THE SITE-SPECIFIC INFLUENCE OF GENE-BY-DIET INTERACTIONS ON TRABECULAR BONE IN MALE MICE

by

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To my mom and dad

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ABBREVIATIONS

$1,25(OH)_2D_3$	1,25 dihydroxyvitamin D_3
$25(OH)D_3$	25 hydroxyvitamin D_3
B6	C57BL/6J
BMC	bone mineral content
BMD	bone mineral density
BV/TV	bone volume fraction
Ca	calcium
Chr	chromosome
CIM	composite interval mapping
DBA	DBA/2J
eQTL	expression quantitative trait locus
GxD	genetic-by-diet interactions
IBD	identity by descent
KO	knock out
LOD	logarithm of odds
LRS	likelihood ratio statistic
QTL	quantitative trait locus
RCR	physiologic response to dietary Ca restriction
RI	recombinant inbred
SNP	single nucleotide polymorphism
Tb.TMD	trabecular tissue mineral density
Tb.N	trabecular number
Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
μCT	micro-computed tomography

ABSTRACT

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Osteoporosis and fractures are debilitating skeletal problems. Accumulating the highest peak bone mass in both cortical and trabecular bone (Tb) as well as developing strong Tb microarchitecture play an integral role in preventing bone loss and osteoporotic fractures later in life. Because Tb is modulated by genetics (G) and environment (e.g. diet, D), my dissertation research focuses on the influence of dietary calcium (Ca) intake, genetics as well as GxD interaction controlling Tb phenotypes in two clinically relevant skeletal sites, i.e. the femur and the L5 vertebra. Male mice from 11 in bred lines and 51 BXD recombinant inbred (RI) lines were fed either adequate (Basal, 0.5%) or low (0.25%) Ca diets from 4-12 weeks of age. We used micro-computed tomography (μCT) to measure Tb mass and microarchitecture phenotypes. We systematically proved that there are site-specific effects of diet, genetic, and GxD interactions influencing Tb phenotypes. This indicates that there are unique genetic effects modulating Tb at each bone site. Therefore, we conducted a genetic mapping experiment using the 51 BXD RI lines separately for each bone site. We coupled genetic mapping analysis with bioinformatics analysis to identify novel genetic variation and candidate genes accounting for the variation in each phenotypes. The findings from this work serve as a foundation for future research to identify novel pathways and genes underlying the development of Tb as well as an adaptation to Ca insufficiency.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Osteoporosis is a growing public health problem worldwide. It is characterized by low bone mass, disruption of bone microarchitecture, compromised bone strength, and an increase in fracture risk.[1] In the U.S. alone, almost 10 million people have osteoporosis and more than 40 million people have low bone density.[2] Osteoporotic fractures are associated with significant morbidity, mortality, loss of independence, and financial burden.[3] Most pharmacologic treatments primarily focus on reducing bone resorption and slowing adult bone loss but they do not restore the bone that has been lost.[4] As a result, strategies that promote the development of peak bone mass so that the impact of adult bone loss is reduced become important.

Bone consists of two main compartments that coordinately support bone strength: cortical bone and trabecular (or cancellous) bone.[5] Cortical bone is the dense outer shell of bone that surrounds a central cavity where bone marrow is stored. The main function of cortical bone is structural and protective. Within the central cavity, primarily at the end of long bones, there is another skeletal compartment called *trabecular bone*. Trabecular bone (Tb) consists of connecting plates and rods of bone that give Tb a sponge-like appearance. This highly connecting microarchitecture helps distribute forces to the cortical bone, provides tissue elasticity and serves as a main site for calcium (Ca) ion exchange.[6]

Tb is the focus of my dissertation research because of its strong relevance to bone mechanical strength.[7] Several association studies in humans have shown that osteoporotic patients have lower Tb volume fraction (BV/TV) and weaker Tb microarchitecture.[8,9] Generally, osteoporotic patients have significantly thinner, more rod-like Tb and less connectivity with less axially aligned trabeculae. When compared to healthy individuals with similar BV/TV, this

structure results in compromised bone strength as reflected as reduced maximum modulus,[9] bone stiffness[8] and less uniformly distributed strain over Tb microarchitecture.[10] Thus, several lines of evidence suggest that weaker Tb is highly associated with increased risk of osteoporotic fractures.

Attaining the highest possible bone mass and strong microarchitecture is clinically relevant for preventing fractures later in life. Peak bone mass (PBM) is defined as the maximal bone mineral density that is acquired during growth and development. Depending on the skeletal site, PBM occurs around the age of 25-35 years.[11,12] Mounting evidence supports the idea that attaining high bone mass early in life is a strong predictor of low osteoporosis risk.[13-15] In support of this position, Hui et al.[16] conducted a longitudinal study in healthy white postmenopausal women and observed that for each 0.5 standard deviation increase of PBM at the radial midshaft, the lifetime fracture risk is lowered by 40%.[16] Similarly, Tabensky et al.[13] studied the resemblance of vertebral volumetric bone mineral density (vBMD) between mothers and daughters to study whether vBMD in women with fractures is caused by the abnormality in growth or aging. The authors observed that the deficit in vertebral bone accrual in the daughters of women with fractures (-0.36 SD relative to age-matched controls) was almost one-half their mothers' deficit (-0.98 SD relative to age-matched controls). This evidence suggests that developing higher PBM is likely to reduce the risk of osteoporotic fractures later in life.

Bone development is subject to both genetic and environmental influences.[17] The role of genetics in bone was first suggested by early twin studies[18,19] and family studies.[20-23] For example, Krall and Dawson-Hughes[22] measured familial resemblance of bone density of female and male members of 40 families. They reported that 46-62% of variance in bone density was attributable to heredity. However, the fact that genetics does not explain all of the variation in bone

mass suggests that bone mass is also influenced by other environmental factors as well as the interaction between genetics and extrinsic factors.[24]

Studying genetics and environmental effects independently may not allow us to fully understand the regulation of bone mass.[24] Environmental influences such as diet, physical activity, smoking, alcohol consumption and age at menarche have been reported to modulate bone mass accrual.[17,25] However, studies investigating the effect of these environmental factors are commonly conducted without the control of genetic background. To study the impact of both genetics and environment altogether, we need to classify individuals by their genetic profile. The impact of gene-by-environment (GxE) interactions has been clearly demonstrated in controlled calcium balance studies conducted in the lab of Dr. Connie Weaver. Her research showed that black adolescents have higher calcium (Ca) absorption and lower Ca excretion than their white counterparts.[26,27] As a result, black adolescents showed greater mean skeletal C retention than did whites.[26] This evidence highlights the importance of underlying genetic factors that regulate differential response to dietary Ca intake (as known as "gene-by-diet (GxD) interactions")

In this review, I will provide conceptual foundations for my dissertation research. First, I will provide an overview of the developmental processes and basic biology of bone. Second, I will summarize what is known about the individual influences of dietary factors and genetics on the acquisition of PBM and the development of trabecular microarchitecture. Third, I will discuss key findings from studies on GxD interactions affecting the acquisition of PBM. Finally, I will identify research gaps that this dissertation aims to answer.

1.2 Overview of skeletal growth, acquisition of bone mass and trabecular bone development

Skeletal development begins in the first trimester of gestation and continues to develop until the maximum bone mass is attained in late adolescence/early adulthood.[11,12,28] During this process, bones gradually change resulting in longitudinal and radial growth through the process called bone modeling where bone formation and bone resorption occur on different bone surfaces (uncoupled process). Subsequently, skeletal consolidation occurs by depositing minerals on to bone matrix. After an individual achieves his/her PBM, the bone mass is maintained through bone remodeling which is a coupling process between bone formation and bone resorption. Bones undergo constant remodeling process where bone resorption occurs, followed by bone formation on the same bone surface. In late adulthood, this balance shifts and a slow, but steady decline in bone mass occurs. As bone resorption predominates over formation in the elderly and more aggressively in postmenopausal women, a negative balance between formation and resorption leads to dramatic bone loss. This highly coordinated process relies on Ca and phosphorus (P) metabolism, multiple bone cells as well as specific signaling pathways. This section will cover these fundamental topics before we delve into how diet, genetics and GXD interactions affect bone phenotypes.

1.2.1 Calcium and bone homeostasis

Ca is an indispensable mineral for the human body as it builds teeth and bones, involves in blood clotting, nerve signaling, skeletal muscle and heart contractions, and the release of hormones and other chemicals in the body. Therefore, the body needs to maintain the serum Ca level within the normal physiologic range (8.5-10.5 mg/dL) through a multi-tissue axis, i.e. the intestine, bone, kidney and parathyroid gland. The communications at the tissue level are carried out by regulatory hormones, i.e. growth hormone (GH), sex hormones, parathyroid hormone (PTH), calcitonin and

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). GH and sex hormones regulate growth in general but also have specific functions in bone development. On the other hand, PTH, calcitonin and $1,25(OH)_2D_3$ are hormones that distinctively work to maintain bone and Ca metabolism.

At the beginning of puberty, sex hormones, i.e. estrogen and testosterone, activate the GH-IGF-I axis to stimulate longitudinal bone growth and later lead to growth plate closure at the end of puberty.[29] Girls experience these growth spurts on average 1.5 years before boys. Estrogen and testosterone stimulate the secretion of GH that leads to the activation of IGF-I to stimulate proliferation of growth plate cartilage. In addition, both estrogen and testosterone promote bone formation and suppress bone resorption.[29] Though testosterone mainly is preferentially stimulates periosteal bone expansion while estrogen inhibit periosteal expansion. As a result, girls at puberty gain cortical thickness through endosteal expansion. At the end of puberty, estrogen signaling through estrogen receptor-alpha is necessary for epiphyseal closure in both sexes. Therefore, estrogen, testosterone and GH work together to promote skeletal formation and maturation.

The body has a tightly regulated system to maintain serum Ca level. This system works through three hormones, PTH, calcitonin and $1,25(OH)_2D_3$. In chronic inadequate Ca intake, low serum ionized Ca level reduces calcium-sensor receptor (CaR) signaling and allows active PTH to be secreted from parathyroid gland.[30] PTH acts on the bone, kidney, and small intestine to regain the serum Ca homeostasis. First, PTH promotes bone resorption by indirectly stimulating the formation of bone-resorbing cell osteoclasts. PTH binds to its receptor and leads to an increased transcription of the receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL binds to its receptor RANK on osteoclasts and leads to subsequent signaling pathways that promote osteoclast formation and activity. As a result, Ca is liberated from the mineralized bone matrix, in turn, increases serum Ca level. Second, PTH stimulates intestinal Ca absorption and renal Ca

reabsorption. It upregulates a renal enzyme 1α-hydroxylase and this enzyme then activates the conversion of prehormone 25-hydroxyvitamin D_3 ($25(OH)D_3$) to an active hormone $1,25(OH)_2D_3$. As a consequence, $1,25(OH)_2D_3$ promotes active Ca absorption in the proximal small intestine by upregulating intestinal Ca transporters and Ca-binding protein (e.g. TRPV6, PMCA1 receptors and calbindin D_{9K}) as well as stimulates renal Ca reabsorption.[30] To maintain the balance, PTH also stimulates the production of a phosphorus-regulating hormone fibroblast growth factor 23 (FGF23) in bone cells. FGF23 then increases the excretion of P in urine as well as negatively regulates PTH secretion. An increase in serum Ca level feeds back on the parathyroid glad to reduce PTH secretion. However, if serum Ca level is markedly higher than a physiological range, calcitonin will act on this multi-tissue axis in opposition to PTH in order to reduce the serum Ca level to the normal physiologic range. In sum, the strict regulation of Ca and P homeostasis exists in order to ensure that the serum ionized Ca level is within the normal range.

1.2.2 The critical cellular events necessary for bone growth and mineralization.

There are two essential processes that occur during fetal bone development: intramembranous and endochondral ossification. While intramembranous ossification is rudimentary for flat bones of the skull and clavicles, endochondral ossification is the process by which mesenchymal stem cells (MSCs) develop into cartilaginous templates, which are shaped roughly in the form the fully developed bones will be.[31] Unlike in intramembranous ossification where MSCs directly differentiate into osteoblasts, MSCs in the central parts of these cartilaginous templates condense and differentiate into chondrocytes.[31] These chondrocytes undergo proliferation and hypertrophy, followed by apoptosis. Chondrocytes deposit collagen and noncollagenous matrix proteins (NCPs) in the matrix around them and the matrix along the cartilaginous template subsequently calcifies.[32] New vascularization invades the calcified

cartilage and matrix metalloproteinases in the blood digest the structural components in the matrix, thereby allowing the matrix to degrade and regenerate.[33] The calcified cartilage is gradually replaced by new bone called trabeculae. This process occurs until the growth plate becomes ossified and closed and results in longitudinal growth.[34] At this point, trabeculae at the periphery are coalesced to form metaphyseal cortical bone. Tb at the metaphyseal regions of long bones establishes structural foundation for Tb microarchitecture in adulthood.

Once the initial structure of bone is in place, bone grows by the process called *modeling*. This is how bone changes its overall shape in response to physiologic and mechanical forces during childhood and adolescence.[35] This process promotes longitudinal growth at ends of long bones as well as radial expansion commonly occurred in the diaphysis (midshaft). Bones widen by simultaneously addition of bone (formation) on periosteal surface and removal of bone (resorption) on endosteal surface. Consequently, bone diameters expand, the cortices thicken, and the bone matrix are eventually mineralized.

Type I collagen and NCPs, e.g. osteocalcin, alkaline phosphatase, matrix gla protein, are synthesized and secreted from bone-forming cell osteoblasts. Type I collagen is the backbone of bone matrix. NCPs play vital roles in skeletal development, regulate matrix formation and dictate size of collagen fibrils.[6] Mineral is rapidly deposited as an amorphous calcium carbonate within the collagen framework. This initial deposition or primary mineralization achieves two third of its total mineralization within 3 weeks. Subsequently, the bone matrix continues is mineralized at a slower rate (requires a year or more) until it reaches a physiologic limit. As bone matures, mineral crystals grow and form hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$). HA in the bone provides mechanical rigidity and strength, whereas the organic matrix provides elasticity and flexibility.

Bone mass accrual occurs during growth can be measured by dual X-ray absorptiometry (DXA). Bone mineral density (BMD) and bone mineral content (BMC) provide information about the overall amount of mineral deposited in the bone. BMD captures the amount of mineral presents per square meter (g/cm^2) whereas BMC represents the total mineral mass observed in the bone cross-section.[36] DXA is clinically valuable as it reflects overall bone mass of the area of interest and it is easily accessible. Because DXA captures bone mass from two-dimensional imaging, a major drawback is that it is unable to evaluate three-dimensional (3D) structure of bones. The 3D structure of bones provide crucial information in terms of bone development because, during growth, bones also radially expands via the bone formation on the outer surface as known as periosteal expansion as well as trabecular bone develops its microarchitecture.[5]

As Tb microarchitecture also contributes to bone strength, it is important to assess its structure in terms of mass and the connection of trabeculae. Newer technologies such as computed tomography (CT) and magnetic resonance imaging (MRI) overcame the limitations of the 2D evaluation by DXA. In a given region of interest or a volumetric section of the bone, we can measure several aspects of trabecular bone such as its mass (bone volume/total volume, BV/TV), trabecular number (Tb.N), thickness (Tb.Th), separation (Tb.Sp), connectivity density (Conn.D), structure model index (SMI) and trabecular tissue mineral density (Tb.TMD). The reported values are a mean of measures within the specified volume of interest, e.g. the whole lumbar spine, the distal femur. In addition, we can use CT to measure bone mass and density of the cortical bone as well. Though the main disadvantage of CT is radiation exposure which limits its use in clinical settings, CT has become a very powerful and widely used tool in bone research.

1.2.3 Bone Cells

The process of bone patterning and structural development are modulated by multiple bone cells including chondrocytes, osteoblasts, osteoclasts and osteocytes. The following section will briefly summarize basic function, origin of the cell, the development and major regulators of each bone cell.

1.2.3.1 Bone Forming Cells: Chondrocytes and osteoblasts

Because chondrocyte and osteoblast originate from mesenchymal stem cell (MSC) or the osteochondrogenic precursor, specific transcription factors like Runx2 is required for OB differentiation while Sox9 promotes the differentiation of MSCs into chondrocytes. The development of chondrocytes are governed by several signaling pathways including Wnt/β-catenin and Indian Hedgehog (Ihh).[34,37-39] This is critical in the development of bone cartilage during growth as well as fracture healing. Similar to chondrocyte, the development of mature osteoblast from the MSC is stimulated by the Wnt/β-catenin canonical pathway while differentiation of pre-OBs to mature osteoblasts is mediated by transcription factors like Runx2. OBs synthesize and secrete abundant type I collagen and noncollagenous matrix proteins.

Wnt proteins are a family of secreted factors that bind to a complex form between the Frizzled (Fz) receptors and a low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). In the presence of Wnt, Axin (a scaffold protein) binds to the LRP co-receptor and subsequently leads to the recruitment of glycogen synthase kinase 3 (GSK3). Instead of forming a complex with Axin, the adenomatosis polyposis protein (APC), GSK3 and casein kinase 1 (CK1), β-catenin is freely stabilized and accumulates in the cytoplasm. As a result, it can translocate into the nucleus and forms a complex with the TCF/LEF transcription factors to initiate the activation of downstream genes involved in the regulation of OB differentiation, and survival (e.g. Runx2, Osx).[40]

Disruptions of Wnt signaling pathway are associated with changes in bone mass, OB maturation, mineralization and bone turnover.[41]

1.2.3.2 Bone Resorbing Cells: Osteoclasts

Osteoclasts (OCs) are differentiated from hematopoietic stem cells. In the bone marrow, the macrophage-colony stimulating factor (m-CSF) promotes the proliferation and survival of OC precursors. OC differentiation and maturation are primarily governed by the RANKL/RANK/OPG pathway.[42] The binding of RANKL to RANK receptor on OC cell surface induces the activation of the nuclear factor kappa B (NFκB), c-Fos and the nuclear factor of activated T-cells (NFATc1) and activates the maturation and multinucleation of osteoclast. To balance out the activity of osteoclasts, osteoprotegerin (OPG) which is produced by osteoblast competitively binds to the RANKL to counteract the effect of RANKL. OCs resorb bone by attaching to the bone surface and secrete cathepsin K, hydrochloric acid, and acidic proteases to degrade the mineralized matrix.

1.2.3.3 Osteocytes

Osteocytes are the most abundant cells (90%) in the bone. They are former osteoblasts that become entrapped in the bone matrix during the process of mineral deposition.[42] Osteocytes develop projections that connects among themselves and with other cells. This network enables osteocytes to work as a sensor for chemical and mechanical stimuli. As a result, osteocytes can modulate the activities of OBs and OCs through the excretion of endocrine factors. Thus, osteocytes maintain the balance between bone formation and resorption in response to external stimuli.[43]

1.2.3.4 Communications among bone cells

Bones are dynamic tissues that undergo formation and resorption. This requires a rigorously controlled communication among OBs, OCs, and osteocytes. Here, I reviewed some critical regulatory pathways among these cells required for coordination between bone formation and resorption. First, sclerostin secreted by osteocytes binds to its receptors on osteoblasts and inhibit osteoblastic bone formation. In contrast, mature OBs produces OPG to competitively bind to RANKL. This reduces the amount of freely available RANKL to bind to RANK on the cell surface of OCs, thus counteracting the excessive activation of the RANKL/RANK pathway. During bone resorption, transforming growth factor beta-1 (TGF-β1) and insulin-like growth factor 1 (IGF-1) are released from the bone matrix. TGF-β1 induces migration of MSCs to the bone resorption site resulting in increased pool of osteoprogenitor, while inhibits OB maturation, mineralization, and transition into osteocyte. In addition, TGF-β1 regulates osteoclasts activity in two ways. First, it induces the production of OPG and in turn indirectly inhibits OC differentiation. Second, it binds to its receptors on the osteoclasts and directly promotes osteoclastogenesis.[44] IGF-I is a critical regulator in the coupling process of bone formation and resorption. IGF-1 provides a microenvironment that favors OB differentiation and its activity.[45] As a result, promoting the function of OBs in the bone resorption site. Through the coordination among OBs and OCs, bone formation occurs at bone resorption sites to maintain the bone mass and structure.

1.2.4 Trabecular bone dynamics over a lifetime

Tb of the growth plate origin is the starting structure that coalesces into the cortex in the metaphyseal region as well as secondary Tb that remain as Tb in the adulthood. Because of this, Tb development during growth plays such an immense role in setting a foundation for bone

integrity of an individual. Wang et al.[46] studied distal radial and tibial structure of 61 daughter-mother pairs using high resolution peripheral quantitative CT. They found that the daughter's Tb phenotypes strongly predicted the corresponding traits in their premenopausal mothers. In addition, their BV/TV was also a strong predictor of their mothers' cortical thickness at the metaphyseal regions. Therefore, Tb abnormalities at an early age is likely to determine the trajectory of both Tb and cortical bone development.

In the adulthood up until before menopause in women and 50 years old in men, bones are undergone remodeling. Unlike bone modeling occurred during growth, OBs and OCs work coordinately to remove old bone and add new bone to the same surface. Tb has a large surface-to-volume ratio that benefits the maintenance of normal serum Ca level.[35] When the body Ca is imbalanced, e.g. due to dietary deficiency, Tb is more sensitive to Ca withdrawal and this makes Tb more vulnerable to structural deterioration. Riggs et al. assessed bone loss at the distal radius and distal tibia in a longitudinal, population-based study in Rochester, MN and showed that both women and men experience a larger percentage of their total lifetime trabecular bone loss before age 50 (about 40% of their total lifetime Tb loss) compared to cortical bone (6% and 15% in women and men, respectively).[47] This supports the notion that Tb continuously undergoes remodeling. Since bone formation and mineralization take several months to over a year to complete, bone resorption is the main driver of overall bone status. This negative balance worsens as an individual gets older. Lastly, it is evident that bone loss occurs more aggressively in the elderly with the earlier age of onset in women after the menopause. Five to ten years post-menopause, the rate of bone loss is more severe in Tb (4-6% per year) than in cortical bone (1-2% per year).[42] Afterward, bone loss occurs in cortical bone as much as trabecular bone.[48].

1.2.5 Skeletal site-specific characteristics: responses of trabecular bone to intrinsic and extrinsic factors.

Tb at the femur and vertebrae have been observed to have distinct characteristics both in young and older individuals. First, Tb at the femoral neck achieved highest vBMD many years before lumbar vertebrae.[49-51] In addition, Berenson et al. demonstrated that lumbar vertebral peak BMC occurred at around age of 30-33 in whites, blacks and Hispanics, but in the femur, whites peaked earlier than others (6 and 13 years earlier than blacks and Hispanics, respectively).[49] This implies genetic impact on the acquisition of bone at different sites.

Tb at each site bears different types of mechanical loads resulting in differences in their characteristics. Based on Wolff's law, bones that bear more load are likely to adapt for example by developing greater trabecular bone mass. Therefore, specific types of loading will lead to site-specific differences in bone gain. Heinonen et al.[52] conducted a peripheral quantitative CT study in long-term female weightlifters and showed that cortical density did not differ between the weightlifters and controls at either radial shaft, distal femur or tibial midshaft, whereas Tb density was greater in the weightlifters in the distal radius and femur. However, the change in bone structure is site-specific. Specifically, bones subjected to exceptionally high bending-loading like distal radius and radial shaft get larger in bone size compared to the control. However, sites experiencing axial, compressive loading like distal femur adapts by increasing the connectivity density of Tb and no increase in cortical bone site is needed. Similarly, treadmill exercise in rats promotes Tb bone gain at the femur or tibia, but not at the vertebra.[53,54] These studies support the notion that there is differential responses of Tb in each site to specific types of mechanical loading to ensure a functional competent bone microarchitecture.

In addition to the mechanical factor, physiologic needs such as lactation and estrogen deprivation have been shown to affect Tb at each site differently. Liu et al.[55] found that lactation

primarily turned trabecular plates into rods while it reduced trabecular connectivity at the proximal tibia and distal femur. Because the microarchitecture in the vertebra was still in place, the lactating mice were able to gain back Tb bone mass 28-day post lactation while they lost some trabeculae in the femur. Similarly, mature ovariectomized rats lost greater mass of Tb at the proximal tibia and distal femur than in the lumbar vertebrae (75% vs. 36%).[56] In addition, while Shin et al.[57] found similar OVX-induced loss of BV/TV at the L4 vertebra and femur in rats, the main effect in the vertebra was reduced trabecular thickness while in femur it was a 6-fold reduction of trabecular number (Tb.N).

Intrinsic factors like genetics has been suggested to play a role in determining bone mass accrual in a site-specific fashion. First, Chesi et al. observed skeletal site specificity of genetic determinants in human genome-wide association study (GWAS), i.e. with *TBPL2* being more strongly associated at the aBMD of total hip and femoral neck, and *IZUMO3* and *RBFOX1* affected the spine aBMD.[58] Second, two mouse genetic mapping studies (one in femoral and vertebral BMD,[59] and the other in vertebral Tb[60]) identified site-specific genetic loci unique from those identified using whole body BMD as a phenotype. This suggests that genetic regulation of skeletal traits may exhibit skeletal-site specificity. Lastly, Varanasi et al. provided molecular evidence to support the premise that genetics regulates Tb in a site-specific manner by showing skeletal site-related variation in the transcriptome of human Tb from iliac crest and lumbar spine.[61] We can imply from these studies that genetics potentially contributes to site-specific characteristics of Tb. However, no study has proven the impact of genetics and identify genes regulating trabecular bone at different sites. Further research directly compares the impact of the aforementioned regulators on Tb at two or more Tb-rich sites are warranted.

In the developmental period, bone undergoes longitudinal growth, periosteal expansion, and mineralization. A complex network of hormonal regulations and cell signaling ensures that the development of bone structure and the acquisition of PBM occurs properly given that an individual has adequate supplies of nutrition. In addition, existing research suggested that intrinsic and extrinsic factors may govern Tb in a site-specific manner. In order to further evaluate the complexity of bone development, we need to understand the fundamental role of each of these factors. In the following section, I will first discuss in details the role of key dietary factors in regulation of bone and Ca homeostasis during growth.

