between the 0.5% and 0.25% Ca diet.

However, this method is limited by the small sample size of the BXD RI panel in relation to the large number of comparisons present in transcriptome analysis (>24,000). The choice of target tissue is also limiting. Duodenal mRNA is an intuitive choice to relate to intestinal Ca absorption, and while it will likely be informative one must not assume is will be all-inclusive. The choice of tissue for comparison to vitamin D metabolite QTLs is slightly more problematic because multiple tissues participate in vitamin D metabolism (e.g. parathyroid, liver, kidney, bone, and intestine). I would profile and map the kidney transcriptome because it is downstream of most vitamin D metabolic signals, and is therefore most likely to harbor transcript variation in candidate genes.

In summary, eQTLs represent genetic loci linked to differential gene expression of a specific transcript. Our expected results would be overlap between an eQTL and a QTL identified in previous studies for Ca absorption or serum vitamin D metabolites. With this link between a specific transcript and our trait of interest we can begin to assign biological function to the variation driving Ca homeostasis QTLs and hypothesize specific interactions and pathways influencing our trait of interest.

2. Examine Transcription Factor Binding Sites for Variation

The results of eQTL mapping show genetic loci that contain genetic variation which influences a specific transcript level. If those eQTLs co-localize in the genome and correlate to our traits of interest (i.e. “candidate eQTLs”), it gives an indication of what gene pathway may be affecting our traits of interest, but will not definitively determine the causal variation. In order to more clearly understand the mechanisms

altering candidate transcript levels, I would examine transcription factor binding sites within candidate eQTLs.

This process would begin with a detailed literature search for known transcription factors of the candidate transcript. The genome sequence underlying candidate eQTLs will be queried for the binding motifs of these transcription factors. Numerous software packages exist to retrieve binding motifs and align sequences. Alternatively, transcription factor binding sites for 119 human transcription factors have been characterized genome-wide by ChIP-seq in 72 cell types as part of the ENCODE project.[9] This dataset is valuable because it represents actual binding of transcription factors to the genome, but is limited by the selection of transcription factors and cells chosen by the investigators. Transcription factor binding motifs are highly conserved evolutionarily[9], but data gathered from human sources would of course need to be confirmed in mouse.

Following identification of transcription factor binding sites in candidate eQTLs, sequence polymorphisms between the BXD parent lines B6 and DBA will be examined. We would expect to see polymorphisms in the transcription factor binding sites which lead to dysregulation of the candidate transcript with downstream effects on our trait of interest. Ultimately, the hypotheses generated from these genomic tools and databases will need to be directly tested.

5.3 References

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