1.3 Key nutritional factors proven to promote the development of peak bone mass

The early stage of life where bone is rapidly developing is an opportune period that one can intervene to build a solid skeletal foundation.[17,62,63] Nutrition has proved to play a vital role in bone development. The nutrients that have been most extensively studied and shown to have significant impacts on bone health are Ca and vitamin D.[64] We will summarize key impacts of these two nutrients on bone development as well as studies examining specifically on Tb. In addition, we will discuss emerging roles of dietary fatty acids on osteoblasts and bone metabolism.[65,66] Later in this review I will discuss how these nutrients work in conjunction with genetics to affect bone.

1.3.1 Calcium is the key nutrient for bone mineral accrual

Ca and P are key minerals required for bone mineralization. As discussed earlier, the mineral component is important in supporting bone strength by forming HA crystal and mineralizing collagen fibrils.[5] Since our body cannot produce these mineral elements on its own, adequate consumption is indispensable for building strong bone. Fortunately, P is ubiquitously

abundant in most food, so it is not a limiting nutrient. On the other, most children in the U.S.[67,68] and other countries worldwide[69-71] do not achieve calcium intake requirements. This is a concern given that Ca is the main constituent of bone structure.

Low dietary Ca intake has been shown negatively associated with bone mass accrual. Because 99% of Ca is stored in bone, measurement of Ca utilization in the body can be used as a crude measure of bone accrual. Ca balance represents the net difference between calcium intake and output in the body in steady state. This balance is typically positive during skeletal growth in children, zero in adults, and negative in the elderly.[72 2019] Matkovic and Heaney studied Ca balance, which is an intermediate biomarker of bone mass accrual, from birth to early adulthood, calcium balance values at lower intakes were highly correlated with intake and this relationship flattened and became constant at higher intakes.[73] This emphasizes the consequence of low Ca intake on bone development as confirmed by increased BMD and BMC in several Ca-supplement studies in pre-pubertal boys[74,75] and girls[74-76] with habitually low Ca intake.

In 2016, the National Osteoporosis Foundation issued a position statement on peak bone mass development and lifestyle factors.[77] This scientific statement systematically summarized evidence primarily from 16 randomized controlled trials (primarily in white subjects) since 2000 and concluded that dietary Ca intake has strong evidence on its benefit on bone.[77] Briefly, eight[75,78-84] out of the nine RCTs in whites and adolescents from other racial backgrounds (i.e. Indian, Gambian, Jewish and Arab) using supplement pills (ranged from 0.5-4 years intervention) found a small, but positive impact on aBMD and/or BMC accrual (ranging from 0.57-5.8% gain). In addition, two studies with a supplement of Ca (800 mg/d) and vitamin D (400 IU VD_3/d) for 1 year (in whites from the U.S)[85] and 6 months (in female twins in Australia, race not specified)[86] observed significant gain in tibial trabecular vBMD. However, another study in females from

Finland (race not specified) supplemented with 1000-mg Ca and 800-IU VD_3 found no significant change in tibial BMC, while no 3D pQCT measurement was performed.[87] Three RCTs[88-90] using Ca fortification of food or beverages and two RCTs[91,92] using supplementation with dairy in white and Chinese found a significant, positive effect on BMD or BMC gain in spine (+1.5% BMD, +4.6% BMC), femur (+2.7 to 4.6% BMD, hip (+3.1% BMD, +3.8% BMC) and total body (+1.85 and +35.9% BMC). The data from these studies support the hypothesis that chronic Ca deficiency during growth compromised cortical and Tb mass development and that supplementation of Ca in the form of pills, fortified food, or dairy improve bone parameters.

In rodents, studies of low Ca intake in male (0.2% vs. 0.5% Ca in AIN-93G diet)[93] and female (0.25% vs. 0.5% Ca in AIN-76 diet)[94] growing Sprague –Dawley rats consistently showed that low Ca intake led to compromised peak bone mass and microarchitecture as representing in reduced BMC, decreases in Tb volumetric BMD and BV/TV. More importantly, the study in male rats also showed significantly reduction in bone strength of rats fed low Ca diet.[93] Peterson et al. followed female SD rats that received either low (0.25%) or adequate (0.5%) Ca diet during growth (from 4-12 wks) and were either maintained on the same diet or switched to the counterpart until 37 weeks old of age. They reported that the impact of Ca intake during the adulthood on bone mass depends upon Ca intake during childhood and the negative effect of a deficient calcium Ca intake during childhood and adolescences on PBM is not improved by a higher Ca intake after young adulthood[94]. Recently, two interventional studies in female[95] and male[96] outbred CD1 mice compared the effect of low Ca at 0.35%, 0.30% and 0.25% with 100 IU or 400 IU VD to the standard AIN-93G diet (0.5% Ca with 1,000 IU VD). The authors reported that using the modified AIN-93G with 100 IU vitamin D/kg diet, Ca can be lowered to 0.35% in male and to 0.25% in the female rats without detriment to BMD or Tb structure. This contradicted the previous findings in

SD rats. This conflicting evidence suggested that genetic background of rats used in these study as well as genetic variation within the same strain of rats, which are outbred, may affect the response of bone development to low dietary Ca intake.

Several studies examined the impact of dietary Ca intake on trabecular bone at different site. Early studies of Ca supplement in adolescents suggested differential effect of Ca on BMD gain at different bone sites.[76,77,89,97,98] However, none of these clinical studies have systematically and statistically prove the site-specific effect of dietary Ca intake on bone traits. Since there is a possibility of site-specific response as discussed in 1.2.5, this area deserves further investigation. In summary, an adequate intake of Ca influences cortical and trabecular bone development and leads to improved bone strength. Therefore, it is critical to maintain adequate Ca intake during developmental years of life. Supplementation and food fortification have been incorporated to increase Ca intake.

1.3.2 Dietary vitamin D serves as a precursor for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)

Vitamin D (VD) is a fat-soluble vitamin that is naturally present in a limited number of foods like fatty fish and fish liver oils as well as certain VD-fortified foods (e.g. dairy products, cereals and fruit juices) and supplements. Unlike other nutrients, VD can be synthesized in the skin via the exposure of sunlight. Ultraviolet (UV) B radiation facilitates the conversion of 7-dehydrocholesterol in the epidermis to previtamin D. Thus, there is no nutritional VD requirement in the presence of UV light. Unfortunately, different regions in the world do not have sunlight all year. In addition, nowadays more and more people work indoor and do not expose to adequate sunlight. Relying on VD from this source may not be sufficient for physiologic needs. The natural form of vitamin D for mammals is cholecalciferol or vitamin D_3 (VD_3). VD_3 is converted into its active form $1,25(OH)_2D_3$ via a 2-step metabolism. First, vitamin D is hydroxylated at the carbon

position 25 to 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver. The second occurs primarily in the kidney to form the active $1,25(OH)_2D_3$ or calcitriol.

Vitamin D has a principal function in Ca homeostasis by promoting Ca absorption from the intestine.[99,100] The active hormonal metabolite of VD_3, $1,25(OH)_2D_3$, activates the vitamin D receptor (VDR) to increase transcription of genes involved in active Ca absorption such as Calbindin D9k and TRPV6.[101] In the events of decreased serum Ca levels or increased demand of Ca during growth, the renal production of $1,25(OH)_2D$ is increased to promote intestinal Ca absorption (as described in 1.2.1). This complex metabolic pathway involves several proteins (e.g. VD binding protein) and enzymes (e.g. Cyp27B1, Cyp24A1). Therefore, genetic variations affecting those protein and enzymes can lead to differential response in bone formation and development to VD deficiency.[102]

Several studies in humans support the role of VD intake in bone mineralization. According to the Institute of Medicine (https://www.ncbi.nlm.nih.gov/books/NBK56056/), serum $25(OH)D_3$ level should be ≥ 50 nmol/L in order to maximize BMD, prevent risk of VD-deficiency rickets and osteomalacia. In order to achieve the serum level ≥ 50 nmol/L, the dietary recommended allowance for VD was set to 600 IU/day for children from 1-18 years old and adults from 19-50 years old. Epidemiological data from different countries worldwide found positive associations between VD deficiency and rickets (in children) and osteomalacia (in adults).[103,104] More recently, Yang et al. showed that higher serum VD concentrations in adolescence (measured at age of 16) have positive association with BMD and bone microarchitecture in early adulthood (age of 25).[105] In support of this, four RCTs in Chinese,[92] Lebanese,[106,107] and white[108] Ca-replete, VD-deficient adolescent girls and boys with high compliance demonstrated a positive effect of VD_3 supplementation on bone size-corrected BMC and bone area. In contrary, some RCTs in multi-

ethnic,[109] Pakistani,[110] and Danish[81] VD-deficient subjects showed no effect of VD_3 supplementation on BMC gain. Collectively, literature supports the bone promoting benefit of vitamin D, yet the effect might be dependent on racial background.

Rodent models are widely used to model human response to dietary interventions. Yoshizawa et al. used VDR KO mouse and demonstrated that the VDR is mediating the bone protective effects of vitamin D.[100] After weaning, mutant mice failed to thrive and they developed hyperparathyroidism, hypocalcemia, and osteomalacia. This demonstrates that vitamin D signaling through its nuclear receptor is necessary for bone health. To determine the exact intake of dietary vitamin necessary to permit normal growth and bone mineralization, Fleet et al. investigated the impact of AIN93G diet modified to contain either 25, 50, 100, 200, 400 or 1000 IU vitamin D/kg diet on BMD and BMC of growing male C57Bl/6 mice.[111] They found that femur BMC and BMD were significantly reduced when dietary vitamin D was less than 100 IU/kg and serum 25OHD level was less than 60 nmol/L. This dietary value was significantly less than the requirement listed in the NRC Nutrient Requirements of Laboratory Animals.[112]

Altogether, evidence in both humans and animal models emphasizes the importance of vitamin D sufficiency in bone development. Most studies assessed bone mass accrual, not specifically trabecular bone microarchitecture. However, BMD and BMC data from sites such as lumbar spine where approximately 75% or more of bone are trabecular bone infer the positive impact of vitamin D on trabecular bone development.[106,108]

1.3.3 Essential fatty acids (omega-3 and omega 6) support bone development

Fatty acid (FA) consumption has been initially associated with bone health in studies of osteoporosis and fracture risk. For example, saturated FA (SFA) is correlated with increased fracture risk of hip whereas monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) are

linked with decreased total fracture risk.[113,114] While different types of FA intake have been examined with regards to bone metabolism, particular attention has been given to specific polyunsaturated essential FA (EFA), i.e. omega-3 (n-3) FA and omega-6 (n-6) FA[115] (see a review on PUFA and bone and muscle health in adults by Mangano et al.[116]). EFAs are precursors for prostaglandins that involve in the production of pro- and anti-inflammatory cytokines. Overconsumption of omega-6 FA decreases the ratio of n-3:n-6 FA which associated with higher secretion of pro-inflammatory cytokines like IL-6.[117]

Several studies have shown that an appropriate ratio of n-3:n-6 FA intake is critical for bone development. In addition, the amount of EFA intake can modify the FA composition of cortical and trabecular bone. Watkins et al.[118] showed that chicks given a blend of oil high in n-3 FA compared to corn oil (high in n-6) had lower cortical bone n-6 levels but higher n-3 levels. Consistently, Korotkova et al.[119] studied the effects of diets with different n-3: n-6 ratios during the perinatal period on adult bone parameters in rats and found that femur length, cortical cross-sectional bone area and BMC were significantly higher in the n-3+n-6 group than in the groups received only high n-3 or n-6 FA. These represent that a proper mixture of dietary n-3 and n-6 potentially leads to increased bone development.

It has been proposed that EFA intake may influence bone development by modulating prostaglandin (PG) E_2 biosynthesis. Studies in animal models and cell cultures unanimously demonstrated that a high ratio of n-3: n-6 FA intake is negatively associated with PGE_2 production, but positively associated with bone formation rate.[118,120-122] PG are the principle mediators produced locally in the bone cells and PGE_2 is a primary PG that regulates bone modeling. PGE_2 has been shown to be a primary PG affecting bone metabolism. It is a potent stimulator of bone resorption.[123] Furthermore, several cell culture studies identified the role of PGE_2 in stimulating

collagen synthesis and increasing the proliferation of osteoblasts.[124,125] These results demonstrated that the dietary ratio of n-6: n-3 influences PGE_2 production in the bone. Thus, the higher ratio of n-3: n-6 FA, which leads to lower PGE_2, may favor bone formation.

Further studies have shown that potential mechanism by which a high ratio of n-3: n-6 FA might reduce the activation of peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a ligand-activated transcription factor that mostly involved in the regulation of the adipogenesis, energy balance, and lipid biosynthesis.[126] This involves bone cell regulation because, once activated, PPARγ acts as a metabolic switch for stem cell fate in the MSC to favor adipocyte production, instead of differentiating to osteoblasts.[127] Studies in animal models have shown that excessive activation of PPARγ may shift the cell differentiation to favor adipocytes and in turn inhibit osteoblast differentiation and the expression of several anabolic mediators involved in bone formation.[128,129]

Currently, there is no studies investigating whether the binding of n-3 FA to PPARγ leads to the suppression of PGE_2 and affects bone development. One can only speculate from the data discussed above that the higher ratio of n-3: n-6 FA attenuate the activation of PPARγ. As a result, the MSC favors the differentiation to osteoblasts, and an optimal level of PGE_2 (synthesized from EFA) might directly or indirectly promote osteoblast differentiation. However, further mechanistic experiments are needed to prove this hypothesis.

In summary, mounting evidence has substantiated the impact of diet in promoting bone development in terms of structure and molecular regulations of bone development. The role of calcium and vitamin D are strongly supported by multiple RCTs as well as rodent studies. EFA is an emerging nutrient that has foundational studies to support their functions in the development of bone. However, traditional human dietary intervention studies do not account for genetic

differences among the individuals in their study population while animal studies use inbred lines where genetic variability is minimal. Thus, the data presented in this section provides insight the effect of diet that works independent of genetics. In the following section, I will explain how bone traits are controlled by genetic factors and I will discuss the principal candidate genes that have been identified to regulate bone development.

1.4 Genetics control bone and Ca metabolism

Recently, the concept of personalized nutrition or precision nutrition has gained popularity in the nutrition community.[130] Personalized nutrition is the idea that each person may have unique dietary requirements based on how their distinct genetic make-up influences their biochemical and physiological need for specific nutrients. Inherent in the concept of personalized nutrition is the need to understand genetics. Bone mass and the development of PBM are a complex traits regulated by accumulative effect of genetic variation affecting multiple genes.[131] As a result, before I delve into how diet interacts with genetics to control PBM, I will review how genetics influences PBM and I will describe the technologies used to study bone.

1.4.1 Race, Family, and Twin Studies Suggest Genetic influences on bone development

1.4.1.1 Twin studies

It is well established that genetic differences are smaller the more closely related two individuals are. Based on these early genetic studies looked to determine whether bone phenotypes were more similar in families and twins than in unrelated individuals. Pocock et al.[18] and Dequeker et al.[19] examined bone mass of spine of premenopausal monozygotic and dizygotic twin pairs. Both studies reported that monozygotic twins had a higher correlation among their

BMD than those from dizygotic twins. As monozygotic twins are more genetically related than dizygotic twins, these results show that genetics is a major contributor of healthy adult BMD.

1.4.1.2 Family Studies

In addition to twin studies, family studies use familial resemblance among parents and offspring as a genetic indicator and correlate the genetic indicator with a measured bone trait. Matkovic et al.[132] studied the hereditary influences on bone mass by comparing female adolescents' and their parents' bone mass and observed that strong familial association of BMD between offspring and their premenopausal mothers. Choi et al.[20] confirmed this finding in an adolescent Korean cohort (10-18 years old) and extended the finding that the association was even stronger between BMD of offspring aged 19-25 and maternal BMD. Additionally, aBMD[23] and trabecular vBMD[21,23] in daughters of mothers who had osteoporotic fractures is lower than in daughters of women who have not fractured. These observations strongly suggest the existence of genetics of bone mass accrual.

Family studies are the basis for linkage analysis in humans. Linkage analysis is a statistical method that identifies a chromosomal region that is implicated in a trait by observing which region is inherited from the parents to offspring that carry the same trait of interest.[133] Initially they started with family trees to track the phenotype of interest to find the genetic roots for rare, inherited diseases. With large enough studies using sequence-tagged site markers derived from genes, expressed-sequence tags, microsatellites, random sequences, or bacterial artificial chromosome end sequences along with further refinement of a chromosomal region, linkage analysis has also been used to identify genes responsible for extreme bone phenotypes, e.g. mutations in *LRP5*[134] and *SOST*[135] that cause high bone mass.

1.4.2 Racial Studies

Large scale observational studies of populations from different ethnicities/races substantiates the contribution of genetics on bone mass. Ethnic or racial background can be viewed as a surrogate for genetic differences in unrelated individuals. Kalkwarf et al. showed that BMC and BMD were higher in blacks compared to non-black (white and Hispanic) children (aged 6-16 years old).[136] Moreover, Berenson et al. found that, in young women (16-33 years old), age at PBM was similar among white, black and Hispanic women at the spine (up to 30-33 years of age), while PBM at femoral neck was achieved at least 6 years earlier in white.[49] Henry and Eastell examined the effects of ethnicity on BMD in young African-Caribbean and Caucasian adults (aged 20-37 years) and found that blacks had higher BMD (total body, spine and femoral neck) compared with white counterparts.[137] Lastly, Novotny and Davis[138] examine calcaneal bone mass of Asian, white and Asian-white girls (mean age: 11 years) and found that at the age of 10, Asian girls had higher bone mass measured by ultrasound for the speed of sound than white girls. In cross-sectional studies, cortical BMD and BMC in all Tanner stages of boys and girls,[139] and trabecular vertebral BMD[140] only in late puberty of girls were significantly greater in African American. Most recently, McCormmick et al. reported that, in a 7-year longitudinal study of children aged 5-19 years, there is race-dependent differences in age and magnitude of peak height velocity as well as whole body and skeletal site-specific BMC. African American girls and boys had greater whole-body BMC than their non-African American counterparts.[141] Collectively, these studies confirmed that the age of achieving PBM and PBM are race-specific.

Studies in twins, families and populations with different racial backgrounds have some challenges. First, they can exaggerate the proportion of genetic effects controlling the trait of interest. With the assumption that the environment is well-controlled, geneticists have defined heritability as the proportion of phenotypic variation (i.e. variation in PBM) that can be explained

by genetic factors.[142] Unfortunately, the aforementioned types of studies do not have full control over environmental factors. Therefore, we cannot determine absolute genetic effects on the observed variability in PBM. Many shared environmental factors such as dietary components/patterns, level of physical activity and culture can contribute to the observed phenotypic variation. Second, we cannot identify the causal polymorphisms in genes underlying the trait from these studies. While twin and family studies are based on approximate proportion of shared genetic background among related individuals, population-based studies use racial or ethnic background typically reported by subjects to classify individuals into genetic categories. As a result, we cannot use these approaches for gene discovery. These limitations led to alternative approaches that enable bone biologists to identify genes regulating PBM.

1.4.3 Identification of candidate genes underlying PBM

In an attempt to identify the genes underlying bone mass acquisition, linkage analysis of bone-related traits in multi-generational families or sibling pairs offers an unbiased alternative. Phenotypic data (e.g. BMD) inherited from parents to the offspring are associated with the inheritance of genetic markers (e.g. microsatellite markers, SNPs)[143,144] The identified genetic markers reveal potential gene locations regulating the trait of interest as known as *quantitative trait loci* (QTLs). Historically, this approach has been successful in identifying genes that are responsible for rare, monogenic bone diseases. More recently, much denser maps of SNPs allow researchers to perform genome-wide linkage analysis for complex traits like bone phenotypes. However, several difficulties preventing the discovery of causal genes include genetic heterogeneity in a studied population combined with the possibility of genes with small to modest effects. Thus, linkage analysis has a very low statistical power to detect modest genetic effects of

the trait of interest, even when a large sample size is used.[145] As a result, linkage analysis has had limited success in identifying potential causal genes underlying bone development.

1.4.3.1 Candidate Gene Studies

An alternative approach for determining genes underlying complex traits is through candidate gene approach. This approach has been used to relate polymorphisms in the gene encoding bone-related protein to bone phenotypes. This process begins by identifying proteins or enzymes that have been shown to regulate bone development. The polymorphisms of the gene encoding that essential protein/enzyme are hypothesized to cause the interindividual variation of bone phenotypes. Early candidate gene studies used common genetic markers that are based on restriction fragment length polymorphisms (RFLPs). RFLPs are variations in specific endonuclease cut sites occurred from random mutations.[146] One of the genes that have been extensively scrutinized may have been the *VDR* gene. Polymorphisms in *Fok*I, *Bsm*I, *Taq*I, and *Apa*I restriction enzymes in the *VDR* gene were associated with variability in whole body, vertebral and femoral BMD as well as bone size-adjusted vertebral and femoral trochanteric BMC.[147-149] In addition, two RFLPs within the intron 1 of the estrogen receptor α (*ESR1*), XbaI and PvuII, are significantly associated with bone mass accrual in Chinese adolescent girls,[150] young Caucasian adolescent boys[151] and Japanese premenopausal women (mean age=44.5 years).[152] However, these polymorphisms were not associated with the development of PBM in young Finnish men (18.3-20.6 years).[153]

Numerous studies have reported associations between skeletal phenotypes and genetic polymorphism in genes known to function in bone biology. The most widely studied bone-related molecular pathways (gene names in parentheses) are: 1) Bone matrix related genes (*COL1A1*);[154,155] 2) Regulators in Wnt signaling pathway (*WNT, LRP5/6, SOST, DKK,*

FZD1);[156,157] 3) Members of the RANK/RANKL/OPG pathway; 4) Calcium regulating hormones and receptors (*VDR, PTHR1, IGF-1, ESR1*); and 5) Cytokines (*TGF, IL-6*[150]).

Though a candidate gene approach has shed some light on genes regulating PBM, the fact that candidate genes are selected from only known mechanistic pathways limits new gene discovery. To expand our understanding in genetic regulation on bone traits, genome-wide genetic screening method has become widely used.

1.4.3.2 Genome-Wide Association Studies (GWASs) have revealed important genes associated with high PBM

GWAS enables us to identify new candidate genes associating with a trait with an unbiased means[24] and those that might be neglected by candidate gene studies. GWAS evaluates associations between genetic variants identified from genetic scanning in unrelated individuals and a trait of interest. The genetic scanning is performed using either high-throughput genotyping or next-generation sequencing technologies. The ultimate goal is to identify causal functional variants and elucidate the mechanisms by which they affect the trait of interest.

GWASs in children and adolescent populations have unraveled new candidate genes associated with bone development. Kemp et al. first identified polymorphisms in the two candidate genes, *CPED1* and *WNT16*, to be associated with the BMD of skull, upper limb and lower limb in children and adolescent cohort from the Avon Longitudinal Study of Parents and Children (ALSPAC) study.[158] In 2015, Chesi et al. confirmed the association between *CPED1* (rs7797976) with BMD and BMC at the distal radius in another young females (aged 5-20 years old).[159] Moreover, Koller et al [160] conducted a meta-analysis of GWASs of early adulthood and premenopausal white women (aged 20-45 years old) and reported a single nucleotide polymorphism (SNP) in *WNT16* (rs3801387) to be associated with BMD at the lumbar spine. In addition to this SNP, Koller et al. found multiple SNPs in *ESR1* achieved genome-wide

significance levels for lumbar spine BMD. The SNPs in *WNT16* and *ESR1* were also validated in seven replication cohorts of premenopausal women of European, Hispanic-American and African-American descents.[160]

Though GWAS is a powerful tool for gene discovery, it has some weaknesses worth mentioning. First, GWAS only suggests the association between genetic variants and the trait of interest but does not prove a causal relationship. Second, unlike QTL mapping study in animals, genetic data are obtained from unrelated individuals and environmental factors are not well controlled. As a result, environmental factors can confound the correlations observed between genetics and the trait. To mitigate the confounding issue, GWAS requires an extremely large sample size which can in turn limit the use of GWAS in studying traits developed in an early age like PBM. Lastly, some phenotypes (e.g. bone strength, 3D microarchitecture) are impractical or unethical to measure in a large human cohort. Thus, we cannot use GWAS to identify genetic determinants for those phenotypes. Other tools that overcome these limitations can provide more insight in genetics of bone development.

1.4.4 Mouse models as complementary tools for studying the role of genetics in bone

Mouse models have been widely used to examine the contribution of specific genes to the development of bone mass and microarchitecture. Genetic manipulations through gene knockouts and targeted mutations and their relation to a large number of traits are well catalogued by the International Mouse Phenotyping Consortium (IMPC).[161] When scaled up to a genome-wide study, mouse models are a very powerful tool for bone biologists for many reasons: a) the strong homology between human and mouse genomes, particularly within the protein-coding regions;[162] b) Whole genome sequencing data of both human and several mouse strains are complete and publicly available; c) the ease and rapidity of conducting crosses among inbred strains and

accessing commercially available recombinant inbred strains; d) specific crossing and breeding schemes can be used to generate large variation in bone traits;[163] e) a rigorous control that investigators have over environmental factors that affect genetic impact on bone traits; f) certain phenotypes that are difficult or infeasible to assess in humans (e.g. bone strength test using 3-point bending, *in vivo* 3D trabecular microarchitecture or histomorphometry at the end of growth period) can be easily obtained from mice; g) a plethora of publicly available bioinformatic resources facilitates candidate gene prediction; and h) the advanced knockout and transgenic technology allowing investigators to perform functional validation of putative genes controlling bone trait(s) of interest.

A similar approach used for linkage analysis in humans can be applied to mice and referred to as "quantitative trait locus (QTL)" mapping study. QTL studies inform us regions on the chromosome where existing polymorphisms or SNPs are highly correlated with variation of the trait of interest. With the advancement in DNA sequencing, whole genome database of several mouse strains as well as gene expression data from several tissues are available. This allows us to use bioinformatic tools to identify candidate genes with greater confidence for further functional validations.

1.4.4.1 Mouse Genetic Populations

There are several mouse resources available for genetic mapping analysis. Here, I classified the mouse resources into two main categories: (1) those used mainly for association (GWAS) studies and (2) the resources used for linkage analyses. I will provide an overview of how each mouse genetic resource is generated, strengths and limitations.

1.4.4.1.1 Mouse Resources for Association Studies

i. *Hybrid Mouse Diversity Panel (HMDP)* is a collection of approximately 100 well-characterized inbred strains that were derived from 30 classic inbred strains and 70 or more RI strains derived primarily from C57BL/6J (B6) and DBA/2J (DBA), and A/J and B6.[164] The HMDP has a high density of genotyping information from the 30 inbred parental lines. Thus, the HMDP has a high resolution genetic map that can detect a QTL of one to two megabase (Mb) regions where a lesser number of candidate genes exist, e.g. a QTL accounting for 5% of the phenotypic variance can be detected (power=0.8) using 100 strain (8 mice/strain).[133] This panel of mice is also readily available for purchase through the Jackson Laboratory. However, this population does not carry alleles from wild-derived strains resulting in limited genetic diversity. Currently, there are two studies using the HMDP to identify genetic components for BMD.[165,166]

ii. *Diversity Outbred (DO) mouse population* is a heterogeneous stock derived from partially inbred Collaborative Cross (CC) strains (originated from eight inbred strains) and followed by outcrossing. Thus, the DO and CC captures the same set of natural genetic variation. Of these parental strains, five represent the classical inbred strains from *Mus mus musculus* (129S1/SvImJ, A/J, B6, NOD/ShiLtJ, and NZO/H1LtJ), with the remainders are wild-derived strains (CAST/EiJ, PWK/PhJ and WSB/EiJ). Thus, this population covers more variations compared to other strategies resulting in a broad range of phenotypes.[167] High level of allelic heterozygosity is uniformly distributed in the coding or regulatory regions of essentially all known genes that can help to avoid the identification of idiosyncratic reaction that occurs only in a limited genetic context.[168] Because of population structure in the DO, more complex procedures in genetic mapping are required to account for the eight founder haplotypes. The most important disadvantage of the DO is that each DO mouse is a unique individual, so each individual needs to be genotyped and phenotyped for each experiment.[169]

iii. Heterogeneous stock (HS) mouse population is created from the breeding of eight inbred strains, followed by circular or random breeding for over 50 generations that results in an average distance between recombinants of < 2 cM.[170] The HS mice descended from eight inbred founder strains (A/J, AKR/J (AKR), BALBc/J, CBA/J, C3H/HeJ (C3H), B6, DBA and LP/J). Because the HS mice are outbred offspring, they are genetically unique. This population can be used to for genome-wide, high-resolution mapping with an average of 95% confidence interval of 2.8 Mb and QTLs explaining 5% or less of the phenotypic variation can be mapped into intervals of <1 cM.[171] Because of the success of HS mice, the HS rat colony has been created using the following inbred rat strains: ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, WN/N.[172,173] The downside of this population is that it requires ~100 times more markers and ten times more animals as compared with an inbred strain cross. The population structure in this population necessitates more complicated analysis and reduces power. As a result, this population is mainly used for fine-mapping of single QTL intervals.

1.4.4.1.2 Mouse Resources for Linkage Analyses

i. F2 intercrosses from two inbred strains: This is a classic breeding protocol used for QTL mapping by breeding two inbred lines to obtain a filial (F1) with heterozygosity at all polymorphic loci. The F1 mice can be crossed back to one of the two parental lines (backcross) or can be intercrossed among their siblings to generate an F2 intercross.[142] The F2 will be genotyped and measured for the phenotype of interest and these information can be used for QTL mapping. This breeding strategy was useful in the past to identify several bone-related loci,[59,60,174-178] However, it has low mapping resolution so it has been gradually replaced by new mouse genetic resources with better mapping resolutions.[24]

ii. Recombinant inbred (RI) population is a panel of mouse lines derived from two inbred strains followed by an F1 intercross and 20 or more generations of repeated sibling mating to create new inbred lines. After 20 generations of inbreeding, individuals from each RI strain carry the same genomic structure with a mosaic of haplotypes derived from the two parental strains.[179] Compared to classic inbred strains, the genotype in an RI strain is somewhat limited because there are only two possible alleles (from each parental line) at each locus and there is limited recombination.[180] For example, the BXD RI panel was developed from a cross between B6 and DBA inbred strains. However, there are many strengths to the BXD RI strains. It is the largest, commercially available mouse reference panel (191 independent strain and 7 substrains[181]). The important advantage of this resource is that both parents have been sequenced by which 4.8 million SNPs are different between the 2 strains (www.genenetwork.org/mouseCross.html). This makes this panel a remarkable resource for genetic mapping as well as for studying GxE interactions. In January 2017, the new set of 7321 genotypic markers was released, which is twice of the number of previous set of markers (released in 2011). This will increase the resolution of mapping analysis resulting in a more accurate prediction of QTLs. Because of the inbred nature of this population, research with RI strains tends to be cumulative and collaborative. A great example for this is microarray data from a large amount of studies in a variety of tissues using the BXD panel are available on the GeneNetwork (http://genenetwork.org). With the data from the tissue of interest, we can utilize this resource for predicting expression QTL (eQTL). Other RI sets of mice include AKR X DBA, A/J X B6, and B6 X C3H that are also readily available. However, they are much less commonly used in bone research and will not be included in the following review of QTL studies.

iii. The Collaborative Cross (CC) population is a panel of RI strains developed from the same eight founder inbred strains as the DO strains to capture approximately 90% of genetic diversity in mouse genome.[168,182,183] This population is suitable for the identification of genetic risk factors of various pathophysiological states because it is designed for high resolution analysis of complex traits.[184] The CC is genetically stable, reproducible, and contains fixed novel mutations introduced by wild-derived strains and genetic drifts.[185] Thus, the CC can be a valuable resource for genetic mapping and the study of GxE in bone phenotypes in the future.[186]

These mouse populations have different advantages and limitations. Majority of studies in the past two decades had used F2 intercrosses and BXD RI strains. In the following section, I will summarize what we have learned from the QTL studies using these populations.

1.4.4.2 QTL identified from Mouse Genetic Mapping Analysis

In 1998, Klein et al. first used a panel of 24 BXD RI strains to perform QTL analysis and identified 10 chromosomal sites linked to the acquisition of peak, whole body(WB)-aBMD in 12-week-old female mice.[187] In 2001, Orwall et al. studied male and female mice from 16 BXD RI strains and first reported that there are gender-specific loci controlling peak BMD (6 male-specific, 5 female-specific loci, and 9 loci in both).[188] This emphasizes the importance of controlling gender in genetic studies. Moreover, Masinde et al. used 4-month old female F2 mice from MRL/MPJ and SJL/J intercross and demonstrated that there is little overlap between QTLs mapped to whole body BMD and femoral BMD.[189] This brought the interest to the study of phenotypes at specific skeletal sites[176,177] as well as specific compartment like Tb that may decrease the variation in bone phenotypes resulting in better and more accurate mapping analysis. Subsequently, several other QTL studies have reported a large number of loci controlling WB-aBMD as well as aBMD of specific skeletal sites like femur and vertebrae using not only BXD RI strains, but also

F2 intercrosses of B6 and DBA, and B6 and C3H (see a comprehensive review by Xiong et al.[190]). Though genetic loci controlling for aBMD are distributed across the genome, there are apparent segregation of QTLs on chr 1, 2, 4, and 11 (i.e. BMD-associated genes within the QTL ≥ 10 genes and polymorphisms within the QTL ≥ 5 genes).[190]

Previous work mostly used aBMD of either whole body or specific bone sites as phenotypes for genetic mapping. This can hinder our ability to dissect genetic contribution to bone traits. DXA assessment has drawbacks and may not provide accurate bone mass evaluation as discussed earlier in this review. In addition, osteoporosis is defined by not only reduced bone density, but also altered Tb microarchitecture. 3D bone assessment with greater resolution may provide a solution for extending our understanding. A mapping study by Bouxsein et al.[60] in the F2 mice of B6 and C3H intercross is the first study examining genetic regulation at Tb. Using genome-wide quantitative trait analysis of high-resolution μCT in the fifth lumbar (L5) vertebra, the authors reported novel QTLs unique to vertebral BV/TV and Tb microarchitecture. These include QTLs on chr 9, 10 and 12. At that time, this was the first genetic mapping study of Tb phenotypes because a similar type of study in humans was limited by the imaging technology, ethical concerns of high amount of radiation required to obtain Tb phenotypes and lack of statistical power. Other subsequent studies[191,192] revealed additional novel QTLs with improved resolution (narrower regions).

Mouse GWASs have been used to discover novel genetic loci and candidate genes for several complex traits like metabolic and cardiovascular trats;[164,168] however, only one study specifically investigated BMD. Using HMDP, Farber et al. conducted a GWA of WB-BMD, lumbar vertebral and femoral BMD and identified four significant associations on chr 7, 11, 12, and 17. The associations implicated 26 functional candidates with the most significant BMD SNP

on chr 12 was a non-synonymous variant in *Asxl2*. This finding led to multiple functional validation studies that confirmed the role of *Asxl2* in osteoclast differentiation.[165] Although the high cost and the requirement for sophisticated statistical analysis of conducting a mouse GWAS may limit its use in bone field, the study by Farber et al. demonstrated the power of mouse GWAS populations as a gene discovery tool.

The ultimate goal for using mouse genetic resources is to discover loci and candidate genes that we might have missed with linkage or GWASs in humans. Thus, the most important concern researchers have in using mice is whether the information can be translated to human genome. The early QTL studies of aBMD by Klein et al.[187] and vertebral Tb by Bouxsein et al.[60] showed that several loci identified in the mouse are homologous with the regions identified in humans genome-wide scans. In 2010, Ackert-Bicknell et al. remapped QTLs for BMD from 11 archival mouse data sets using the new, denser genetic map.[162] The authors also conducted a comprehensive comparison between the mouse QTLs and human GWAS BMD loci and demonstrated the concordance between 26 (out of 28) human GWAS loci for BMD and a mouse QTL recalculated on the new genetic map.[162] This is critically important because it means that QTLs discovered from mouse genetic studies have the potential to be translated to aid identification of phenotype altering loci in humans. Therefore, mouse genetic resources can serve as a useful model for genetic study of human bone biology.

Mouse genetic resources offer additional great advantages such as the use for functional validation of candidate genes. We can manipulate the mouse genome by breeding schemes that transfer a portion of a chromosomal region of one mouse strain into another inbred strain. These congenic lines allow investigators to validate the functional role in bone trait QTLs identified from mapping studies. Multiple studies have revealed the usefulness of this tool[193-195] and groups like

Davis et al.[196] have developed genome-wide congenic strains for B6 and DBA strains that limit the need for individual investigators to generate their own lines. A final important aspect of mouse models is that investigators can have complete control over environmental factors that might influence bone. As a result, these mouse populations permit researchers to conduct controlled studies on GxE interactions. Multiple examples of gene-by-diet (GxD) studies on bone traits using mouse resources will be discussed in the following section.

1.5 The effect of GxD interactions on peak bone mass development

Skeletal development is a complex trait influenced by genetic determinants and environmental factors. Neither genetic (G) nor environment (E) alone can completely explain the variation in PBM. We have discussed about the impact of diet on this trait as well as how genetic polymorphisms modulate it. Unfortunately, what we have learned so far does not provide a complete insight in the regulation of PBM. Even though a large number of candidate genes have been identified, human GWASs have failed to explain a significant amount of heritability of the bone phenotypes.[197] The proposed reasons for the missing heritability includes the fact that low frequency or low effect size genetic polymorphisms and epistasis (gene-gene interactions) cannot be detected via GWASs, as well as the fact that the effect of GxE interactions may mask or hide genetic contributions in free-living individuals.[198] An example of how genetic effects may be conditional upon the environment is that polymorphisms in the estrogen receptor1 gene (*ESR1*) affects bone phenotypes in premenopausal white women[160] but do not impact the development of PBM in young Finnish men.[153] This is why many groups feel that the study on the GxE interaction merits extensive evaluation.[24,131,199]

In a GxE interaction study, the effect of genetics on a trait of interest is hypothesized to vary depending on the condition of the environment.[24] For example, the *ESR1* gene polymorphism

example above suggests that environmental factors that differ between men and women (likely differences in circulating sex hormones) modify the impact of this variant on bone mass. Studies by McGuigan et al.[200] and many others[201,202] support the notion that the impact of genetics on bone depends on gender. Physical activity is another factor that has been shown to interact with genetic factors to impact the attainment of PBM.[200,203,204]

Dietary factors deserve special attention as an environmental factor that interacts with genetics because we are exposed to our diet every day and we can modify it to our own benefit. The findings from several Ca intervention trials in children and adolescents demonstrated that there is a large variability in the acquisition of bone mass, despite the control of age range and pubertal maturation of participants.[28] Weaver et al.[102] conducted a 3-week long, controlled feeding trial (Ca intake ranging from 760-2195 mg/day) in 105 girls (50% black, aged 11–15 year). They reported that Ca intake and race were strong predictor for net Ca absorption and retention, with black girls absorbing Ca more efficiently at low calcium intakes than white girls. This suggests that the effect of diet on bone might not be straightforward.[24,28]

To date the number of studies that have investigated possible interactions between genotypes and dietary factors are limited. In this section, we will review two examples of GxD interaction studies that affect bone health. The first will be a discussion of how genetics and EFA interact to influence bone. This example is being presented because it has led to the identification and testing of specific polymorphisms within candidate genes in humans. As such, it is a successful paradigm for this line of research. The second example relates to the impact of genetics on the bone response to dietary Ca. This second example is the foundation for my dissertation research.

1.5.1 Gene-by-EFA interactions

In recent years convincing evidence has emerged regarding the association between dietary FA and bone. As discussed earlier, n-3 FA may act through PPARγ, and recent evidence indicates there is an interaction between polymorphisms in the *PPARγ* and dietary FA on PBM. Ackert-Bicknell et al.[205] studied the effects of a 13-week (from the age of 3-16 weeks) treatment of three different levels of fat (11%, 32%, and 60% kJ) in the B6.C3H-6T (6T) congenic mice compared with wild-type B6 mice. The 6T congenic mice was created to carry a region of chr 6 (52-116 Mb mm10) that contains a large chromosomal inversion that is also polymorphic between C3H and B6 mice.[206] Total body aBMD and trabecular bone mass (BV/TV) were the traits of interest. Dietary fat intake was associated with decreased aBMD and BV/TV in the 6T mice, but not in B6 mice. Using block haplotyping, expression profile, and by examining the existence of the coding region SNPs, the investigators identified 5 candidate genes known roles in bone biology including *Gpnmb*, *Adamts9, PPARγ, Raf1* and *Zfp422*.

Out of these five candidate genes, Ackert-Bicknell et al.[205] selected *PPARγ* as their most likely candidate gene because previous data showed that *PPARγ* expression was significantly increased in calvarial osteoblasts of the 6T congenic mice as compared to the WT.[207] The author used the Framingham Offspring Cohort (FOC) as a validation cohort to verify polymorphisms implicated in the FA-by-*PPARγ* interactions identified in the mouse study. The total of 13 SNPs within the *PPARγ* gene were used in this study. These include 11 tag SNPs chosen from a dense SNP map (constructed using data from the population of the Center for the Study of Human Polymorphism) and 2 common SNPs previously shown to be associated with cardiometabolic disease.[205] They showed that the interaction of dietary fat by *PPARγ* polymorphisms was strong in both men (60.1 ± 9.5 years) and women (59.5 ± 9.2 years) for the phenotypes of trochanteric,

femoral neck, and lumbar vertebral aBMD. Because the *PPARγ* polymorphisms were associated with aBMD and BV/TV of growing mice, the significant effect observed in the validation cohort might explain that the interactions between *PPARγ* polymorphisms and dietary FA are implicated in both the bone development and the maintenance of bone in the elderly.

However, some limitations of the study need to be considered. In the animal study, dietary fat was administered based on the calorie without indication of types of FA. Thus, we cannot dissect whether the effect detected in this study was the consequence of the increased amount of fat intake or by the increased proportion of certain types of FA. Second, the validation cohort used in this study consists of elderly individuals which is the different age range from those in the mouse discovery cohort. Lastly, the dietary data used in the FOC were based on food frequency questionnaires which might not provide accurate amount of dietary FA intake as well as other nutrients. While there were certain weaknesses to the study, this study clearly demonstrated the effect of gene-by-FA interactions on bone and the candidate genes identified from the animal study are worth validating in the future.

Because the previous study showed that *PPARγ* polymorphisms may interact with dietary FA to affect bone during aging, Bonnet et al. investigated the role of *PPARγ*-by-(n-3) FA interactions on bone mass in aging 6T congenic mice. Omega-3 PUFAs are of particular interest because they are natural ligands for PPAR*γ*. Bonnet et al.[208] postulated that there might be a GxD interaction between fish oil (high in n-3 FA) intake and *PPARγ* genotypes. The fish oil diet was fed to mice from the age of 3-12 months old and an isocaloric high safflower oil diet (rich in n-6 FA) served as the control diet. Bonnet et al. found that the fish oil diet preserved vertebral BMD and microstructure in B6, but not in 6T mice. On the other hand, the fish oil intervention was also associated with a decrease in femoral BMD of the 6T mice.[208] This study confirms that the effect

of fish oil may be varied depending on genetic polymorphisms in the 6T congenic region, presumably those in the *PPARγ* gene. However, as the adult mice were studied, we must use this knowledge with caution when applying these results in populations during growth.

Bonnet et al. tested the effect of the 6T congenic region, not a specific gene. In addition to *PPARγ* gene, *Alox5*, *Cidec* and *Zfp422* genes are also within the tested region and their polymorphisms could be responsible for the negative effects on bone observed in the 6T congenic mice fed the HFD. Le et al.[209] examined whether the gene that encodes 5-lipoxygenase, *Alox5*, interacts with a high-fat diet (HFD) to impact BMD. *Alox5*$^{-/-}$ and wild-type (WT) mice were fed either a HFD (45% fat by kcal) containing high n-6 FA or a control diet (10% fat by kcal) from the age of 3-13 weeks. When fed HFD, *Alox5*$^{-/-}$ mice had lower areal bone mass, had lower cortical parameters, and had reduced BV/TV and Tb number compared with the WT. Histomorphometry showed that *Alox5*$^{-/-}$ mice had an increase in osteoclast number on the bone surface. The effect of HFD on osteoclast activity was confirmed in an *in vitro* study showing that the rate of osteoclastogenesis from bone marrow stromal cells was higher in the knockout mouse. Collectively, this study shows that the *Alox* gene interacts with the HFD to regulate bone mass during growth.

These studies on gene-by-EFA interactions well exemplified how mouse models were utilized and successfully identified genetic polymorphisms whose effects on bone depend on the level of dietary fat consumption. Since genetic modification can easily be done in mouse models, it is a useful platform for validating the functional role of candidate genes. Using a similar paradigm, in the following section I will discuss research that have been done on gene-by-calcium interactions.

1.5.2 Gene-by-calcium interactions

Ca is the principal mineral for bone mineralization so it is the target nutrient that researchers attempt to optimize in the diet. However, there are some physiological differences among racial groups that suggest dietary Ca requirements may be influenced by genetics. For example, blacks develop a higher PBM than whites and this is associated with a reduced risk for bone fracture.[210] To address the racial differences in mineral metabolism, Dr. Connie Weaver conducted controlled clinical studies in black and white adolescents.[26,27] Her studies show that black adolescents have greater skeletal Ca retention and that they adapt better to low dietary Ca intake than white adolescents. Her data shows that there is no universal dietary requirement for Ca intake during growth that is adequate for all individuals.[211] Thus, genetics may determine the impact of Ca intake on bone phenotypes.

Ca absorption is mediated by two main pathways, a passive paracellular pathway and a VD-mediated, active intestinal Ca absorption pathway. To stimulate the active pathway, $1,25(OH)_2D_3$ binds to VDR to stimulate the genomic actions necessary for Ca uptake and release from the enterocyte.[101,212] Because of this, genetic polymorphisms in VDR were among the first to be tested for their interactions with Ca intake on bone and calcium metabolism. Two longitudinal studies have prospectively tested the association between interactions of VDR genotypes with dietary Ca and BMD changes during childhood.

*Bsm*I polymorphisms in the VDR gene have shown to interact with Ca to influence BMD in prepubertal girls. *Bsm*I is the RFLPs at the 3'-end region of the VDR gene that has been associated with bone mass traits in human GWAS.[213-215] Ferrari et al. randomized prepubertal Caucasian girls (7-11 years old) based on their *Bsm*I genotypes to two milk extract-supplemented products providing 850 mg/day Ca or placebo. By increasing dietary Ca intake, girls with *the Bb* genotype showed increased BMD accrual at lumbar spine (LS) and femoral neck (FN), (with a

trend also found in girls with the BB genotype) compared to girls wtih the *bb* genotype.[216] In contrast, in the absence of Ca supplements, *bb* girls gained higher BMD than other genotypes. These results suggest that *Bsm*I genotypes interact with Ca supplementation to modulate bone development in growing girls.

Previous studies showed that coding sequence differences identified by digesting DNA with the restriction enzyme *Fok*I can shorten the VDR protein by three amino acids and reduce its activity.[217] These *Fok*I polymorphisms have been associated with adult bone mineral density (BMD) in Caucasian American and Japanese premenopausal females [217-219] and so they might interact with dietary Ca to regulate BMD in adolescence. To study this, Ferrari et al. gave 137 prepubertal girls (aged 6.6–11.4 years) either 850-mg Ca supplement or placebo and measured BMD changes in response to the Ca supplement. They found that in girls with the *FF* genotype there was a trend towards higher gains in BMD with Ca supplementation compared to girls with the *ff* genotype.[220]

Human studies targeting certain candidate gene polymorphisms allow us to test the effect of GxD interactions, but this is limited to only genes whose functions in bone are already established. As a result, we cannot use this approach to discover new candidates. Furthermore, uncontrolled factors in human studies may affect the outcomes by reducing statistical power to detect differences between genotype groups. Conducting QTL mapping studies in a well-characterized animal model under a well-controlled environment provides a solution to these problems.

The Fleet lab has conducted a series of experiment to investigate the role of GxD interactions on Ca absorption, bone mass accrual and hormonal regulation. First, Replogle et al.[211] used 4-week male mice from 11 common laboratory inbred strains that were chosen to represent

the genetic diversity in the mouse genome: B6, 129S, CAST, DBA, C3H, SWR, A/J, AKR, CBA, PWK and WSB. Mice were fed either 0.5% (adequate) or 0.25% (low) Ca diets until 12-weeks of age, i.e. the point where mice achieved PBM.[221,222] The mice treated with the 0.5% Ca diet had large variability in BMD and Tb mass among the inbred lines. This confirmed the findings in previous human and animal studies that genetics regulates bone. In addition, the Replogle et al. study showed that the degree that mice responded to low dietary Ca intake depended on their genetic background. For example, the low Ca diet had a strong negative effect on peak femur BMD in AKR, C3H and CBA mice and a moderate negative effect on peak femur BMD in B6, A/J and WSB, while 129S, CAST, DBA and SWR lines were resistant to the effect of low Ca intake.[211] The differences in the response to low dietary Ca intake clearly demonstrates that GxD interactions affecting the development of PBM in male mice.

Because we saw the effect of GxD interactions on Ca absorption, the Fleet lab conducted a follow-up genetic mapping study to identify genetic loci affecting serum $1,25(OH)_2D_3$, $25(OH)D_3$ and FGF23 levels and their response to low dietary Ca intake.[223] Male mice from 51 BXD recombinant inbred (RI) lines were fed either 0.5% (adequate) or 0.25% Ca diet from weaning until 12 weeks of age and the impact of diet, genetics, or their interaction on these serum hormone levels was assessed. As expected, genetics strongly regulated these phenotypes in both mice fed adequate and low Ca diet. In addition, unique genetic loci were found to regulate the physiologic adaptation of these hormones to dietary Ca restriction. Reyes-Fernandez et al.[224] carried this concept of gene-by-dietary Ca interactions affecting bone and mineral metabolism one step further. By examining the femurs of the BXD RI mice from the GXD study above they identified QTLs underlying basal bone mass as well as the response of bone mass to the low Ca diet. This study revealed novel genetic loci on chr 1, 3, 4, 8 and 9 controlling the response to Ca

restriction for Ca absorption and BMD/BMC. Collectively, these experiments by the Fleet lab have proved the existence of GxD interactions on of Ca absorption, $1,25(OH)_2D_3$, and the acquisition of bone mass in the femur.

To date, our knowledge on genetic variants affecting bone mass and structural acquisition is still incomplete, particularly our understanding in genetics and dietary influence on Tb development. Moreover, there is still a gap in our understanding of GxD interactions affecting Tb mass and microarchitecture. Since the integrity of Tb is strongly associated with the risk of osteoporotic fractures, it is essential for us to understand how this specific bone compartment develops and how we can help each individual develops the best version of their Tb early in life.

1.6 Aims for Dissertation Research

This dissertation aims to fill a significant knowledge gap in our understanding of how genetics influences dietary requirements for optimal bone health. Specifically, in my dissertation I will characterize the influence of genetics, dietary Ca intake and their GxD interactions on the development of Tb mass and microarchitecture. To do this, I will utilize bone samples from previous studies by our research group that used a set of 11 inbred mouse lines[211] and a panel of 51 BXD RI strains.[224] These studies fed mice with semi-purified AIN93G diets (with 200 IU of VD_3/kg diet) that contained either adequate (0.5%) or low (0.25%) levels of dietary Ca during growth. My work will extend this earlier research by evaluating the impact of dietary Ca intake, genetics and GxD interactions on Tb phenotypes in the distal femur and L5 vertebra.

First, I will systematically test the effect of dietary Ca intake on Tb in each skeletal site by combining data from the 11 inbred and 51 RI lines of mice and using this data to reflect a genetically diverse population. This will allow me to test two important questions, i.e. “is low calcium intake harmful to the acquisition of peak Tb mass in a diverse population similar?” and

"do two Tb rich sites have a similar response to inadequate dietary Ca intake?" Moreover, I will use the genetic structure among 62 inbred lines to determine the heritability of Tb phenotypes and to test if there are differential effects of genetics on Tb in the two sites (i.e. gene-by-site (GxS) interactions. Finally, I will test for the existence of GxDxS interactions. The results from this experiment will establish whether dietary Ca intake, genetics and GXD interactions influence Tb in a site-specific manner.

Second, I will conduct genetic mapping analysis using a population of 51 BXD RI lines in order to identify QTL controlling femur and vertebral Tb traits. Because we used diets with two different levels of Ca, I will be able to determine the genetic loci responsible for basal Tb phenotypes as well as the loci that are sensitive to low Ca intake. In this experiment, I would like to identify: 1) genetic loci affecting Tb phenotypes in both diet group (robust effects) or either in the basal or low Ca group (i.e. suggesting the existence of genetic effects that are sensitive to diet), 2) genetic loci that affect multiple interdependent Tb phenotypes (i.e. suggesting genetic control of a central regulator), and 3) whether genetic loci responsible for Tb phenotypes in response to low Ca intake are unique from the loci controlling basal Tb phenotypes (i.e. a direct reflection of GxD interactions). Identification of genetic loci will be followed by careful bioinformatics analysis to identify potential candidate genes that contribute to Tb mass and microstructure. This research will provide new insight into genetic control of Tb phenotypes both in a homeostatic state and under low dietary Ca stress.

In the end, I will integrate all the genetic loci identified from a previous study[224] by our group in femoral BMC and BMD with genetic loci I newly discovered from genetic mapping of femoral Tb phenotypes. This will provide more insight whether there are common genetic factors that modulate overall bone mass and Tb phenotypes and whether there are genetic loci that are

unique to Tb phenotypes. Finally, I will use the same research paradigm from the second part of my dissertation to determine genetic loci regulating Tb phenotypes in the L5 vertebrae. When all of these findings were integrated, I will address whether loci identified in the femur are co-localized with the loci from the vertebrae as well as whether the loci that control the phenotypes only under the low Ca environment are common or unique to only one site.

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CHAPTER 2. DIETARY CALCIUM INTAKE AND GENETICS HAVE SITE-SPECIFIC EFFECTS ON PEAK TRABECULAR BONE MASS AND MICROARCHITECTURE IN MALE MICE

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2.1 Abstract

Trabecular bone (Tb) is used for rapid exchange of calcium (Ca) in times of physiologic need and the site-specific characteristics of Tb may explain why certain sites are more vulnerable to osteoporosis. We hypothesized that peak trabecular bone mass (PTBM) and Tb microarchitecture are differentially regulated by dietary Ca intake, genetics, or Gene-by-Diet (GxD) interactions at the distal femur and the fifth lumbar (L5) vertebra. Male mice from 62 genetically distinct lines were fed basal (0.5%) or low (0.25%) Ca diets from 4-12 wks of age. Afterwards, the right femur and L5 vertebra were removed and trabecular bone was analyzed by μCT. In mice fed the basal diet, bone volume fraction (BV/TV), trabecular number (Tb.N), and connectivity density (Conn.D) were significantly higher in the L5 vertebra than femur. Femur Tb had a weaker, more rod-like structure than the L5 vertebrae while mice fed the low Ca diet developed rod-like structures at both sites. Dietary Ca restriction also caused a greater relative reduction of Tb.N and Conn.D in the femur than L5 vertebra, i.e. it was more harmful to the integrity of Tb microarchitecture in femur. Genetics was a major determinant of Tb at both sites, e.g. heritability of BV/TV on the basal diet = 0.65 (femur) and 0.68 (L5 vertebra). However, while GxD interactions altered the impact of dietary Ca restriction on Tb parameters at both sites, the effect was not uniform, e.g. some lines had site-specific responses to Ca restriction. The significance of

our work is that there are site-specific effects of dietary Ca restriction and genetics that work independently and interactively to influence the attainment of PTBM and Tb microarchitecture.

2.2 Introduction

Osteoporosis affects millions of individuals worldwide[1] and approximately 50% of osteoporotic fractures occur at trabecular bone-rich sites like the lumbar vertebrae and at the ends of long bones.[2] Consistent with the idea that trabecular bone-rich sites are sensitive to fracture, cadaver studies on the mechanical strength of trabecular bone from proximal femur[3,4] or the thoracic ninth vertebrae[5] show that bones with lower trabecular bone volume (BV/TV), fewer trabeculae, and reduced connectivity are more susceptible to structural failure. Trabecular bone may be more sensitive to fracture because it has a large surface-to-volume ratio that provides a rapidly exchangeable calcium (Ca) pool that is drawn upon in times of need.[6] For example, studies have shown that Ca is liberated from trabecular bone resulting in a decline in BV/TV during lactation[7,8] and in ovariectomized (OVX) rat[9] despite the fact that no significant change was observed at cortical bone-rich sites.

Previous studies suggest that some trabecular bone-rich sites are more sensitive to physiologic challenges. For example, OVX-induced bone loss in mature rats was greatest at the proximal tibia and distal femur (~75%), moderate in lumbar vertebrae (36%), and negligible in cranial and jaw bones (<5%).[10] In addition, while Shin et al.[11] found similar OVX-induced loss of BV/TV at the L4 vertebra and femur in rats, the main effect in the vertebra was reduced trabecular thickness while in femur it was a 6-fold reduction of trabecular number (Tb.N). Similarly, Liu et al.[12] found that lactation changed trabecular bone microarchitecture in vertebra from plates into rods while it reduced trabecular connectivity at the proximal tibia and distal femur.

Thus, physiologic changes known to influence bone mass and fracture risk can have differential effects depending on the bone site examined.

Research shows that adequate dietary Ca intake is necessary for maximizing bone mass accumulation and structural development during growth.[13] However, some studies show that Ca supplements increase BMD more at the femoral neck than at the lumbar vertebra[14][15,16] and others show that Ca fortified foods[17] increase BMC at the humerus and radius-ulna but not lumbar vertebra, femur, or tibia. Inconsistencies regarding the impact of dietary Ca on bone in clinical trials could result from a number of factors including the length of treatment, the level of Ca intake, pubertal status, or race. Carefully controlled studies in animal models over the whole growth period may be able to clarify whether there are site-specific effects of dietary Ca on trabecular bone.

In addition to environmental factors, previous studies have shown that distinct genetic determinants define trabecular characteristics at various skeletal sites.[18-21] We previously showed that the genetic diversity in inbred mouse lines can be used to identify the role of genetics in calcium and bone metabolism.[22] Using C57BL/6, C3H/HeN, and BALB/c inbred mouse lines, Buie et al.[23] found that the gain in L3 vertebral BV/TV from 6-12 wks of age was similar across lines but that tibial BV/TV gains were low in C57BL/6, intermediate in BALB/c, and high in C3H/HeN mice. In another study, C3H/HeJ mice had higher distal femur bone volume but lower L5-6 vertebral BV/TV and Tb.N than C57BL/6 mice.[21] Thus, in order to determine the site-specific impact of dietary Ca restriction on bone, we also need to consider the effect of genetics and gene x diet interactions on these responses.

Here we conducted a study to systematically examine how dietary Ca restriction affected the accumulation of trabecular bone mass at the femur and L5 vertebra of growing mice. In

addition, we used a large population of inbred and recombinant inbred mouse lines to interrogate whether genetics affects how trabecular bone responds to low Ca intake at each site. We hypothesized that peak trabecular bone mass and microarchitecture at the distal femur and L5 vertebra are differentially regulated by dietary Ca restriction and the site-specific response to low Ca intake is regulated by genetics.

2.3 Materials and Methods

2.3.1 Experimental Design

To model the genetic diversity present in humans, we used a large, genetically-diverse population of mice for our experiment. This population included 11 common laboratory 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)) and 51 BXD recombinant inbred (RI) mouse lines that are defined by a fixed recombination pattern of alleles from the B6 and DBA inbred mouse lines.[24] This population of mice encompasses three mouse subspecies (*Mus musculus domesticus, M.m. musculus,* and *M.m. castaneus*), includes classical inbred strains as well as more genetically divergent wild-derived inbred lines, and represents the parental strains of available genetic mapping resources.[25] Four-week-old male mice from the 62 lines were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). An equal number of mice from each line (n = 8 for all lines except BXD36 where n=4) were randomly assigned to either a 0.5% (basal) Ca or 0.25% (low) Ca diet (AIN93G base with 200 IU vitamin D_3/kg diet, Research Diets, New Brunswick, NJ, USA). The dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca)[26] or to model the low level of dietary Ca intake seen in the U.S. population (0.25% Ca).[27,28] Mice were group-housed (2-4 mice/cage) at the Purdue University animal facilities in conventional shoebox cages, maintained in rooms with

UV-blocking filters over lights and a 12-hour light/dark cycle, and provided food and distilled water *ad libitum*. At 12 wks of age, at a point where Buie et al. have shown that mice reach peak trabecular bone mass,[23] mice were fasted overnight. Right femora and L5 vertebrae were harvested and prepared for analysis as previously described.[29] Investigators were blinded to genotype and dietary treatment during allocation, animal handling, bone sample collection and endpoint measurements. All animal experiments complied with the ARRIVE guidelines and the Purdue Animal Care and Use Committee approved the experimental protocol.

2.3.2 Micro-computed Tomography (μCT) Evaluation

Femora and L5 vertebrae were analyzed using μCT (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) using settings reported elsewhere[22] with one exception. For all samples, the regions of interest were binarized using a global threshold (474.3 mg HA/cm^3 for femur and 559.6 mg HA/cm^3 for L5 vertebra). For the femur, the region of interest was defined as 56 slices starting from the first slice containing no evidence of distal growth plate. The L5 vertebra was selected for evaluation over the other lumbar vertebrae because it is the most common vertebra reported in the literature, particularly in genetic studies.[30-32] The entire L5 vertebra was scanned at a voxel size of 16 μm and trabecular bone was evaluated in a 50-slice region distal to the cranial growth plate. This region was chosen as it correlates well with the trabecular bone parameters of the entire L5 vertebra.[33,34] For both sites, we manually contoured trabecular bone every 10 slices with the outline 2-3 pixels away from the cortical bone, and the intermediate slices were interpolated with the contouring algorithm in the Scanco's 3D analysis software to create a volume of interest. We reported the measurements for bone volume fraction (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn.D, 1/mm^3) and structure model index (SMI) as recommended elsewhere.[35] SMI is

an indicator of the shape of trabeculae; a value of 0 reflects trabeculae shaped like plates while a value of 3 reflects trabeculae shaped like cylindrical rods.[35]

2.3.3 Statistical Analysis

Statistical analysis was conducted using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC). Data points with a Z-score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. **Supplemental Table S2.1** reports the number of mice in each genotype and dietary treatment group for each µCT parameter after outlier removal. In addition to the data obtained from each mouse on each diet, a parameter reflecting the response to dietary Ca restriction (RCR) was calculated as the percentage difference between the phenotypic value for an individual fed the 0.25% Ca diet and the line mean from the 0.5% Ca diet, standardized to the line mean from the 0.5% Ca diet.[22] Analysis of covariance (ANCOVA) was used to test the main effects and interaction effects on each parameter and their RCR while controlling for the covariate effects of body weight (BW) and/or femur length (FL).[36] Repeated measure ANCOVA was used when site was a main effect in the model to account for within-subject covariance. When the residuals from ANCOVA were not normally distributed, the following transformations were used: BV/TV and Conn.D ($\sqrt{y}$), Tb.Th and Tb.Sp (log 10), and SMI ($[y]^2$).

Data were initially analyzed as a genetically diverse population and one-way ANCOVA was used to determine the impact of diet or site on the µCT parameters. Next, we used two separate approaches to assess whether there was a site-by-diet (SxD) interaction affecting µCT parameters. First, we used two-way, repeated-measure ANCOVA to examine the main effect of diet and site as well as their interaction. Second, we assessed the effect of site on the RCR parameters using one-way, repeated-measure ANCOVA.

Relationships between parameters were determined using Pearson's correlation tests. First, we investigated correlations among µCT parameters without regard for the genetic structure of our population. This analysis was performed separately for each site as well as for each diet group. Second, we examined the correlations between the basal femoral and vertebral µCT parameters. Third, we accounted for the genetic structure of our population by using covariate-corrected least square means (LSMeans) of each genetic line as measure of the parameter for each "genetic individual" (n=62 mouse lines). We then used these line means to assess the correlation between parameters of "genetic individuals" fed the basal diet or the 0.25% Ca diet (LSMeans shown in **Supplemental Table S2.2**). Finally, we used this data to assess the correlation between the µCT parameter of mice fed the basal diet and the RCR parameters.

Lastly, we used the genetic line (genotype) as a predictor variable. Initially we assessed the narrow-sense heritability (h^2) of each µCT parameter using the r^2 from a one-way ANCOVA (main effect = genotype); this was conducted separately for each bone site and for each diet group as well as for their RCR. Interactions among the main effects were investigated for each µCT parameter (i.e. genotype-by-diet (GxD), site-by-genotype (SxG) and GxDxS). The GxDxS interaction affecting each parameter was confirmed using a two-way ANCOVA of SxG interaction on RCR parameters. When site was a main effect, we used a general linear mixed model with the restricted maximum likelihood approach to estimate the main effects and their interactions while accounting for the within-subject covariance.[37] The covariance structure in this analysis was defined as compound symmetry (i.e. constant variance, constant off-diagonal matrix). This analysis assumes that the variance of each uCT parameter is the same at both sites.

The study was powered based on variance estimates for the effect of genetics on femoral µCT parameters in B6 mice (n=8, 30% difference between dietary groups, SD=20% of mean,

α=0.05, power=0.797). Differences were considered significant when $p \leq 0.05$. When a significant F statistic for an interaction was detected, post hoc pairwise comparisons were made using Tukey's HSD with $p \leq 0.05$ considered significant. Data are reported as LSMean ± SEM. For parameters that required data transformation, we report the back-transformed LSMean ± SEM for inclusion in tables and figures.

2.4 Results

2.4.1 Effect of Dietary Calcium Restriction on Trabecular Characteristics in Femur and Vertebra of Growing Mice

In this genetically diverse population of mice, μCT analysis showed that the baseline values for all of the parameters except Tb.Sp were significantly different between femur and L5 vertebra (**Table 2.1**). For example, BV/TV was 18% higher and Conn.D was 23% greater in vertebra compared to femur. In addition, the SMI value showed that vertebral trabeculae were more plate-like than in femur (L5 vertebra = 1.3, femur = 2.3).

At each bone site, BV/TV, Tb.N, Tb.Th, and Conn.D were significantly lower, and Tb.Sp was significantly higher, in mice fed the low Ca diet (**Table 2.1**). In addition, the SMI for both femur and vertebra was higher, reflecting weaker, more rod-like trabeculae at both sites, because of inadequate Ca intake. A significant positive correlation existed between femoral and vertebral RCR for Tb.Th ($r=0.63$, $p<.0001$) and BV/TV ($r=0.55$, $p<.0001$). However, weaker correlations were seen for Tb.Sp ($r=0.27$, $p<.0001$), Tb.N ($r=0.26$, $p<.0001$) and Conn.D ($r=0.17$, $p=0.0004$).

Table 2.1 Trabecular bone of mice fed a low Ca diet was significantly impaired compared to mice fed a basal Ca diet at both the femur and L5 vertebra

μCT Parameter	Femoral Basal Ca Diet	Femoral Low Ca Diet	Vertebral Basal Ca Diet	Vertebral Low Ca Diet
BV/TV	0.1973 ± 0.0038 (n=496)	0.1704 ± 0.0036[a] (n=466)	0.2346 ± 0.0029[c] (n=456)	0.2124 ± 0.0029[a] (n=451)
Tb.N (1/mm)	4.8016 ± 0.0401 (n=468)	4.5204 ± 0.0400[a] (n=469)	4.9046 ± 0.0029[c] (n=454)	4.7754 ± 0.0029[a] (n=454)
Tb.Th (mm)	0.0672 ± 0.0005 (n=467)	0.0646 ± 0.0005[a] (n=467)	0.0600 ± 0.0003[c] (n=456)	0.0573 ± 0.0003[a] (n=454)
Tb.Sp (mm)	0.2043 ± 0.0025 (n=465)	0.2197 ± 0.0027[a] (n=464)	0.2067 ± 0.0018 (n=451)	0.2122 ± 0.0018[a] (n=454)
Conn.D ($1/mm^3$)	96.4854 ± 2.4315 (n=471)	82.6608 ± 2.2572[a] (n=469)	128.3378 ± 1.9635[c] (n=454)	123.2279 ±1.9657[b] (n=453)
SMI	2.2951 ± 0.0267 (n=469)	2.4623 ± 0.0247[a] (n=473)	1.3045 ± 0.0276[c] (n=452)	1.4721 ± 0.0275[a] (n=454)

[a] Significant at $p \leq 0.05$, [b] a trend at $p \leq 0.10$ between the two diet groups.
[c] Significant at $p \leq 0.05$ between sites in the basal diet group.
Values are reported as LSmeans ± SEM (n) from one-way ANCOVA adjusted for BW and FL. Analysis of Tb.Th and SMI were adjusted only for FL.

ANCOVA revealed the existence of significant site-by-diet (SxD) interactions for Tb.N, Tb.Sp, and Conn.D (**Figure 2.1**); a trend for a SxD interaction was seen for SMI (p=0.0658). Consistent with the existence of SxD interactions, we observed significant differences in RCR at femur and spine for all of the μCT endpoints (**Figure 2.2**). The negative femoral BV/TV RCR reflected a retardation in trabecular bone development that is due to loss of trabecular number and connections but the negative vertebral BV/TV RCR was due to a large change in the geometry of trabeculae (i.e. an SMI reflecting a more rod-like structure) as well as a 30% greater decrease of Tb.Th (**Figure 2.2**).

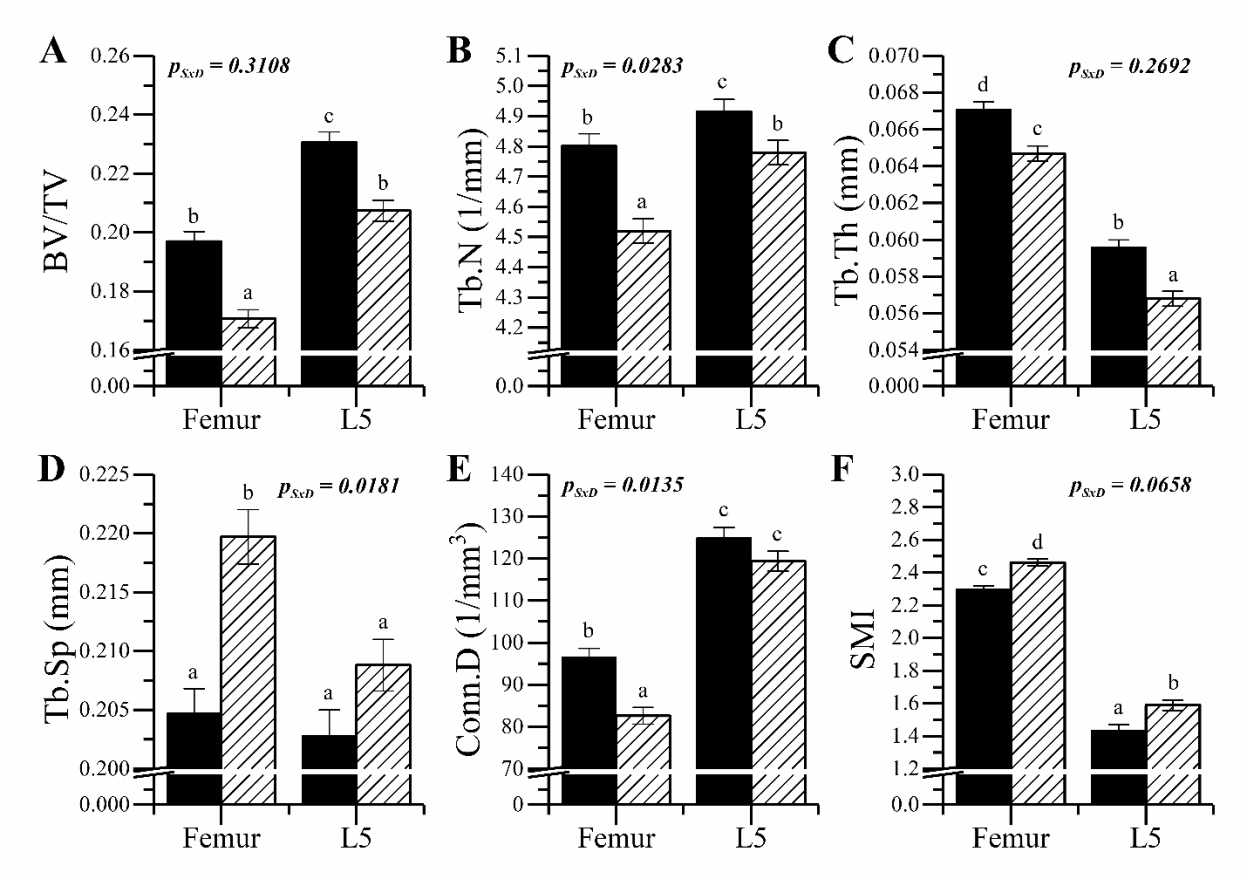

Figure 2.1 *Site-by-diet (SxD) interactions affecting (A) BV/TV, (B) Tb.N, (C) Tb.Th, (D) Tb.Sp, (E) Conn.D, and (F) SMI.* Symbols represent the LSmeans ± SEM from two-way, repeated measure ANCOVA. Filled bar = basal (0.5%) Ca diet group. Hatched bar = 0.25% Ca diet. Values with different letter superscripts are significantly different from one another (Tukey's HSD, $p \leq 0.05$). p_{SxD} = p-value for SxD interaction.

2.4.2 The Relationships between Trabecular Parameters is Altered by Diet and Bone Site

Several well-known relationships between trabecular parameters exist [38] and we observed these relationships among these parameters in the basal diet group at both sites (**Supplemental Table S2.3** (unadjusted), **S2.5** (body-size corrected)). We found similar relationships in mice receiving the 0.25% Ca diet **(Supplemental Table S2.4, S2.6**). There were also correlations between the μCT parameters of the two bone sites but these were generally weak to moderate (e.g. for femur vs. vertebra BV/TV, r = 0.43 in the basal Ca diet group; Supplemental Table S7). Within each site, there was a tight correlation between the basal and the 0.25% Ca diet values for each

trabecular parameter **(Supplemental Table S8**). For example, the correlation in BV/TV between the two diet groups was 0.85 for femur ($p < 0.001$) and 0.88 for vertebra ($p < 0.001$). This shows that dietary Ca intake accounts for approximately 28% and 23% of the variation in femoral and vertebral BV/TV, respectively.

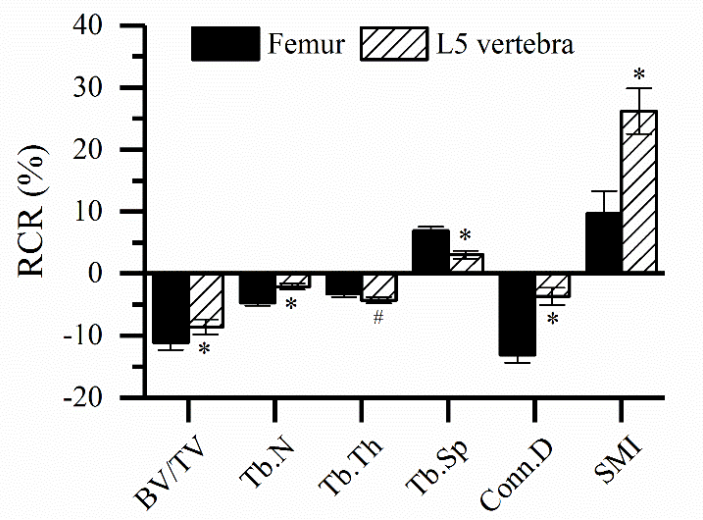

Figure 2.2 *Site-specific differences in the response to dietary Ca restriction (RCR) for trabecular bone µCT in the distal femur and L5 vertebae.* RCR for each µCT parameter in a mouse was calculated using the equation $(X_{\text{low Ca diet}} - X_{\text{line mean, basal Ca diet}} / X_{\text{line mean, basal Ca diet}})*100$. Bars = Mean ± SEM (n= 436-445). Repeated measures one-way ANCOVA was conducted; femur length, but not body weight, was significantly correlated with BV/TV RCR, Tb.Th RCR and Conn.D RCR and was used as a covariate in these analysis. * Significant at $p \leq 0.05$ and # a trend at $p \leq 0.10$.

We next examined the relationship between the µCT parameters of mice fed the basal diet (basal values) and their RCR using LSMeans from each line as measure of the parameter for each "genetic individual" (n=62; **Supplemental Table S2.9**). There was no correlation between basal and RCR values for BV/TV or Conn.D. However, a significant negative correlation was observed between the basal and RCR values at both sites for Tb.Sp (**Figure 2.3A, B**) and Tb.N (**Supplemental Table S2.9**) and for vertebral Tb.Th (**Figure 2.3D**) and SMI (**Supplemental Table S2.9**). This indicates that lines with a high basal phenotype were more sensitive to the low

Ca diet during growth. However, because this relationship was not uniform across two sites, this suggests a site-specific impact of dietary Ca restriction on some µCT parameters.

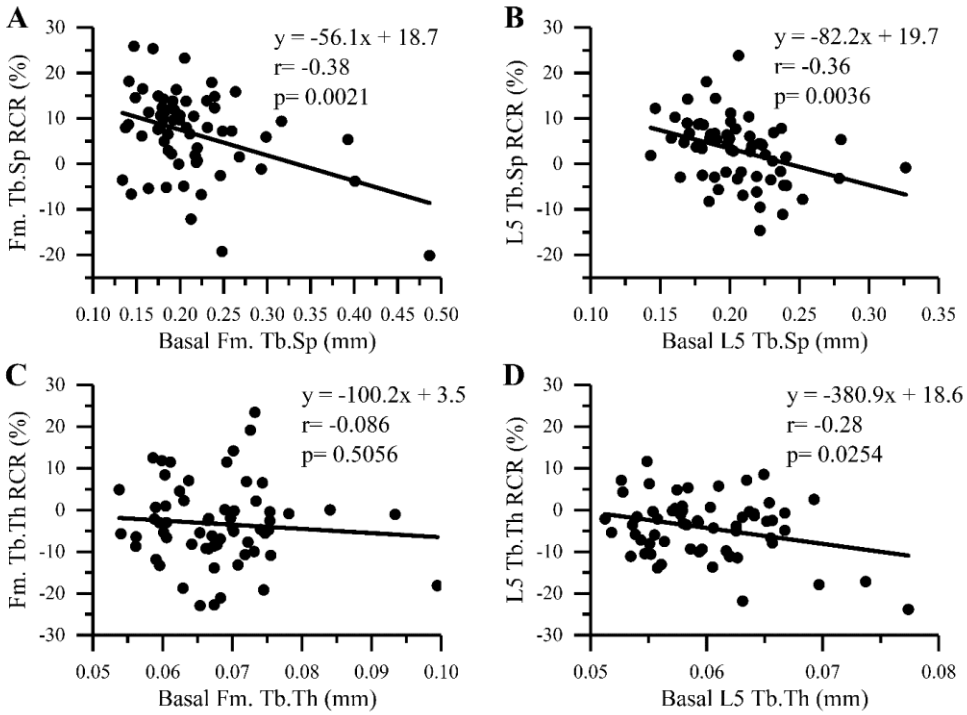

Figure 2.3 *Correlations between the µCT parameters and their corresponding response to dietary Ca restriction (RCR) for (A) femoral Tb.Sp, (B) vertebral Tb.Sp, (C) femoral Tb.Th, and (D) vertebral Tb.Th (D).* Pearson's correlation tests were conducted on body size-corrected LSMeans (n=62 genetic lines). A slope and an intercept for each linear correlation are reported. x = the µCT parameter of mice fed the basal diet, y = the corresponding RCR, r = correlation coefficient. p = p value of the regression line. Fm. = femur; L5 = L5 vertebra.

2.4.3 Genetics regulates basal trabecular parameters as well as the ability to adapt to dietary Ca restriction at each bone site

Because our mouse population has a defined genetic structure reflected in the mouse lines, we investigated the influence of genetics on the basal µCT parameters or their RCR. Each µCT parameter was significantly influenced by genetics ($p<0.0001$). The narrow sense heritability (h^2) was high for all of the µCT parameters in both femur ($h^2= 0.56$ to 0.82) and vertebra ($h^2= 0.59$ to 0.71) and these heritability estimates were not significantly reduced in mice fed the 0.25% Ca diet

Table 2.2 Heritability estimates (h^2) of trabecular parameters and their RCR*

µCT Parameter	Diet	Femoral h^2	Vertebral h^2
BV/TV	0.50%	0.65	0.68
	0.25%	0.63	0.64
	RCR	0.37	0.31
Tb.N	0.50%	0.79	0.70
	0.25%	0.80	0.71
	RCR	0.32	0.30
Tb.Th	0.50%	0.56	0.59
	0.25%	0.58	0.51
	RCR	0.44	0.36
Tb.Sp	0.50%	0.82	0.71
	0.25%	0.84	0.69
	RCR	0.33	0.30
Conn.D	0.50%	0.74	0.69
	0.25%	0.70	0.69
	RCR	0.28	0.36
SMI	0.50%	0.58	0.69
	0.25%	0.56	0.67
	RCR	0.41	0.58

*RCR = Response to dietary Ca restriction. Values were r^2 from one-way ANCOVA (main effect = genotype) adjusted for femur length; the analysis of L5 Tb.N, Conn.D, and their RCR adjusted for body weight.

(**Table 2.2**). In contrast, the heritability of the RCR parameters were more moderate than basal estimates both in the femur (h^2=0.28 to 0.44) and the vertebra (h^2=0.30-0.58).

Two-way ANCOVA revealed a genotype-by-diet (GxD) interaction affecting BV/TV, Tb.Th, Tb.N, Conn.D, and SMI in the femur and L5 vertebra. In addition, there was a trend towards an interaction for Tb.Sp (p=0.0685). Consistent with the two-way ANCOVA analysis, a significant genotype effect was observed for all of the RCR parameters at both sites. The Z-scores for µCT RCR parameters revealed significant variation across lines at both sites (representative data for BV/TV RCR and Conn.D RCR are shown in **Figure 2.4**; data for other parameters are shown in **Supplemental Figure S2.1**).

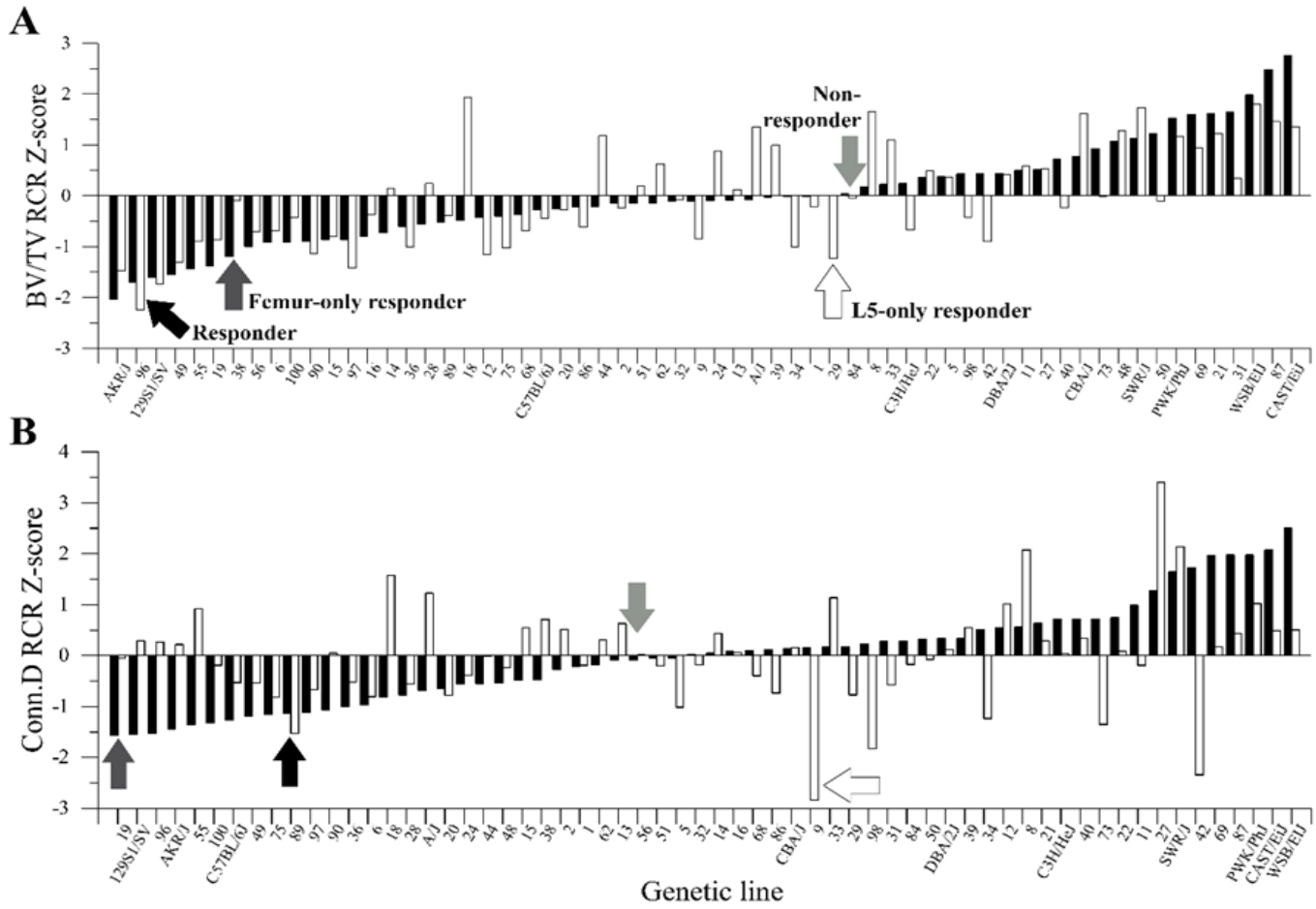

Figure 2.4 *Site-by-genetic interactions affect the response of trabecular parameters to dietary Ca restriction (RCR). (A) BV/TV RCR, (B) Conn.D RCR.* Data are presented as Z-scores. The x-axis reports the 51 BXD RI lines as numbers along with abbreviations for the 11 inbred mouse lines. Filled bar = distal femur. Open bar = L5 vertebra. Arrows identify four mouse lines that represent the site-specific responses to low dietary Ca intake at each bone site. Light grey arrow = neither site responds. Black arrow = both sites respond. Dark grey arrow = femur-only responder. White arrow = L5 vertebra-only responder

Finally, we tested whether complex interactions between diet, genetics, and site were affecting μCT parameters using two different approaches: three-way ANCOVA on trabecular parameters and site-by-genotype (SxG) interactions on RCR parameters. Three-way ANCOVA showed that a site-by-diet-by-genotype interaction affected BV/TV ($p=0.0073$), Tb.Th ($p=0.0137$), Conn.D ($p=0.0111$), and SMI ($p=0.0108$). A representative example of this complex interaction is illustrated for Conn.D in Figure 5; data from other significant parameters are shown in **Supplemental Figure S2.2**. Z-scores from the baseline and the 0.25% Ca group varied across the 62 genetic lines and the patterns of variation differed between the femur (**Figure 2.5A.a.) a**nd the vertebra (**Figure 2.5A.b.**). For example, the low Ca diet reduced Conn.D equally at both sites in BXD20 mice, while BXD56 mice were resistant to Ca restriction at both sites (**Figure 2.5B.a.**). In contrast, we observed differential tissue responses for BXD28 (femur only) and BXD98 (L5 only) (**Figure 2.5B.b.**). This demonstrates the complexity of how diet and genetics interact to influence trabecular bone in each site during growth. Consistent with this, a significant SxG interaction was observed for all of the RCR parameters (data not shown).

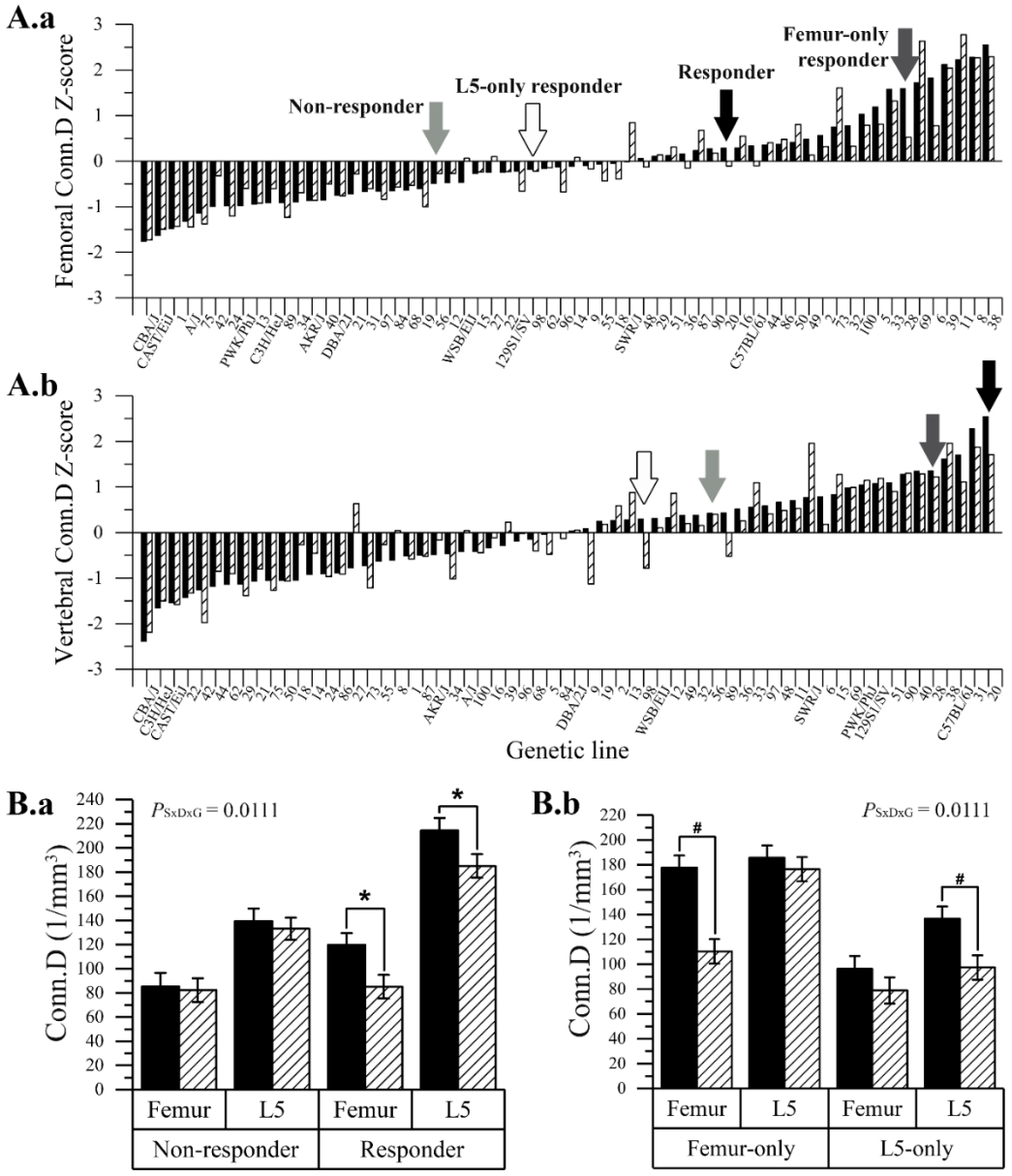

Figure 2.5 *Representative data from Conn.D for the complex Site-by-Diet-by-Genetics interaction regulating trabecular parameters.* (A) Z-score from each genetic line of femoral Conn.D (A.a.) and vertebral Conn.D (A.b.) are shown. The x-axis reports the 51 BXD RI lines as numbers along with abbreviations for the 11 inbred mouse lines. Black bars = 0.5% Ca diet group. Hatched bars = 0.25% Ca diet group. Arrows identify four mouse lines that are representative of the types of responses to low dietary Ca intake at the two sites. Light grey arrow = no response to diet at either site. Black arrow = lower response to diet at both sites. Dark grey arrow = a response to diet a just the femur. White arrow = a response to diet at just the L5 vertebra. (B) Bar charts showing the Conn.D values for lines identified with arrows in panel (A). Bars represent LSMeans of Conn.D from each group. Black bars = 0.5% Ca diet group. Hatched bars = 0.25% Ca diet group. (B.a.) A non-reponder line (BXD56) and a responder line (BXD20). (B.b.) A L5-only responder line (BXD98) and a femur-only responder line (BXD28). p_{SxDxG} = p-value for SxDxG interaction. P-values for pairwise comparisons between diet groups within each site and line: * $p<0.005$, # $p<0.05$.

2.5 Discussion

There are several important findings from our study. First, we show that dietary Ca is a critical factor regulating the development of peak trabecular bone mass, even in a genetically diverse population. Thus, this study overcomes a weakness of other mouse studies of inbred mice, where the effects may not be generalizable, and addresses a question that cannot be ethically tested in human children. Second, we show that there are site-specific differences in the impact of inadequate dietary Ca on trabecular bone. Finally, we report that genetics has a significant impact on trabecular bone at both spine and femur and that these genetic effects influence how trabecular bone responds to low Ca intake. Thus, our data reveal that while maintaining an adequate dietary Ca intake is a good general strategy for maximizing peak bone mass, they also show that the benefit of adequate Ca is not uniform across individuals or across bone sites.

Other groups had attempted to determine the impact that low Ca intake has on trabecular bone microarchitecture in controlled animal studies using Ca restriction in the context of low protein diets[39] or with very low dietary Ca intake.[40] However, our study is unique in that it captures the effect of a modest, and human relevant, dietary Ca inadequacy (i.e. 50% of the requirement[27,28]) on attainment of trabecular bone during growth. We found that dietary Ca restriction attenuated trabecular bone accrual in both femur and vertebra but that the impact on trabecular bone microarchitecture was distinct at each site, i.e. lower Tb.N and Conn.D in the femur but reduced Tb.Th with increased SMI in the vertebra. Reduced Tb.N and Conn.D has a greater consequence on the mechanical integrity of trabecular bone than does an equal reduction in Tb.Th and the shape of trabeculae. This because as trabeculae and their interconnectivity are lost bone structure is significantly weakened.[12,41,42] Thus, the reduction of femoral Tb.N and Conn.D we observed due to low dietary Ca intake could make this site more sensitive to future fracture compared to the vertebra. Fortunately, it may be possible to restore bone strength

following a period of diet-compromised bone development provided a substantial number of trabeculae are still intact.[42] For example, although lactation induced the thinning of trabeculae and the perforation of trabecular plates in the vertebrae of mice, this effect was reversed because of the improved calcium balance that results from weaning.[12,43] In contrast, weaning did not improve the loss of Tb.N and connectivity that was caused by lactation in femur.

Another unique feature of our study is that we used 62 lines of mice to represent the high level of genetic diversity observed in humans. This allowed us to address whether genetics modulates the response of each skeletal site to low dietary Ca intake. Here, we found significant genotype-by-diet-by-site interactions affecting trabecular parameters (**Figure 5**). This extends our previous report in 11 inbred mouse lines, where we identified significant genetic effects on the response of BMD and trabecular parameters to dietary Ca restriction in the distal femur.[22] The complex interaction among diet, genetics, and site was seen in two ways: in the pattern of variation of the femoral and vertebral RCR parameters across lines (Figure 4 and Supplemental Figure S1) and in the differential heritability estimates for the RCR parameters between the two sites (e.g. Conn.D: L5 vertebra= 0.36, femur= 0.28; **Table 2**). Collectively, this indicates that genetics influences the site-specific effect of Ca insufficiency on the development of trabecular bone during growth.

Our study is the first to carefully test the independent and interactive effects of dietary Ca restriction and genetics on trabecular bone mass accrual and microarchitecture at two clinically relevant skeletal sites. There are many strengths to our study. First, we conducted our study under strictly controlled environmental conditions that included the use of a well-defined, semi-purified experimental diet. Second, by coupling the controlled dietary intervention to a large, genetically diverse population of mice, we could assess the effects of site and diet on trabecular bone in ways

that are difficult to do in humans. Finally, we could take advantage of the genetic structure of our population to identify relationships between genetics and the site-specific response of trabecular bone to low Ca intake. Nonetheless, there were certain limitations for our study. First, we only measured trabecular parameters at the 12 week of age. Therefore, we do not know what happens in each stage of growth or whether low dietary Ca intake delays, rather than blocks, peak bone mass accretion. Furthermore, our study was conducted only in male mice. Previously, we showed that female C57BL/6 mice have a more robust physiologic response to dietary Ca restriction, and are more responsive to changes in serum 1,25-dihydroxyvitamin D, than males.[44] As a result, our current findings may not apply directly to trabecular bone mass accrual in females. Lastly, we did not test for mechanical properties of our bone samples. Future studies using finite element analysis may provide more insight in this matter.

The critical finding of our study is that dietary Ca deprivation during growth leads to site-specific differences in the development of trabecular bone microarchitecture at the femur and L5 vertebra. In addition, we report for the first time that genetics has a site-specific influence on how trabecular bone responds to low dietary Ca intake. The significance of our work is that dietary Ca restriction and genetics work both independently and interactively to influence the attainment of peak trabecular bone mass and its microarchitecture. Furthermore, these interactions are not uniform across bone sites. In the future, it will be important to identify the genetic variants that underlie the response to low dietary Ca intake at each skeletal site.

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2.7 Author's roles

Study design: JCF; Study conduct: JCF; Bone analysis: PRF and KC; Data management, QTL analysis, and statistical analysis: KC; Data interpretation: JCF, PRF, and KC; First draft of manuscript: JCF and KC; Revision of manuscript and approval of the final version of the manuscript: JCF, PRF and KC. JCF takes responsibility for the integrity of the data analysis.

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CHAPTER 3. QUANTITATIVE TRAIT LOCI FOR TRABECULAR BONE MASS AND MICROARCHITECTURE TRAITS AND THEIR DIETARY RESPONSES TO CALCIUM RESTRICTION IN MALE BXD RECOMBINANT MOUSE STRAINS

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Supplemental data have been included with the submission.

3.1 Abstract

Genetics and dietary calcium (Ca) are key regulators for the development of trabecular bone (Tb). We have reported that the impact of low dietary Ca on femur BMD varies depending on genetic background in mice. Here, we extend this work by examining the impact of diet and genetics on Tb parameters in the distal femur. Mice from 51 BXD recombinant inbred lines were fed either adequate (Basal, 0.5%) or low (L, 0.25%) Ca diets from 4-12 wks of age. We used micro-computed tomography (μCT) to measure: bone volume fraction (BV/TV), Tb tissue mineral density (Tb.TMD), thickness (Tb.Th), separation (Tb.Sp), number (Tb.N), connectivity density (Conn.D), and structure model index (SMI). A "response to dietary calcium restriction" (RCR) parameter was calculated for each trait in each line. Body size (BS)-corrected residuals were used for statistical analysis, genetic mapping, and to estimate narrow sense heritability (h^2). Genetics had a strong, independent impact on Tb traits (e.g. BV/TV-Basal, h^2=0.60) but a more modest influence on the RCR (e.g. BV/TV, h^2=0.32). Using quantitative trait loci (QTL) mapping (CIM, 2244 markers, 1000 permutations, WinQTLCart), we identified 1-6 loci affecting each trait and 1-3 loci affecting the RCR for each trait. Loci on chr 6 (BV/TV, Tb.N, Tb.Sp, SMI, Conn.D), chr 9 (Tb.Th, Tb.TMD) and chr 12 (Tb.N, Tb.Sp) were seen in both diet groups providing replication of environmentally robust genetic effects. In addition, several loci control multiple phenotypes suggesting the existence of latent traits. Some of these loci are insensitive to dietary Ca environment (i.e. the chr 6, 9, and 12 loci above and chr 19 (BV/TV-Basal, Conn.D-Basal); many are sensitive to dietary Ca condition including the loci on chr2 (@161.4 Mb: Conn.D-L, Conn.D-RCR, @176.6 Mb: Conn.D-RCR and BV/TV-RCR), chr 7 (SMI-RCR, Tb.Th-Basal), chr 8 (Tb.TMD-RCR, Tb.N-RCR), and chr 15 (SMI-RCR, Tb.TMD-RCR) Candidate genes underlying select loci were identified with PROVEAN (protein coding effects) or expression QTL (eQTL) analysis in The GeneNetwork. In the chr 6, there is a deleterious coding variant in *Sumf1*, which

is a regulator of proteoglycan desulfation, and an eQTL in *Cidec* which regulate cell apoptosis. In the chr 12 there were three non-synonymous coding variants in *Serpina3n*, a serine protease inhibitor that suppresses osteoblast phenotypes. In the chr 19 loci was a cis eQTL for *Papss2*, a protein that regulates the sulfation of extracellular matrix in bone and whose mutation causes spondyloepimetaphyseal dysplasia in humans. In the first locus on chr 2 (@161.4 Mb), there were a non-synonymous mutations in *Wisp2* and *Cd40*. The former promotes osteoblast differentiation while the latter mediates OPG production which regulates bone remodeling In the other locus on chr 2 (@176.6 Mb) were a cis eQTL and a frameshift mutation affecting *Ogfr*, a gene that suppresses osteogenesis. In the chr 15 locus, there were three deleterious non-synonymous coding variants in *Cyp2d11*, a 25-hydroxylase that convert vitamin D_3 into 25-hydroxyvitamin D_3 in the liver. Our work shows that genetics works independently and interacts with dietary Ca to influence Tb phenotypes in distal femur.

3.2 Introduction

Overall bone strength is a strong predictor for osteoporotic fractures. In the clinical setting, bone mineral density (BMD) and bone mineral content (BMC) are the gold standard for evaluating the risk of osteoporosis and fractures. However, bone mass is only one aspect of whole bone strength. Bone strength is also determined by bone material properties as well as bone geometry and microarchitecture.[1] In addition, fractures commonly occur at trabecular bone (Tb)-rich sites such as femur and spine.[2,3] Tb consists of connecting plates and rods and connects to bone cortex. Cadaver studies of Tb parameters have shown that reduced Tb volume fraction, number and connectivity are strongly associated with susceptibility to structural failure or bone breakage.[4,5] Consistently with this line of evidence, postmenopausal women with fractures had significantly weaker, less connecting Tb than postmenopausal women without fractures.[6] Because Tb has a

high surface-to-volume ratio it is very responsive to physiologic needs for maintaining serum Ca, e.g. during lactation,[7-9] and in ovariectomized rats.[10] In order to prevent osteoporotic fractures later in life, it is of paramount to accumulate high peak bone mass as well as establish a highly connected Tb microarchitecture.

Bone phenotypes, including Tb microstructure, are under genetic regulation.[11-13] These phenotypes display continuous distributions within the population and are considered to be quantitative traits. These traits are influenced by several genes in concert; genome-wide association studies (GWAS) in humans and genetic mapping studies in mouse models[13-19] have reported many chromosomal locations or quantitative trait loci (QTLs) underlying normal variations of bone features. QTL mapping studies in mice revealed genetic loci controlling Tb phenotypes that are not observed in the studies of BMD and BMC.[15,16] Recently, Paternoster et al. used peripheral quantitative computed tomography (pQCT) to conduct the only GWAS that examined Tb volumetric BMD (vBMD) in humans. This study found that genetic loci related to cortical vBMD differ from those that were linked to trabecular vBMD. Consistent with this observation, Lu et al. confirmed that strong QTLs mapped to cortical bone did not overlap with QTLs controlling Tb in a genetic mapping experiment using BXD recombinant inbred (RI) lines.[20] Collectively, these studies suggest there are unique genetic regulators for Tb microarchitecture that are hidden in the genetic analysis of BMD or distinct from those genetic regulators controlling cortical bone phenotypes.

Environmental factors like dietary calcium (Ca) play an important role in bone development.[21] Though dietary Ca has an independent impact on bone by supplying Ca for bone mineralization, its effect is modified by genetics. For example, in two experiments where subject were fed diets with varying amounts of Ca (760 to ~2000 mg/d), adolescent African American

girls had greater skeletal Ca retention, higher 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and higher Ca absorption than tanner stage-matched white girls.[22,23] Similarly, we have shown that both basal bone and Tb phenotypes as well as their response to low dietary Ca intake are highly variable across 11 genetically diverse inbred mouse lines.[24] Because a majority of the U.S. population has inadequate Ca intake,[25] identifying genetic variants that regulate the differential responses of individuals to low Ca intake is an integral step toward developing personalized dietary interventions that promote optimal bone mass. Towards this end, Reyes-Fernandez et al.[26] conducted a genetic mapping in 51 BXD RI mouse lines and identified candidate genes underlying the genetic response of Ca absorption, BMD and BMC to dietary Ca restriction during growth. Unfortunately, no studies have examined the GXD interactions affecting the development of trabecular bone.

Here we have used a panel of 51 genetically distinct BXD RI mouse lines raised under a strictly controlled environment and fed diets with adequate or low Ca levels. By using a large diverse population of isogenic lines, we were able to control both the genetics and the dietary environment that influence the development of Tb mass and microarchitecture. Thus, this study allows us to systematically test whether GxD interactions influence Tb phenotypes and to identify QTLs that influence these Tb phenotypes. Finally, by coupling our QTL analysis to bioinformatic analysis, we were able to identify candidate genes that control these phenotypes.

3.3 Materials and Methods

3.3.1 Experimental Design

We used a population of 51 BXD recombinant inbred (RI) mouse lines that are defined by a fixed recombination pattern of alleles from the C57BL/6J (B6) and DBA/2J (DBA) inbred mouse lines.[27] Four-week-old male mice from the 51 lines, as well as mice from the two parental lines,

were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, an equal number of mice from each line (n = 8 for all lines except BXD36 where n=4) were randomly assigned to either a 0.5% (basal) Ca or 0.25% (low) Ca diet (AIN93G base with 200 IU vitamin D_3/kg diet, Research Diets, New Brunswick, NJ, USA). The dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca)[28] or to model the low level of dietary Ca intake seen in the U.S. population (0.25% Ca).[25,29] Mice were group-housed (2-4 mice/cage) at the Purdue University animal facilities in conventional shoebox cages, maintained in rooms with UV-blocking filters over lights and a 12-hour light/dark cycle, and provided food and distilled water *ad libitum*. At 12 wks of age, at a point where others have shown that mice reach peak trabecular bone mass,[30] the mice were fasted overnight afterwhich the right femora were harvested and prepared for analysis as previously described.[26] Investigators were blinded to genotype and dietary treatment, animal handling, bone sample collection, and endpoint measurements. All animal experiments complied with the ARRIVE guidelines and the Purdue Animal Care and Use Committee approved the experimental protocol.

3.3.2 Micro-computed Tomography (µCT) Evaluation

Femora were analyzed using µCT (µCT 40, Scanco Medical AG, Bassersdorf, Switzerland) with scanning parameter settings reported elsewhere.[24] Briefly, femur was scanned at 16-µm resolution (isotropic voxel size) using an energy level of 55 kVp, an integration time of 300 ms, and an intensity of 145 µA.

Femur length was measured using an electronic digital caliper and then placed in groups of 9 bones with similar lengths (within 0.5 mm) for scanning. Each group of nine bones was placed in a 15-mm-diameter poly-ether-imide (PEI) sample holder filled with 70% ethanol and immobilized with cotton balls. A 2D scout view of the chamber was examined from multiple

angles (0, 90, 180 and 270 degrees) to determine the location of the apex of distal condyles among the 9 bones. The scan captured 1.664 mm (104 slides) proximal to this point. This larger region ensured that we captured all slices required for morphometric evaluation of each bone in the chamber.

Using Scanco evaluation software (version 6), each femur was assessed for bone volume fraction (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn.D, $1/mm^3$), structure model index (SMI), and trabecular bone tissue mineral density (Tb.TMD) as recommended elsewhere.[31] The region of interest was defined as 0.896 mm (56 slices) proximal to the first slice containing no evidence of distal growth plate. We manually contoured trabecular bone every 10 slices with the outline 2-3 pixels away from the cortical bone, and the intermediate slices were interpolated with the contouring algorithm in the software to create a volume of interest. Morphometric parameters were evaluated using a Gaussian filter = 0.8 and a threshold of 220 in the 1/1000 unit (474.3 $mgHA/cm^3$).

3.3.3 Statistical Analysis

Statistical analysis was conducted using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC). Data points with a Z-score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. **Supplemental Table S3.1** reports the number of mice in each genotype and dietary treatment group for each µCT parameter after outlier removal. In addition to the data obtained from each mouse on each diet, a parameter reflecting the response to dietary Ca restriction (RCR) was calculated as the percent difference between the phenotypic value for an individual (i) fed the low Ca diet (x) and the line (j) mean for the phenotype value from the basal Ca diet (y), standardized to the line mean for the phenotypic value from the basal Ca diet and multiplied by 100, i.e., $\left[\left(x_{ij} - \bar{y}_j\right) / \bar{y}_j\right] * 100$.[24] For each phenotype, the covariate effect of body

weight (BW) and/or femur length (FL) was determined by Pearson's correlation and removed by linear regression when the effect on a parameter was significant.[32] When the body size (BS)-corrected residuals were not normally distributed, the following transformations were used: BV/TV ($\sqrt{y}$), and Tb.Th and Tb.Sp (log 10).

Line means of BS-corrected residuals (as we called BS-corrected line means) of the 51 BXD RI lines for each dietary condition (basal, low Ca and RCR) were used for genetic mapping (**Supplemental Table S3.3)**. To reflect the variation among lines, Z-scores were calculated from the line means of BS-corrected residuals of the 51 BXD RI lines and their parental lines (**Supplemental Table S3.5)**. We assessed the narrow-sense heritability (h^2) of each Tb phenotype using the r^2 from a one-way Analysis of variance (ANOVA) (main effect = genotype); this was conducted separately for each diet group as well as for their RCR. Two-way ANOVA of BS-corrected residuals was used to test the main effects and interaction effects (i.e. genotype-by-diet, GxD) on each phenotype. GxD interactions were also determined by conducting one-way ANOVA to assess the impact of genetics on the RCR. Using 51 BXD RI lines allowed us to reliably detect QTL accounting for 15% of the variance observed in the population with the power ~ 0.80 and $\alpha = 0.05$.[26,33]

3.3.4 QTL Mapping

BXD genetic markers were downloaded from the GeneNetwork (http://www.genenetwork.org/genotypes/BXD.geno). For the 198 BXD strains, the file contains 7321 markers and provides approximate locations of 10,300 recombinations, an average of 52 per strain (http://www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600). Genetic marker locations are reported in base pairs (bp) based on the mouse genome build GRCm38/mm10. The Mouse Map Converter (MMC) tool on the Jackson Lab Center for Genome

Dynamics (http://churchill-lab.jax.org/mousemapconverter/) was used to convert mm10 coordinates to sex-average centiMorgan (cM) values that were then used for QTL mapping analysis. Many of the original markers were not informative due to the reduction of lines (from 198 to 51 lines). After excluding genotype data of lines outside the 51 lines, we used the findDupMarkers and drop.marker functions available in the R/qtl package (https://cran.r-project.org/) to remove markers with duplicate genetic locations or perfectly correlated genotypes. The final genetic map for the 51 lines contained 2244 markers (available on request).

Composite interval mapping (CIM) was conducted on BS-corrected line means (n=51) using Windows QTL Cartographer v2.5_011 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). 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. Phenotypes for each diet (0.5% or 0.25% Ca) group and the RCR were mapped separately. For each analysis, permutations (n=1000) were used to determine significance threshold in a logarithm (base 10) of odds (LOD) unit. A peak with LOD ≥ the computed permutation threshold was considered significant and a peak with LOD ≥ 2 was considered putative.[34]

In significant or putative QTL, the 1.5-LOD support region was used for candidate gene identification.[35] This region is considered as an equivalent to 95% confidence interval.[36,37] The downstream and upstream boundaries of this region in cM were converted to mm10 base pair positions (GRCm38) using the MMC tool.[38]

3.3.5 Prioritization of QTL Candidate Regions

Because we conducted QTL mapping analysis on multiple correlated Tb phenotypes, we observed several QTLs from multiple phenotypes that co-localized (i.e. the same peak estimate, peaks located within 5 cM of each other, or overlapping 1.5-LOD intervals). We grouped the co-

localized QTLs in each region of a chromosome into a single locus and assigned an identification (loci ID). A comprehensive list of these loci with the assigned loci ID is shown in **Supplemental Table S3.7**.

Using this list of loci, we then selected high-priority loci for further investigation. We prioritized loci based on the following criteria:

1. A significant QTL of a single trait with an LOD >5
2. A significant QTL observed in an RCR phenotype
3. Significant (or one significant with one putative with LOD ≥ 3) QTLs observed for the same phenotype in both the basal and low Ca groups and with a matched parental influence.
4. Two or more QTLs (one must be significant, and the other(s) must have LOD ≥ 3) observed within the same condition (basal, low Ca or RCR) or within the low Ca group and RCR with a matched parental influence.

We gave a score of 1 for each criterion that was met. These criteria are not mutually exclusive, e.g. a single locus may earn a score of two if it has an LOD > 5 and it is a significant RCR QTL. The loci with the score of 2 or greater were considered as *high-priority* loci that were then examined with in-depth bioinformatics analysis.

3.3.6 Bioinformatic Characterization of Loci

For each locus, single nucleotide polymorphisms (SNPs) between B6 and DBA mice were obtained using the Mouse Phenome Database (MPD, https://phenome.jax.org/snp/retrievals)[39] with the Sanger4 data set that contains data on >89 million SNP and indels from 37 inbred strains of mice.[40] MPD annotations (NCBI dbSNP 138) were used to categorize polymorphisms by gene attribute: i.e. intronic and non-coding, insertions/deletions, mRNA untranslated region (5'and 3'

UTR), and exon-associated (i.e. synonymous and non-synonymous codons, stop codons, splice sites, or frameshift mutations). Genes with SNPs that lead to gain or loss of a stop codon, cause a frameshift, and or alter a splice site were automatically considered candidate genes. Effects of non-synonymous amino acid changes were examined for potential functional effects using PROVEAN v1.1 (score < -2.5) [41] and SIFT v4.0.3 (score < 0.05).[42] Genes with deleterious/damaging SNPs identified by at least one tool were considered as candidate genes.

3.3.7 Expression QTL (eQTL) Mapping

Local (*cis*) eQTL analysis was conducted *in silico* using publicly available microarray data from the femur mRNA of the BXD panel (GN accession: GN414) that is available at the GeneNetwork (www.genenetwork.org/webqtl/main.py). Searches were conducted on the 1.5-LOD support interval of each locus. We defined the LRS threshold as > 9 (~ LOD = 2) to capture both significant and putative eQTL. Of these eQTL, we examined the correlation between the abundance of a gene transcript within the cis eQTL and BS-corrected line means of each phenotype mapped to the locus. Genes whose expression level correlated with our phenotypes (Pearson correlations $r > 0.3$, $p < 0.10$) are considered as candidate genes.

3.3.8 Annotation of Potential Candidate Genes

We identified the final list of candidate genes that are likely to have function altering polymorphisms influencing bone microarchitecture by searching the literature and public databases for information that links the candidate genes to bone biology: i.e. those with a significant association with bone phenotypes in GWAS, those shown to have a functional role in bone using KO mice, and those with evidence from animal or cell studies identifying mechanistic role for the candidate gene in bone cell biology or other pathways known to influence bone phenotypes. Associations between gene mutations in candidate genes and bone phenotypes, for

candidate gene expression in limbs or skeletons at different stages of mouse embryo, and for functional annotation of the candidate genes using gene ontology (GO) terms was conducted using the Mouse Genome Informatics database (MGI, www.informatics.jax.org). Tissue expression patterns for each candidate gene were determined in mouse tissues and cell lines using BioGPS (http://biogps.org/). Changes in bone phenotypes resulting from candidate gene deletion were assessed using knockout (KO) mice data from the International Mouse Phenotyping Consortium (IMPC) and the International Mouse Strain Resource (IMSR) databases. Finally, information regarding functional association of the candidate genes to biological pathways relevant to bone metabolism was obtained from Coremine (https://www.coremine.com/medical/).

3.4 Results

3.4.1 The Effect of Genetics, Dietary Ca Intake (D), GXD Interactions on Tb Phenotypes and Their Heritability

All mice were healthy throughout the study. Body weight (BW) and femur length (FL) were significantly different among the lines ($p < .0001$), but they were not affected by dietary Ca intake ($p = 0.36$ and 0.18, respectively). Since there is variation in BW and FL in the BXD population, Tb phenotypic data were examined for the effect of body size and adjustments were made when necessary (see Materials and Methods, **Supplemental Table S3.2**). Body size (BS)-corrected line means for all phenotypes on each diet and RCR are provided in **Supplemental Table S3.3**. For each analysis (basal Ca, low Ca and RCR), there were significant relationships among all phenotypes ($p < .0001$, **Supplemental Table S3.4**) except for Tb.TMD, which is poorly correlated to BV/TV and SMI in the basal and low Ca groups and whose RCR was not correlated with RCR values for Tb.N, Tb.Sp or Conn.D.

A significant effect of dietary Ca intake was found to affect all Tb phenotypes ($p < .0001$) except Tb.TMD ($p = 0.3482$). There was significant variation across the lines for all of the Tb phenotypes in the basal diet group and for the RCR (**Figure 3.1** and **Supplemental Table S3.5**). In addition, the variability for the basal phenotypes were different from the variability of the RCR phenotypes. This suggests that both basal and RCR phenotypes are influenced by genetics but that they are influenced by different genetic factors. Consistent with this, genetics had a strong main effect ($p < .0001$) on all phenotypes with heritability estimates (h^2) ranging from 0.49-0.71 in the basal diet group with the highest and lowest values observed in Tb.N and Tb.Th, respectively. Similarly, Tb phenotypes in the low Ca diet group have high h^2 (0.46-0.72, highest in Tb.N and lowest in Tb.TMD) (**Supplemental Table S3.6**). ANOVA showed that there was a significant GxD interaction effect ($p < 0.05$) for BV/TV, Tb.Th, Conn.D, Tb.TMD and a trend for SMI ($p = 0.0917$). The RCR parameters of all phenotypes were significantly affected by genetics ($p < 0.005$) and had their heritability estimates range from 0.23-0.39 (**Supplemental Table S3.6**).

3.4.2 Genetic Mapping

We identified the total of 56 loci during QTL mapping analysis of the phenotypes from mice fed the basal Ca diet, the low Ca diet, and for the RCR phenotypes, (**Supplemental Table S3.7**). This includes 8 significant loci and 17 putative loci in the basal diet group phenotypes, 18 significant loci and 14 putative in the low Ca diet group phenotypes and 11 significant loci and 11 putative loci for the RCR phenotypes. Some of loci from each analysis overlapped and the loci that contain at least one significant QTL are summarized in **Figure 3.2**.

We prioritized these loci for bioinformatic follow-up and narrowed our interest from 57 to 10 loci. Four of the 10 loci were identified as QTLs for multiple basal phenotypes; three of these loci contained at least two QTLs with LOD > 5: F-6c, F-12c, and F-19b. The first locus, F-6c, has

QTLs controlling BV/TV and Conn.D (LOD > 7), as well as Tb.N, Tb.Sp and SMI. The effect of this locus on BV/TV, Conn.D, Tb.N was positively influenced by B6 alleles, where the effect on Tb.Sp and SMI was influenced by the DBA alleles. The second locus, F-12c, contains significant QTLs controlling Tb.N and Tb.Sp and a putative QTL for Tb.TMD (LOD=2.97). The effect of this locus on Tb.N was positively influenced by DBA alleles while B6 alleles controlled Tb.Sp and Tb.TMD. The third locus, F-19b, contains significant QTLs controlling BV/TV (B6), Conn.D (DBA), Tb.Th RCR (DBA). The fourth basal phenotype locus, F-9a, includes two putative QTL for Tb.Th (LOD=2.75, B6) and Tb.TMD (LOD=3.09, B6).

The loci F-6c, F-9a, and F12c included QTLs for both basal diet and low Ca diet phenotypes. The appearance of QTL in the two diet groups provides independent experimental validation of these genetic effects and suggests they are insensitive to the effects of diet. Conversely, many of the QTLs controlled phenotypes in either the basal diet group or the low Ca diet group only; this shows that some genetic effects are sensitive to the dietary environment.

The 6 remaining high-priority loci contain QTLs that control Tb phenotypes that are sensitive to low Ca diet intake. Two of these loci contain QTLs from the low Ca diet group and the RCR phenotypes: F-2e and F-10c. The first locus, F-2e, contains QTLs controlling Conn.D in the low Ca diet group as well as the Conn.D RCR (LOD > 5, driven by B6 alleles, **Fig.3.3B.b-c**). F-10c contains QTLs for several low Ca diet phenotypes including SMI, Tb.Th, Tb.N as well as a significant QTL for Tb.N RCR and putative one for Tb.Sp (**Fig.3.3A.c and Supplemental Fig. S3.1-3.2**). The four remaining loci contain QTLs that only influence RCR phenotypes: F-2f (**Fig.3.2B.c,** high allele of Conn.D RCR and BV/TV RCR = DBA**)**, F-8a (**Fig.3.2A.c**, Tb.TMD RCR (B6) and Tb.N RCR (DBA)), F-15b (SMI RCR (B6) and Tb.TMD RCR (DBA)), and F-9d.

3.4.3 In-depth Bioinformatic Analyses of High-priority Loci

The ten high-priority loci were subjected to in-depth, bioinformatics analyses. The number of genome features, genes, functional polymorphisms and eQTL at each locus is given in **Supplemental Table 3.8**. We systematically identified candidate genes in each locus and we chose F-6c as an example for a strong genetic effect that is independent from the influence of diet (**Figure 3.4)**. This locus contains QTLs controlling five Tb phenotypes in both the low and basal diet Ca groups: BV/TV, Conn.D, Tb.N, Tb.Sp, and SMI. The 1.5-LOD confidence interval for this locus (94.85-109.79 Mb) encompasses 54 genes and 42,179 polymorphisms. In the coding sequence, there are 4031 SNPs: 33 SNPs in 5'UTR, 127 SNPs in 3'UTR, 3838 indels, and 15 non-synonymous polymorphisms. Of the 15 non-synonymous polymorphisms, only polymorphisms in the coding region of the *Setmar* and *Sumf1* genes were predicted to cause deleterious amino acid substitutions **(Table 3.1** and **Figure 3.4)**. *Setmar* is constitutively expressed in all cell types including osteoblast, osteoclasts, and bone with the highest expression in embryonic stem cells. *Sumf1* is a sulfatase-modifying factor 1 that is highly expressed in osteoclasts as well as the limb and skeleton of mouse embryo. This protein is involved in proteoglycan desulfation. In the non-coding sequence of F-6c, three cis eQTL had maximum LRS > 9. Two of these cis eQTL, *9430088B20Rik* and *Cidec*, were considered as candidate genes because their mRNA levels were significantly correlated with every phenotype that mapped to the locus except Tb.Sp from the low Ca diet (**Table 3.2**). Out of the 4 candidates, *Cidec* and *Sumf1* have published evidence to support their function in bone phenotypes. *Cidec* is highly expressed in differentiating and mature osteoblasts whereas *Sumf1* is highly expressed in both osteoblasts and osteoclasts. *Sumf1* has also been associated with heel BMD in human GWAS.[43] In addition, *Sumf1* KO mice had impaired bone phenotypes including abnormal long bone epiphysis and metaphysis morphology, decreased length of long bones and lost spinal process of dorsal vertebrae (IMSR database).

Following a similar systematic workflow, we identified several candidate genes for each of the other 9 high-priority loci (**Table 3.1** and **3.2)**. The annotations of these candidate genes are given in **Supplemental Tables S3.9 and S3.10**.

3.5 Discussion

We previously reported that the impact of low dietary Ca intake on bone phenotypes is strongly influenced by genetics.[24,26,44] Consistent with our earlier findings, we now report that basal Tb phenotypes and their response to dietary Ca restriction (RCR) are heterogeneous in our population of 51 BXD RI lines. Tb phenotypes had heritability estimates that are similar to or greater than those that we[26,44] and others[15,45] have previously reported for bone phenotypes measured by DXA and μCT in mice. We also found that the genetic regulation of the RCR is independent from the regulation of basal Tb phenotypes, indicating that the loci controlling the RCR phenotypes contain genetic variation sensitive to the dietary Ca environment.

Within our high-priority loci, we observed several loci that influenced basal Tb phenotypes (F-6c, F-9a, F-12c, and F-19b). Our findings corroborate previously published findings from the literature. Hence, this validates the accuracy of our mapping experiment; we are able to capture genetic effects that have been shown to exist in the regulation of bone phenotypes. For example, the locus F-6c (44.81-50.20 cM) overlapped with QTLs previously reported by others to control BMD and BMC in female F2 intercrosses of BXD[19] and B6 x C3H[46] as well as in both young male and female mice from the BXD RI panel.[47,48] In addition, we remapped our earlier femur data from BMD and BMC[26] using the new, denser marker set available for the BXD RI panel and found a QTL at the Tb phenotype chr 6 locus (LOD = 8.33 basal diet, 3.92 low Ca diet) (**Supplementary Figure S3.4**). Thus, we confirmed genetic effects within chr 6 that have been

identified by others for BMD and BMC and expanded it to include many phenotypes for Tb structure. This suggests that the locus influences overall bone development.

Previously Rosen et al. studied the genetic effects on peak femoral cortical and Tb parameters of 16-wk-old B6.C3H-6T (6T) congenic female mice.[49] Although the confidence interval for our F-6c locus overlapped with their congenic region, less than 50% of the alleles between C3H and DBA are identical-by-descent and the two candidate genes Rosen et al. identified, Raf-1 and caveolin 3,[50] are not polymorphic between B6 and DBA. Thus, despite the strong impact of the chr 6 locus on bone phenotypes, no one has reported a viable candidate gene to explain biological impact of B6 or DBA alleles on bone. Using a bioinformatics approach, we have identified two potential candidates for this locus: *Sumf1* and *Cidec*. *Cidec* plays an important role in apoptosis. It is expressed in differentiating (day 14) and mature (day 21) osteoblasts (http://biogps.org/#goto=genereport&id=14311). In a study comparing vertebral BMD and microarchitecture of B6 and 6T congenic mice, Bonnett et al. showed that *Cidec* is one of candidate genes within this locus on chr 6 that may be responsible for protective effect on Tb microarchitecture in B6 against bone loss occurred due to diet with high omega 6:3 ratio. This suggests a potential role in bone phenotypes.[51] We hypothesized that high transcript level of this gene (influenced by the DBA alleles) will increase apoptosis of mature osteoblasts resulting in reduced bone formation and mineralization. Consistent, with our hypothesis, femur *Cidec* expression level is significantly, negatively correlated with BV/TV, Tb.N and Conn.D. However, direct *in vitro* functional tests of this gene in osteoblasts and studies in KO mice are warranted.

The other candidate gene for F-6c is *Sumf1* that encodes a key enzyme necessary for post-translational modification of sulfatases[52] that regulates the proteoglycan sulfation required for normal bone growth and modeling.[53,54] Though *Sumf1* KO mice did not show significant

difference in bone area, BMD, and BMC/body weight (measured by DXA)(https://www.mousephenotype.org/data/genes/MGI:1889844), it had enlarged long bone epiphysis and metaphysis morphology as well as kyphosis and lost spinal process of dorsal vertebrae (http://www.informatics.jax.org/marker/MGI:1889844). A study of chondrocyte biology during skeletal development in *Sumf1* KO embryo and newborn mice showed that Sumf1 activity is necessary for chondrocyte viability that is critical for endochondral ossification. In addition, it favors extracellular matrix production, and promotes proliferation and differentiation of chondrocytes.[53] We hypothesize that impaired function of this enzyme will lead to proteoglycan undersulfation, reduced extracellular matrix production and fewer mature chondrocytes which in turn compromise bone development. Nonetheless, future *in vivo* studies examine the role of Sumf1 on Tb development will be necessary.

In addition to loci affecting bone mass that have been identified by others, our current research shows that there is genetic regulation unique to the control of trabecular bone. Two loci we identified, on chr 9 (F-9a) and 12 (F-12c), co-located with Tb phenotype QTLs previously reported by others. Bower et al. conducted genetic mapping in 200-day-old male and female mice from 23 BXD RI lines and found two co-locating QTLs on chr 9 and chr 12 controlling proximal tibial BV/TV and SMI (female only), respectively.[16] Recently, Lu et al. conducted a QTL mapping experiment on whole bone, cortical bone and Tb phenotypes from the femur and tibia of female and male BXD RI lines (50-63 RI lines, aged 50-375 days).[20] They reported a significant QTL on chr 9 that overlapped with our locus. Collectively, these findings suggested that there are unique genetic loci that control Tb phenotypes and that these two loci may play a role in the regulation of Tb phenotypes in both sexes throughout growth and adulthood.

We identified six candidate genes in F-12c with evidence for their functions in bone biology: *Serpina3n*, *Efcab11*, *Dglycu*, *Syne3*, *Ccdc88c* and *Rian*. However, our bioinformatics analysis suggests that *Serpina3n*, a gene that encodes a serine protease inhibitor and whose mRNA is highly expressed in mature osteoblasts (http://biogps.org/#goto=genereport&id=20716), is the most promising candidate gene within the locus. For example, when Ishida et al. used small interfering RNA to specifically reduce *Serpina3n* levels in primary mouse osteoblasts, they found significantly increased expression of type 1 collagen (*Col1a1*), Runx2, and alkaline phosphatase in differentiated osteoblasts.[55] Conversely, *Serpina3n* overexpression suppressed the expression of mineralization-related genes (*Enpp-1*, *Ank*, *Dmp-1*) in osteoblast-like ST2 and MC3T3-E1 cells.[55] Because we observed three deleterious, non-synonymous coding polymorphisms and one splice site mutation in this gene, we hypothesize that point mutations will disrupt Serpina3n function and lead to increased osteoblast activity. Since the *in vitro* evidence strongly supports functional roles of this gene in bone, future follow-up studies should examine its role on bone biology using *in vivo* models.

We also identified a novel locus (F-19b) controlling Tb phenotypes at the baseline. F-19b controls BV/TV and Conn.D in the basal diet group and Tb.Th RCR. High values of BV/TV and Conn.D in the basal group were driven by DBA alleles, while high values of Tb.Th RCR were influenced by B6 alleles. We found two candidate genes within this locus: *Dkk1* and *Papss2*. *Dkk1* is a well-established negative regulator of Wnt signaling pathway. Dkk1 binds Wnt coreceptors LRP5/6 to inhibit Wnt binding and signaling resulting in decreased bone formation.[56] We observed a deleterious non-synonymous polymorphism in this gene and we hypothesize that DBA allele leads to an amino acid substitution that cause a reduced function of Dkk1. A reduction in Dkk1 activity increases an activation of Wnt signaling pathway and in turn promotes bone

formation. The fact that we detected the locus with genetic variation in a gene that have established functions in bone biology strengthens our confidence in detecting functional candidate genes that have real impact on bone phenotypes.

Another candidate gene in F-19b is *Papss2*. Papps2 (3'-phosphoadenosine 5'-phosphosulfate (PAPS) synthase 2) is responsible for synthesis of the sulfate donor PAPS and works as a cofactor of sulfotransferases including Sult2A1. Sult2A1 has preferential sulfating activity toward dehydroepiandrosterone (DHEA) and results in an inactive sulfate ester, DHEA sulfate (DHEAS).[57] Alternatively, the active DHEA can be converted toward active androgens. However, once DHEA is converted to DHEAS, it is unlikely to get convert back to DHEA.[58,59] This suggests that increased DHEA sulfation can deprive a circulating pool of DHEA; thus, may lead to reduced synthesis of androgen. Androgens have been shown to play a role in skeletal development in young adults and prevent bone loss in aging men through the suppression of osteoclast activity and increase the lifespan of mature osteoblast and osteocytes.[60] PAPSS2 deficiency in patients with gene mutations can manifest in abnormality of bone phenotypes including brachyolmia (short tunk-short stature due to platyspondyly), minimal epimetaphyseal changes and spondyloepimetaphyseal dysplasia (SEMD, brachyolmia plus additional clinical changes in bone epiphyses and metaphyses).[57,61-64] In addition, some patients showed low DHEAS with normal DHEA and androstenedione levels[57,64] while some had normal or high DHEA level.[62] Though observed phenotypes vary depending on allelic mutations, the fact that patients with *PAPSS2* mutations showed abnormality in bone and serum DHEAS level suggests a functional role of this gene on bone phenotypes potentially through the regulation of DHEA sulfation. Most recently, Oostdijk et al. used urinary steroid metabolite analysis and oral administration of DHEA (or DHEA challenge test) to assess the sulfation and androgen synthesis

capacity of DHEA and reported the first direct functional *in vivo* evidence that impaired DHEA sulfation results in androgen excess in two male patients with compound heterozygosity of a frameshift (p.W462Cfs*3) and a missence (p.G270D) mutations of PAPSS2 gene.[57] These two patients showed clinically SEMD and delayed bone age. Because we observed that femur *Papss2* mRNA level was negatively correlated with basal BV/TV and Conn.D (LRS = 43.1, r = -0.35 and -0.43, respectively), we hypothesize that a decreased *Papss2* transcript level will decrease the sulfation of DHEA into DHEAS. As a result, more DHEA is available for conversion into androgens and promote bone formation. Future studies will be necessary to determine the relationship between *Papss2* and normal variation of bone phenotypes as well as to identify specific polymorphism in the regulatory regions that affect the expression of *Papss2*.

Our study is unique because we used two sets of individuals from 51 BXD RI lines; one set received a well-defined basal Ca diet and the other received the same diet with an exception of 0.25% Ca. Because of this, we are able to compare directly QTLs identified from the low Ca diet to the QTLs from the basal phenotypes mapping. The genetic effects of some loci (i.e. F-6c, F-9a, and F-12c) were robust and insensitive to dietary environment. In contrast, several loci were seen only in the genetic mapping of one diet group; these are diet-sensitive loci that reflect GxD interactions (e.g. F-10c mapped only to the low Ca diet phenotypes). Moreover, because the two sets of mice are from 51 well-defined, genetically identical BXD RI lines, we were able to conduct a genetic mapping on the RCR phenotypes. By doing so, we found several loci that were unique to RCR phenotypes. Thus, the RCR phenotypes captured the adaptation of Tb to low Ca diet in ways that are not reflected in the mapping of the phenotypes from the basal or low Ca diet groups. Two of the RCR loci overlapped with the QTLs previously identified by our group: F-9d and F-15b. The first locus, F-9d, contains a QTL controlling Tb.Th RCR (high allele=DBA, peak @

62.92 cM, LOD=6.03) and that overlapped with a QTL controlling BMC-RCR (high allele=DBA, peak @ 58.3 cM, LOD = 4.5).[26] We observe multiple candidates genes but only one of them, *Nbeal2* has evidence suggesting its role to bone. *Nbeal2* KO male mice showed significantly lower BMD and BMC when compared to wild types; however, no significant difference was seen in the female KO mice (https://www.mousephenotype.org/data/genes/MGI:2448554). Because *Nbeal2* is highly expressed in bone and osteoclasts, we hypothesize that *Nbeal*2 promotes the activity of osteoclasts. The premature truncated *Nbeal*2 (DBA allele) will lead to decreased activity of osteoclasts and will in turn increase the ability to retain Tb.Th under the low Ca treatment. Future validation of functional roles of this gene in bone biology will be needed.

The second RCR locus, F-15b, contains QTLs that mapped to the RCR phenotypes of SMI and Tb.TMD (peak @ 40.79 and 44.42 cM, respectively) and it accounts for approximately 15-18% of the variation in these RCR phenotypes. This locus overlapped with QTLs we previously reported for the Ca absorption RCR phenotype (peak @ 32.3 cM, LOD=5.1)[26] and for serum 25(OH)D_3 levels in the low Ca diet group (peak @ 37.57 cM (78.7 Mb), CI: 71.2-86.2 Mb, LOD=4.1).[65] Because we observed this locus consistently in Ca absorption, serum level and Tb phenotypes as the response to low Ca restriction, we believed that the changes seen in Tb may be as downstream consequences of this locus on the regulation of Ca absorption in the low Ca environment. One particular gene is likely a candidate accounting for the influence of this locus: *Cyp2d11*. Within F-15b, we observed four potentially deleterious, non-synonymous coding variants and a frameshift mutation of *Cyp2d11* (at 82389170, 82389553, 82390510, 82392460, and 82390021 Mb, respectively). This gene encodes cytochrome P450, family 2, subfamily d, polypeptide 11. This enzyme is one of 25-hydroxylases that hydroxylates vitamin D_3 at C-25 into 25(OH)D_3 in the liver.[66] We hypothesize that genetic variation that leads to reduced function of

this enzyme will lead to lowered conversion of vitamin D_3 to 25(OH)D_3. Based on this hypothesis, individuals with these variants will have a lower level of 25(OH)D_3, which will not lead to any negative impact on bone when their serum Ca is within the normal range (i.e. when received adequate Ca diet). However, when they are deficient in dietary Ca and their Ca serum level drops, this can be detrimental to their bone. Due to the low availability of 25(OH)D_3 for converting into 1,25$(OH)_2D_3$, their body cannot regain the serum Ca back to normal through increasing 1,25$(OH)_2D_3$-mediated intestinal Ca absorption and renal Ca reabsorption as quickly. Hence, Ca is withdrawn from Tb as we observed reduced Tb phenotypes under dietary Ca stress. Because existing literature on 25-hydroxylases focuses on Cyp2R1, it is critical that future studies examine the impact of Cyp2d11 particularly in Ca-deprived circumstances.

In addition to the two RCR loci discussed above, we discovered several novel genetic loci that only respond to low dietary Ca intake and/or underlie the RCR of Tb phenotypes (F-2e, F-2f, F-8a, and F-10c). All of these loci mapped to more than one phenotype indicating genetic effects that control correlated phenotypes or possibly latent traits. F-2e and F-2f are particularly interesting because each of the loci accounts for more than 20% of variation in the mapped phenotype. F-2e controlled Conn.D both in the low Ca diet and in the RCR phenotype. This provides strong evidence that this locus has a robust effect specifically in the low dietary Ca environment. Several potential candidate genes were identified from the bioinformatic analysis of this locus. However, two particular genes, *CD40* and *Wisp2,* have published evidence that links their function to bone biology.

The most interesting candidate gene in F-2e is *Wisp2* (or *Ccn5*). *Wisp2* encodes a member of the Wnt1 inducible signaling pathway protein subfamily. Using 14-wk old mice overexpressing the high peak bone mass mutated gene product (LRP5 G171V), Robinson et al. reported increases

in expression of *Wisp2* transcript along with other genes involved in Wnt pathway such as *Wnt10B*, *FzD2*. This suggests that Wisp2 may work downstream of Lrp5 to activate the canonical Wnt pathway.[67] The expression of *Wisp2* is high during osteogenic differentiation of bone marrow-derived mesenchymal stem cells[68] and differentiation of osteoblasts into osteocytes[69] with the highest expression of *Wisp2* observed in mature osteoblasts (http://biogps.org/#goto=genereport&id=22403). *In vitro*, Wisp2 promotes the adhesion of primary human osteoblast[70] as well as matrix mineralization.[69] Recently, Jiang et al. generated *Wisp2/Ccn5* KO mice by replacing exons 2-5 by a lacZ cassette ($Ccn5^{LacZ/LacZ}$ mice) but they found no changes in BMD or BV/TV in $Ccn5^{LacZ/LacZ}$ mice compared to wild-type controls.[71] However, the fact that the *Wisp2*-containing locus (F-2e) was only seen in the low Ca group and the RCR suggests that the biological role of Wisp2 may only be revealed by physiologic stress.

Cd40 is another interesting candidate gene in F-2e that encodes a receptor for CD40 ligand (CD40L or CD154). *Cd40* KO mice had decreased BMD, cortical bone mass, and BV/TV (IMRS database) as well as reduced production of bone marrow osteoprotegerin (OPG).[72] Li et al. reported that T cells promote the production of bone marrow OPG from B cells through the binding of CD40L to its receptor.[72] OPG is a decoy receptor for receptor activator of NF-κB ligand (RANKL) to stabilize the RANKL-induced osteoclastogenesis.[73] In an association study of BMD with 15 SNPs in the *CD40/CD40L* genes in a population of 779 women, 2 SNPs in *CD40* gene showed nominal P value ($P < 0.05$) for femoral neck and lumbar spine BMD but the association was not significant after correcting for multiple testing. Women with the homozygous TT genotype for SNP rs1883832 in *CD40* showed a trend to have lower serum OPG levels.[74] Collectively, these data and our finding support the functional role of CD40 in bone. We hypothesize that

impaired function due to a point mutation in *Cd40* will lead to lowerd bone marrow OPG which results in greater production of osteoclasts and higher Tb resorption when dietary Ca intake is low.

Another novel RCR locus, F-2f, controls the RCR phenotypes of BV/TV and Conn.D. We identified two candidate genes with suggestive evidence for their roles in bone: *Lama5* and *Ogfr*. *Lama5* encodes the laminin α5 subunit of laminin-511 and laminin-521 isoforms that are the major non-collagenous extracellular matrix glycoprotein of all basement membranes.[75] Global *Lama5* KO mice are embryonic lethal which suggests an essential role of Lamini-511 in embryonic development.[76] *Lama5* is highly expressed in the intestine and *Lama5* KO mice had the malformed intestine. RNA profiling of embryonic intestinal tissue of *Lama5* KO mice identified laminin α5 regulates both epithelial and mesenchymal cells by inhibiting Wnt and activating PI3Kinase signaling.[77] Lama5 interacts with integrin beta 1 and has been implicated in cell adhesion, differentiation, migration, and signaling in epithelial cells. Another line of evidence suggested the role of α5 subunit-containing laminins in osteogenesis. Endothelial cells (ECs) in embryonic and postnatal long bones mediate angiogenic growth and provide molecular signals acting on osteoprogenitor cells to develop toward osteoblast lineage.[78] The α5 subunit-containing laminins interact with endothelial integrin β1 resulting in cell-matrix signaling that governs the differentiation and functional properties of bone ECs. Endothelial cell-specific *Lama5* mutants showed shortened vessel columns near the femur growth plate, reduced the expression of transcription factor osterix in osteoprogenitor cells, and shorter femur length.[79] Because laminin α5 has specific roles in both intestinal epithelial cells and bone endothelial cells, we hypothesize that a point mutation in the coding region of *Lama5* will reduce Lama5 activity and lead to poorly developed intestine and compromised bone development. The former will likely affect dietary intestinal absorption (e.g. Ca) and the latter will directly impair development osteogenesis as well

as the endothelial cells in long bones. Thus, we observed the detrimental effect of this genetic variation on Tb phenotypes specifically under low dietary Ca environment.

Finally, the second candidate gene for F-2f is *Ogfr*. It encodes a non-canonical opioid growth factor receptor (OGFR) that binds to native opioid ligands less efficiently than the canonical opioid receptors but binds to the OGFR inhibitor naltrexone with high affinity. The *Ogfr* gene is highly expressed in mesenchymal stem cells (MSCs), osteoblasts, reserved zone chondrocytes of growth plate, and metaphyseal bone adjacent to the growth plate[80] but several lines of evidence suggest that Ogfr is a negative regulator of bone formation and bone mass. MSC cultures (from humans) induced to become osteoblasts and treated with the Ogfr inhibitor Naltrexone had increased mineral accumulation and osteocalcin gene expression. Also, systemic administration of naltrexone daily for 28 days increased femoral bone mass, bone formation ratio, and osteoblast number/bone surface compared to the control group.[81] We observed that high *Ogfr* mRNA is negatively correlated to retention of BV/TV and Conn.D on the low Ca diet. In addition, there is a frameshift mutation within the *Ogfr* gene (high allele=DBA). Thus, we hypothesize that the mutation from a DBA to B6 allele will lead to reduced activity of this gene which will increase the bone formation. Thus, even under the low Ca situation, Tb mass and connectivity are better maintained compared to the gene with wild-type allele. Since the area of opioid receptor and bone biology is still in its infancy, more research will be needed to confirm the promising role identified in these earlier findings.

Our study used a forward genetics approach to identify genetic variation that control Tb phenotypes at the baseline as well as under the low dietary Ca condition. Most recently, Lu et al.[20,45] published a preprint on genetic mapping study of femoral and tibial μCT phenotypes in the female and male mice from the BXD RI panel aged 50 to 375 days. Our study is uniquely

different from this study in that we conducted our study to specifically examine genetic impact on bone phenotypes during growth by using only male mice raised under strictly controlled environmental conditions and used a well-defined, semi-purified rodent diet from 4 to 12-wk old across all the 51 lines. By doing so, we had an absolute control over confounding factors known to impact bone phenotypes during the developmental years, i.e. dietary constituents, gender and age. Second, by using mice from the BXD RI panel that have fixed homozygous genotypes, we had genetically identical replicates from each line to test for the influence of GXD interactions on Tb phenotypes during growth. This enabled us to determine genetic polymorphisms that influence the response of Tb to low dietary Ca stress. Third, we leveraged the new, denser genotypic marker for the BXD IR panel that was released in the end of 2017 (i.e. 2244 informative markers vs 1558 informative markers previously) The combination of a large number of BXD line used and a new denser genetic map allowed us to capture narrower QTL regions in comparison to previous reports (e.g. F-12c: 5 vs. 24-76 cM[38]) resulting in fewer candidate genes within each QTL. Nonetheless, some limitations should be noted. First, the BXD RI panel was derived from only two founders so it captures only 20% of the genetic diversity that exists in the mouse genome.[27] Second, though we used a large number of RI lines, the QTL intervals from our analysis are still relatively wide as compared to newer mouse resources like the Collaborative Cross. Third, we studied only male mice so our study may not capture loci that are influenced by female sex hormones; though we did detect a locus with variants in *Serpina3n* gene whose expression in female osteoblasts was 14 times the expression of male mice.[55]. Finally, we only measured Tb phenotypes at the distal femur and likely to miss loci that specifically influence Tb in other skeletal sites like the vertebra.

3.6 Conclusion

In summary, this study is the first to examine genetic variation controlling the response of Tb mass and microarchitecture to dietary Ca restriction during growth. Our study confirmed the existence of GxD interactions influencing Tb phenotypes. Using genetic mapping in a large panel of male mice from BXD RI lines, we revealed that genetic variation controlling for the RCR phenotypes is independent from that controlling basal phenotypes. Most importantly, we expanded our understanding of the genetic regulation on Tb phenotypes particularly during low Ca intake by revealing novel genetic loci. By coupling our QTL analysis with bioinformatics characterization, we uncovered several novel candidate genes for Tb phenotypes (e.g. *Sumf1, Serpina3n)* and the RCR (e.g. *Wisp2, Ogfr*). These genetic loci and candidate genes identified in this study serve as a foundation for future research to identify novel pathways and genes underlying the development of bone as well as an adaptation to Ca insufficiency.

3.7 Acknowledgments

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3.8 Author's Roles

Study design: JCF. Study conduct: JCF, Bone analysis: PRF and KC, Data management and statistical analysis: KC, Data interpretation: JCF, PRF, and KC. Drafting manuscript: JCF and KC. Revising and approving final version of manuscript: JCF, PRF and KC. JCF takes responsibility for the integrity of the data analysis.

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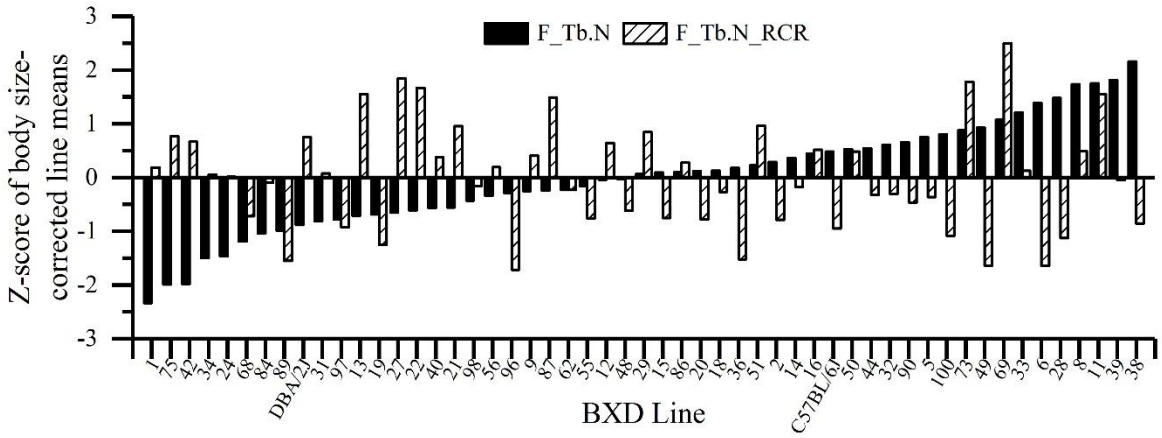

Figure 3.1 *Z-scores of body size corrected Tb.N from the basal Ca diet and Tb.N RCR values from 51 BXD lines.* Lines are ordered for smallest to largest for the Tb.N basal phenotype. Values for the parental lines DBA/2J and C57BL/6J are included for reference.

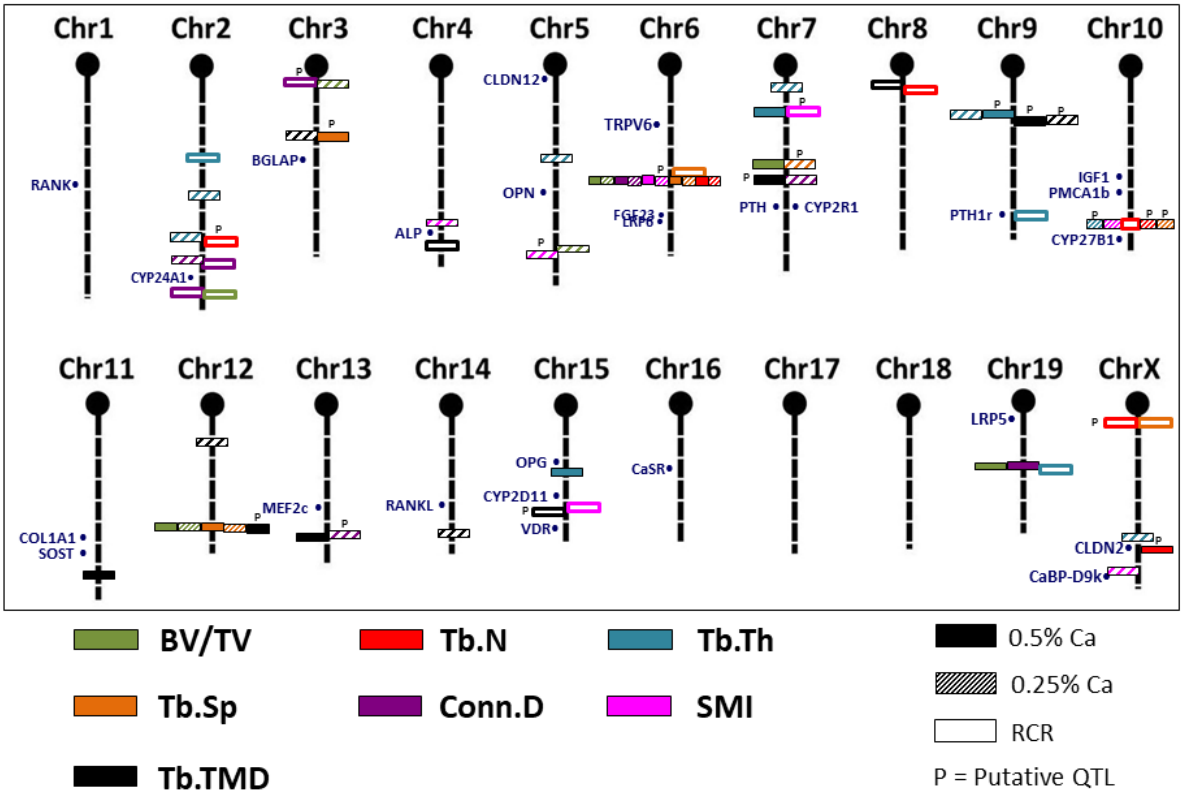

Figure 3.2 *A whole genome summary of QTL identified for femoral Tb phenotypes from 51 BXD mouse lines.* Only loci that has at least one significant QTL are shown in this map (32 loci out of 56). Trabecular bone phenotypes are coded by color and for diet group used. RCR = response to dietary Ca restriction. P = putative QTL.

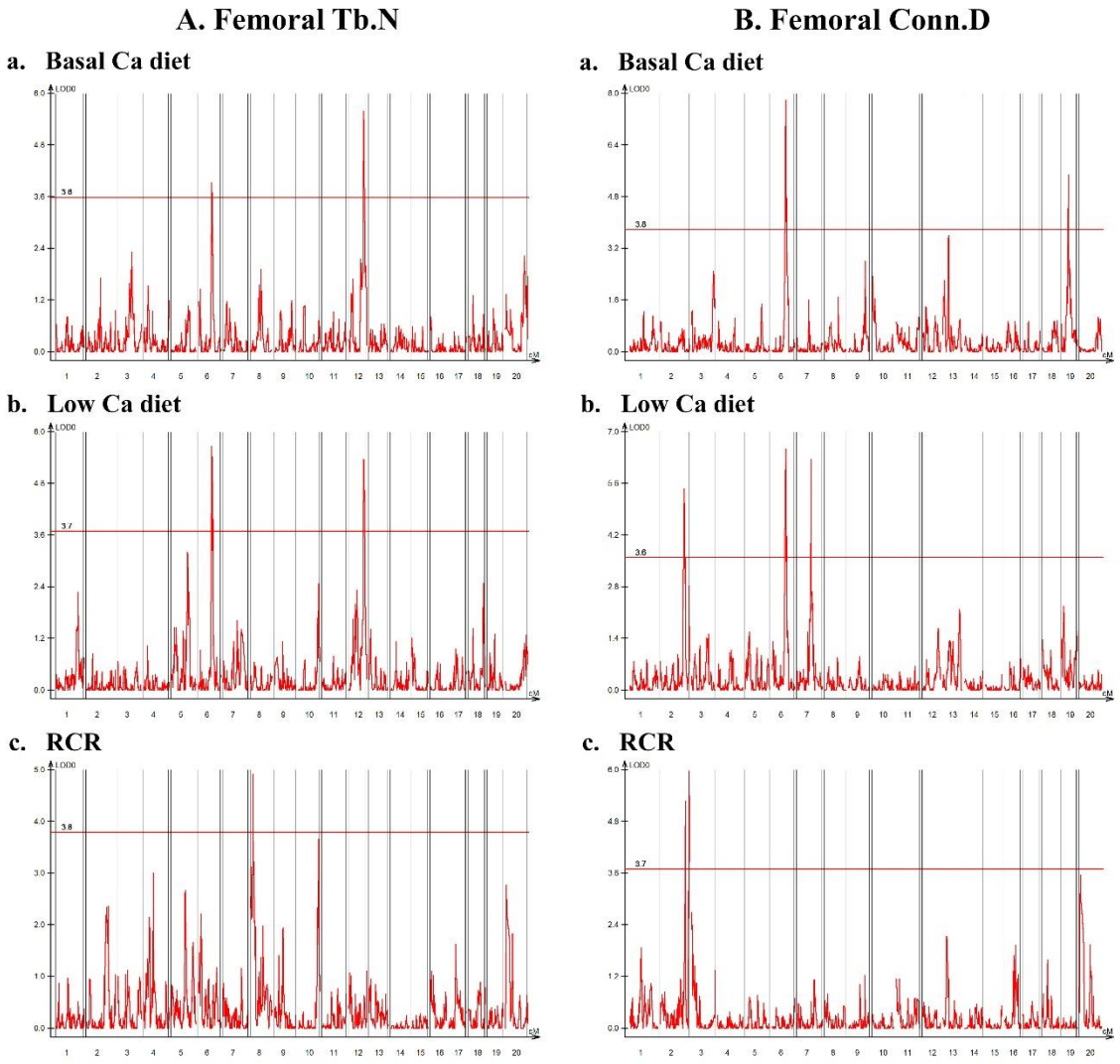

Figure 3.3 *Composite interval maps for Femoral Tb.N (A) and Conn.D (B).* For each phenotype, the maps from CIM of basal, low Ca, and RCR phenotypes are presented in a., b., and c., respectively

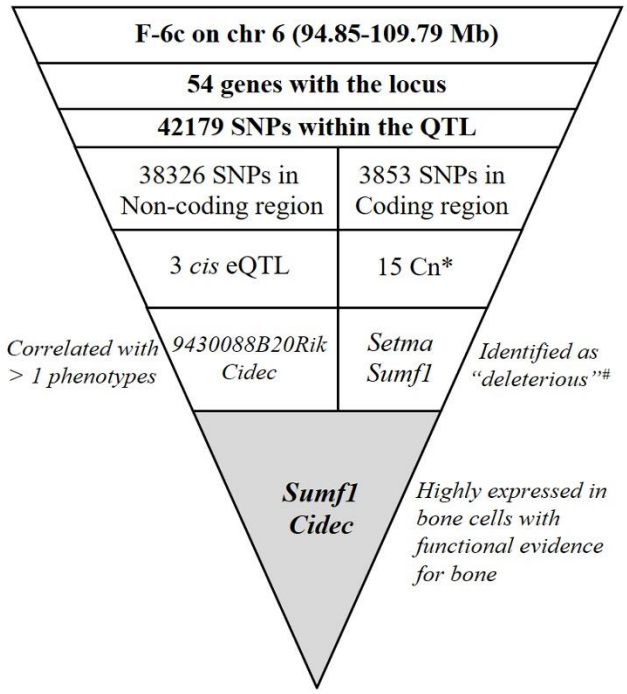

Figure 3.4 *Systematic identification of candidate genes in a representative high-priority locus F-6c.* This locus controls five Tb phenotypes (BV/TV, Conn.D, Tb.N, Tb.Sp, and SMI) in both basal and low Ca diet groups. SNPs = single nucleotide polymorphisms. * Polymorphisms for non-synonymous amino acid substitutions. # Polymorphisms scored as potentially deleterious nonsynonymous amino acid substitutions

Table 3.1 Summary of Potential Functional Candidate Genes within Prioritized Loci in the Distal Femur*

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Types of Consequence in Protein Coding Region[d]			
				Altered Amino Acid Sequence[e]	Premature Stop Codon	Splice Site Mutation	Frameshift
F-2e	2	159.79-165.96	Conn.D_LowCa (B6[b], 5.46[c]), Conn.D_RCR (B6, 5.28)	Plcg1(3), **Wisp2**, Kcnk1, Zfp335(2), **CD40**	-	Wfdc8	-
F-2f	2	175.83-182.11	Conn.D_RCR (DBA, 5.95), BV/TV_RCR (DBA, 3.24)	**Lama5**, Tcfl5, Dido1, Col20a1, Rtel1, Zgpat, Ifih1	-	-	**Ogfr**
F-6c	6	94.85-109.79	BV/TV_Basal/LowCa (B6, 10.42/8.49), Conn.D_Basal/LowCa (B6, 7.79/6.53), Tb.N_Basal/LowCa (B6, 3.93/5.67), Tb.Sp_Basal/LowCa (DBA, 4.02/5.45), SMI_Basal/LowCa (DBA, 4.91/2.53)	Setmar, **Sumf1**	-	-	-
F-8a	8	0.02-15.34	Tb.TMD_RCR (B6, 4.05), Tb.N_RCR (DBA, 4.93)	Arhgef10	-	-	-
F-9a	9	27.26-42.54	Tb.Th_Basal/LowCa (B6, 2.75/3.73), Tb.TMD_Basal/LowC (B6, 3.09/2.29)	Srpr, Tbrg1, Olfr887(2), Olfr893(2), Olfr143, Olfr898, Olfr250(3), Olfr251(2), Olfr902(5), Olfr905, Olfr906, Olfr910, Olfr917, Olfr923, Olfr936, Olfr938, Olfr27(6) Olfr1537, Olfr44, Olfr957(2), 4931429I11Rik, Sorl1, Tecta(2)	Prdm10 Olfr884 Olfr915	LOC105243373 Kirrel3os(2) Ubash3b LOC105245000	Spa17 Olfr908 Olfr911-ps1 Ubash3b(2)

Table 3.1 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Types of Consequence in Protein Coding Region[d]			
				Altered Amino Acid Sequence[e]	Premature Stop Codon	Splice Site Mutation	Frameshift
F-9d	9	110.50-115.06	Tb.Th_RCR (DBA, 6.03)	Als2cl, Lrrc2, Dclk3, Arpp21	**Nbeal2**	Als2cl	-
F-10c	10	115.41-130.69	Tb.N_RCR (B6, 3.66), SMI_LowCa (DBA, 4.08), Tb.Th_LowCa (B6, 3.09), Tb.N_LowCa (B6, 2.47), Tb.Sp_LowCa (DBA, 2.41)	Slc26a10	-	LOC105245294 1700064J06Rik BC048403 Mon2, Myo1a	Avil, Agap2
F-12c	12	99.34-106.09	TbN_Basal/LowCa (DBA, 5.59/5.37), TbSp_Basal/LowCa (B6, 5.45/5.71), TbTMD_Basal (B6, 2.97)	Dglcy(4), Ccdc88c, Serpina1f(2), Serpina1c, Serpina3a(4), Serpina3b(6), Serpina3c, Serpina3i(3), Serpina3k(2), Serpina3m, **Serpina3n**(3), Syne3	Serpina3b^{StopL} Serpina3f Serpina3i	Serpina9 Serpina3a LOC105245053 **Serpina3n**	Efcab11, Tdp1, Serpina3i Serpina3j Serpina3m
F-15b	15	78.32-90.86	SMI_RCR (B6, 3.92), TbTMD_RCR (DBA, 2.94)	Micall1, Cacna1i(2), **Cyp2d11(4)**, Upk3a, Cdpf1, Pkdrej	Bik Ribc2	Kcnj4 Xrcc6 Arhgap8	Sox10 **Cyp2d11**
F-19b	19	27.01-32.38	BV/TV_Basal (DBA, 6.04), Conn.D_Basal (DBA, 5.48), Tb.Th_RCR (B6, 4.62)	**Dkk1**		LOC105243769 LOC105246008	

* This table does not include polymorphisms found in predicted genes. Abbreviation: Chr = chromosome; Mb = megabase; BV/TV = bone volume fraction; Tb.N = trabecular number (mm-1); Tb.Th = trabecular thickness (mm); Tb.Sp = trabecular separation (mm); Conn.D = connectivity density (1/mm^3); SMI = structure model index; Tb.TMD = Trabecular tissue mineral density (mg HA/cm3).

[a] 1.5-LOD confidence intervals in Mb (Build GRCm38/mm10). [b] Parental influence: B6 = C57BL/6J line, DBA = DBA/2J line

[c] Phenotype-specific LOD score for each locus. [d] In parenthesis, number of polymorphisms in that gene if different from 1.

[e] Genes with polymorphisms scored as potentially deleterious nonsynonymous amino acid substitutions. ^{StopL} Genes with polymorphisms cause a lost stop codon.

Bolded gene names are the gene with published evidence for potential functions in bone.

Table 3.2 Summary of Genes with cis eQTL within Prioritized Loci that are Correlated to Femoral Trabecular Bone Phenotypes

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
F-2e	2	159.79-165.96	Conn.D_LowCa (B6[b], 5.46[c]), Conn.D_RCR (B6, 5.28)	Sys1 (37.6)		Conn.D: r = -0.45*	
				Slpi (28.1)		Conn.D: r = -0.51*	
				Eya2 (16.2)		Conn.D: r = -0.38#	
F-2f	2	175.83-182.11	Conn.D_RCR (DBA, 5.95), BV/TV_RCR (DBA, 3.24)	Dnajc5 (29.2)			Conn.D: r = -0.43*; BV/TV: r = -0.43*
				Rtel1 (28.3)			Conn.D: r = -0.36#; BV/TV: r = -0.36#
				Ogfr (12.7)			Conn.D: r = -0.33#; BV/TV: r = -0.33#
F-6c	6	94.85-109.79	BV/TV_Basal/LowCa (B6, 10.42/8.49), Conn.D_Basal/LowCa (B6, 7.79/6.53), Tb.N_Basal/LowCa (B6, 3.93/5.67), Tb.Sp_Basal/LowCa (DBA, 4.02/5.45), SMI_Basal/LowCa (DBA, 4.91/2.53)	9430088B20Rik (13.5)	BV/TV: r = 0.46*; Conn.D: r = 0.41*; Tb.N: r = 0.38*; Tb.Sp: r = -0.37*; SMI: r = -0.50*	BV/TV: r = 0.51*; Conn.D: r = 0.43*; Tb.N: r = 0.37*; SMI: r = -0.49*;	
				Cidec (12.7)	BV/TV: r = -0.48*; Conn.D: r = -0.43*; Tb.N: r = -0.44*; Tb.Sp: r = 0.43*; SMI: r = -0.50*	BV/TV: r = -0.52*; Conn.D: r = -0.48*; Tb.N: r = -0.48*; SMI: r = -0.50*	

Table 3.2 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
F-8a	8	0.02-15.34	Tb.TMD_RCR (B6, 4.05), Tb.N_RCR (DBA, 4.93)	Kbtbd11 (32.6)			Tb.TMD: r = -0.33#
				CDH11 (32.6)			Tb.N: r = -0.31#
				4930435N07Rik (10.6)			Tb.N: r = -0.38*
				Mcf2l (10.1)			Tb.N: r = -0.43*
F-9a	9	27.26-42.54	Tb.Th_Basal/ LowCa (B6, 2.75/3.73), Tb.TMD_Basal/ LowCa (B6, 3.09/2.29)	Kirrel3 (48.8)		Tb.TMD: r = 0.35*	
				Hspa8 (46.5)		Tb.TMD: r = -0.45*	
				Srpr (41.1)		Tb.TMD: r = 0.43*	
				9330161A08Rik (34.1)		Tb.TMD: r = 0.40*	
				9030425E11Rik (27.5)		Tb.TMD: r = 0.41*	
				D230004N17Rik (24.4)	Tb.TMD: r = 0.39*	Tb.TMD: r = 0.49*; Tb.Th: r = 0.34#*	
				C130027E04Rik (23.8)		Tb.TMD: r = -0.48*	
				Cc3d (21.2)		Tb.TMD: r = -0.36#	
				Ets1 (20.2)		Tb.TMD: r = -0.52*	
				Jhy (16.4)		Tb.TMD: r = -0.36#	
				Olfr893 (11.0)	Tb.TMD: r = 0.30#	Tb.TMD: r = 0.36*	
F-9d	9	110.50-115.06	Tb.Th_RCR (DBA, 6.03)	Smarcc1 (11.7)			Tb.Th: r = 0.34#

Table 3.2 continued

Loci ID	C hr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
F-10c	10	115.41-130.69	Tb.N_RCR (B6, 3.66), SMI_LowCa (DBA, 4.08), Tb.Th_LowCa (B6, 3.09), Tb.N_LowCa (B6, 2.47), Tb.Sp_LowCa (DBA, 2.41)	B130020M22Rik (25.2)		Tb.N: r = -0.40*; SMI: r = 0.35*	
				Rassf3 (18.3)		Tb.N: r = 0.33#; SMI: r = -0.44*	
				Shmt2 (16.6)			Tb.N: r = -0.32#
				Lrig3 (15.0)		Tb.N: r = -0.34#	
				Grip1 (13.8)			Tb.N: r = -0.38*
F-12c	12	99.34-106.09	TbN_Basal/LowCa (DBA, 5.59/5.37), TbSp_Basal/LowCa (B6, 5.45/5.71), TbTMD_Basal (B6, 2.97)	Rian (14.6)	Tb.N: r = -0.31#; Tb.Sp: r = 0.32#	Tb.N: r = -0.32# Tb.Sp: r = 0.32#	
F-15b	15	78.32-90.86	SMI_RCR (B6, 3.92), TbTMD_RCR (DBA, 2.94)	Tomm22 (63.1)			Tb.TMD: r = -0.34#
				Cdpf1 (60.0)			Tb.TMD: r = -0.42*
				scl0002487.1_316 (38.9)			SMI: r = 0.39*
				Atf4 (36.8)			SMI: r = 0.34#
				Serhl (36.0)			SMI: r = 0.40*
				Apobec3 (28.0)			SMI: r = 0.32#
				Sult4a1			SMI: r = -0.39*

Table 3.2 continued

Loci ID	C hr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
F-19b	19	27.01-32.38	BV/TV_Basal (DBA, 6.04), Conn.D_Basal (DBA, 5.48), Tb.Th_RCR (B6, 4.62)	Papss2 (43.1)	BV/TV: r = -0.35; Conn.D: r = -0.43		

* This table does not include polymorphisms found in predicted genes. Abbreviation: Chr = chromosome; Mb = megabase; BV/TV = bone volume fraction; Tb.N = trabecular number (mm-1); Tb.Th = trabecular thickness (mm); Tb.Sp = trabecular separation (mm); Conn.D = connectivity density (1/mm^3); SMI = structure model index; Tb.TMD = Trabecular tissue mineral density (mg HA/cm3).

[a] 1.5-LOD confidence intervals in Mb (Build GRCm38/mm10). [b] Parental influence: B6 = C57BL/6J line, DBA = DBA/2J line

[c] Phenotype-specific LOD score for each locus. Significant correlation with Tb phenotypes: *p<0.05, #p<0.10.

CHAPTER 4. NOVEL GENETIC LOCI CONTROL L5 VERTEBRAL TRABECULAR BONE AND THE RESPONSE TO LOW CALCIUM INTAKE IN GROWING BXD RECOMBINANT INBRED MICE

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4.1 Abstract

Epidemiological data show an inverse association between vertebral fractures and trabecular bone (Tb) microarchitecture. Because Tb is modulated by genetics and environment (e.g. diet), we studied the impact of gene-by-diet (GxD) interactions on Tb at the L5 spine. Male mice from 51 BXD recombinant inbred lines were fed either adequate (Basal, 0.5%) or low (L, 0.25%) Ca diets from 4-12 wks of age. We used micro-computed tomography (μCT) to measure: bone volume fraction (BV/TV), Tb tissue mineral density (Tb.TMD), Tb thickness (Tb.Th), separation (Tb.Sp), and number (Tb.N), as well as connectivity density (Conn.D), structure model index (SMI) and Tb tissue mineral density (Tb.TMD). The response to dietary Ca restriction (RCR) was calculated for each phenotype in each line. Body size (BS)-corrected residuals were used for statistical analysis. Genetics had an independent, strong influence on Tb traits (e.g. BV/TV, narrow sense heritability (h^2) = 0.57) but a more modest influence on the RCR (e.g. Tb.N, h^2=0.28). ANOVA revealed significant main effects for genetics and diet for all traits. Similarly, the RCR was affected by genetics indicating a GxD interaction affecting all Tb traits. Quantitative trait loci (QTL) analysis by composite interval mapping (500 permutations, WinQTLCart) identified 2-5 loci affecting each trait and 1-3 loci affecting the RCR for each trait. Loci controlling BV/TV (chr 2, 7, 15), Tb.N (chr 2, 12), Tb.Sp (chr 2, 4), Conn.D (chr 1, 7, 12), and SMI (chr 8) were seen in both diet groups providing replication of environmentally robust genetic effects. Loci on chr 2 (Tb.Sp-Basal, Tb.N-Basal, SM-Basal), chr 12 (Tb.N, Conn.D, Tb.Sp in low Ca diet) and chr 7 (RCR for BV/TV, SMI, Tb.Th) controlled multiple phenotypes suggesting the existence of latent traits. Candidate genes underlying selected loci were identified with PROVEAN (protein coding effects) or eQTL analysis in The GeneNetwork (mRNA level effects). On chr 2, we found non-synonymous (Cn) variants in *Wisp2* and *Cd40,* which promote osteoblast differentiation and suppressed osteoclastogenesis, respectively. In the locus on chr 7 (@37 Mb), we found two eQTL

for *Capns1* and *Nr2h1* (aka *LXRβ*) that promote osteoblast differentiation and activity. A locus on chr 7 also has three Cn variants and a cis eQTL affecting *Lins1*, a gene that regulates Wnt signaling as well as a Cn variant and a cis eQTL affecting *Lrrk1*, a gene that promotes bone resorption. Several basal and RCR loci were found in L5 spine but not distal femur, thus demonstrating the existence of Gene x Site (GxS) and GxDxS interactions affecting Tb phenotypes.

4.2 Introduction

Vertebral fractures are the most common type of osteoporotic fractures worldwide[1] and they are associated with increased mortality[2,3] and significantly lower quality of life.[4-7] The majority of bone tissue in the vertebrae is trabecular bone (Tb). Prospective research in elderly men[8] and several model-based[9] or μCT-based finite element analysis studies[10] of vertebral Tb show that the biomechanical competence of Tb is dependent both the absolute amount of bone and Tb microarchitecture (e.g. Tb number (Tb.N) and connectivity). Tb microarchitecture is considered an independent determinant of fractures from BMD and BMC because of its role in mechanical strength and several studies[8,11] have shown that individuals with osteoporotic fractures have markedly lower Tb connectivity and Tb.N than their healthy counterparts. Therefore, it is critical to understand factors that contribute to the development and maintenance of Tb mass and microarchitecture like genetics.

It is well established that skeletal phenotypes are under strong genetic regulation.[12-15] However, Judex et al. first proposed the concept that there are unique genetic controls affecting trabecular and cortical regions of the femur in a study that compared 4-month-old C57BL/6J (B6), BALB/cByJ, and C3H/HeJ (C3H) female mice.[16] Paternoster et al. recently confirmed the distinct genetic effect between trabecular and cortical volumetric bone mineral density (BMD) of the tibia using three cohorts of young Caucasian individuals.[17] Moreover, a recent genetic

mapping study of μCT phenotypes in BXD recombinant inbred (BXD RI) mice (50-63 RI strains, aged 50-375 days) showed that in the femur and tibia, Tb and cortical bone phenotypes are differentially and independently modulated by genetics.[18] More importantly, it has been suggested by others[19,20] and confirmed by our group[21] that genetics has differential impact on Tb in the distal femur and the L5 vertebra.

While previous genetic studies highlight the importance of identifying the unique genetic controls on vertebral Tb, most genetic mapping studies have examined the genetic contribution to Tb in the femur[18,22] and the tibia,[18,23] while only one study has directly investigated the L5 vertebral Tb traits in the 2-month-old F2 population of B6 x C3H intercross.[24] Moreover, our group found that total bone and Tb phenotypes are influenced by gene-by-diet (GxD) interactions[25,26] that are site specific.[21] These studies show a need for more research on genetic and GxD interactions affecting vertebral Tb, especially gene mapping studies that identify the loci and candidate genes contributing to vertebral Tb phenotypes.

To directly examine the effects of genetics on vertebral Tb phenotypes, we conducted a study using male mice from 51 BXD RI lines raised under rigorously controlled environmental conditions, with strict control of age, and the use of a dietary intervention known to influence bone mass, i.e. dietary Ca. We conducted genetic mapping analysis to identify quantitative trait loci (QTLs) that influence these phenotypes at the baseline and under low dietary Ca stress. Finally, coupling our QTL analysis to bioinformatics analysis, we were able to gain insight in candidate genes that potentially modulate these phenotypes.

4.3 Materials and Methods

4.3.1 Mouse Model

We conducted a forward genetic linkage mapping study to identify quantitative trait loci (QTLs) influencing the fifth lumber (L5) vertebral Tb phenotypes using 51 lines from the BXD recombinant inbred (RI) panel.[27] BXD lines are each defined by a fixed recombination pattern of alleles from the B6 and DBA inbred mouse lines.[27] Whereas individual F2 progeny from standard crosses are genetically unique, each BXD line is inbred and each individual within a line is genetically identical. This allows genetic experiments that include biological replicates and permits investigators to use interventions to test for the existence of gene-environment interactions.[27] A list of the specific lines used for the experiment is provided in **Supplemental Table S4.1**.

4.3.2 Experimental Design

Male mice from the 51 lines, as well as mice from the parental lines, were obtained at 4 weeks of age (the Jackson Laboratory, Bar Harbor, ME). At arrival, an equal number of mice from each line was randomly assigned to either a 0.5% Ca (Basal) or 0.25% Ca (LowCa) diet (AIN93G base with 200 IU vitamin D_3/kg diet, Research Diets, New Brunswick, NJ) (n = 8 per diet per line except BXD36 where n=4). Dietary Ca levels were chosen to meet the rodent dietary Ca requirement (0.5% Ca) or induce an adaptive increase in Ca absorption (+90%) (0.25% Ca).[25] The LowCa diet reflects the low Ca intake commonly seen in the United States (i.e., 50% of the requirement).[28,29] Mice were maintained in an UV-free environment (12 h light, 12 h dark) and given food and water ad libitum. At 12 weeks of age, mice were fasted overnight. Mice were anesthetized with a mixture of ketamine and xylazine and euthanized by exsanguination. The entire lumbar vertebral columns were harvested and preserved for analysis as previously described.[25]

L5 vertebra was extracted from the vertebral column. Investigators were blinded to genotype and dietary treatment, animal handling, bone sample collection, and endpoint measurements. All animal experiments were approved by the Purdue University Animal Care and Use Committee and were in compliance with the ARRIVE guideline.

4.3.3 Micro-computed Tomography (μCT) Evaluation

L5 vertebrae were analyzed using μCT (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) with scanning parameter settings reported elsewhere.[25] Briefly, vertebra was scanned at 16-μm resolution (isotropic voxel size) using an energy level of 55 kVp, an integration time of 300 ms, and an intensity of 145 μA. Since we have a large number of samples to scan, we executed a high-throughput scanning approach to fasten the time required for data collection Three vertebrae were stacked on top of each other and immobilized by inserting a blunt toothpick through the vertebral body. Six of three-vertebra stacks were placed in 15-mm-diameter polyetherimide sample holder filled with 70% ethanol and immobilized with cotton balls. The scan was performed for the entire three-vertebra stack. A 2D scout view of the chamber was examined from multiple angles (0, 90, 180 and 270 degrees) to confirm that all vertebrae were vertically aligned.

Using Scanco evaluation software (version 6), each vertebra was assessed for bone volume fraction (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn.D, $1/mm^3$), structure model index (SMI), and trabecular bone tissue mineral density (Tb.TMD) as recommended elsewhere.[30] The region of interest was defined as 0.800 mm (50 slices) distal to the end of cranial growth plate. This region was chosen as it well represents trabecular bone parameters of the entire L5.[31,32] We manually contoured trabecular bone every 10 slices with the outline 2-3 pixels away from the cortical bone,

and the intermediate slices were interpolated with the contouring algorithm in the software to create a volume of interest. Morphometric parameters were evaluated using a Gaussian filter = 0.8 and a threshold of 246 (559.6 mg HA/cm^3).

4.3.4 Statistical Analysis

Statistical analysis was conducted using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC). Data points with a Z-score in the extreme 2.5% of either end of a line/diet group distribution were removed as outliers. **Supplemental Table S4.1** reports the number of mice in each genotype and dietary treatment group for each µCT parameter after outlier removal. In addition to the data obtained from each mouse on each diet, a parameter reflecting the response to dietary Ca restriction (RCR) was calculated as the percent difference between the phenotypic value for an individual (i) fed the low Ca diet (x) and the line (j) mean for the phenotype value from the basal Ca diet (y), standardized to the line mean for the phenotypic value from the basal Ca diet and multiplied by 100, i.e., $\left[\left(x_{ij} - \bar{y}_j\right) / \bar{y}_j\right] * 100$.[25] For each phenotype, the covariate effect of body weight (BW) and/or femur length (FL) was determined by Pearson's correlation and removed by linear regression when the effect on a parameter was significant.[33] The body size (BS)-corrected residuals were examined for normality and all of them were normally distributed. Thus, no data transformation was required

Line means of BS-corrected residuals (as we called BS-corrected line means) for each dietary condition (basal, low Ca and RCR) were used for genetic mapping (**Supplemental Table S4.3)**. To reflect the variation among lines, Z-scores were calculated from the line means of BS-corrected residuals of the 51 BXD RI lines and their parental lines (**Supplemental Table S4.5)**. We assessed the narrow-sense heritability (h^2) of each Tb phenotype using the r^2 from a one-way Analysis of variance (ANOVA) (main effect = genotype); this was conducted separately for each

diet group as well as for their RCR. Two-way ANOVA of BS-corrected residuals was used to test the main effects and interaction effects (i.e. genotype-by-diet, GxD) on each phenotype. GxD interactions were also determined by conducting one-way ANOVA to assess the impact of genetics on the RCR. Using 51 BXD RI lines allowed us to reliably detect QTL accounting for 15% of the variance observed in the population with the power ~ 0.80 and $\alpha = 0.05$.[34,35] Using n=8/line/diet also allowed us to obtain accurate line estimates of Tb phenotypes.

4.3.5 QTL Mapping

The QTL mapping analysis was performed based on what have been previously described in Chapter 3. Briefly. BXD genetic markers were downloaded from the GeneNetwork (http://www.genenetwork.org/genotypes/BXD.geno) and were reduced to those that were informative among the 51 BXD RI lines used in this study (2244 genetic markers). using Windows QTL Cartographer v2.5_011 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm), we used BS-corrected line means (n=51) to perform composite interval mapping (CIM). Parameter setting was described in the Materials and Methods section of Chapter 3 (5 background markers, 2 cM walking speed, and a 10 cM window). Phenotypes for each diet (0.5% or 0.25% Ca) group and the RCR were mapped separately. For each analysis, permutations (n=1000) were used to determine significance threshold in a logarithm (base 10) of odds (LOD) unit. A peak with LOD $\geq$ the computed permutation threshold was considered significant and a peak with LOD ≥ 2 was considered putative.[36] In significant or putative QTL, the candidate gene region was determined as the 1.5-LOD support region.[37]

4.3.6 Prioritization of QTLs

Because we conducted QTL mapping analysis on multiple correlated Tb phenotypes, we observed several QTLs from multiple phenotypes that co-localized (i.e. the same peak estimate,

peaks located within 5 cM of each other, or overlapping 1.5-LOD intervals). We grouped the co-localized QTLs in each region of a chromosome into a single locus and assigned an identification (loci ID). A comprehensive list of these loci with the assigned loci ID is shown in **Supplemental Table S4.7**. Using this list of loci, we conducted a two-step prioritization. First, ranking the loci within the genetic mapping experiment of L5 vertebra. Second, consider loci identified from the mapping analysis of the distal femur altogether with those from the L5 vertebra. Finally, we included all loci identified from these two steps and considered them high-priority loci

4.3.6.1 Ranking Criteria among L5 Loci

We prioritized loci based on the following criteria:

1. A significant QTL of a single trait with an LOD >5
2. A significant QTL observed in an RCR phenotype
3. Significant (or one significant with one putative with LOD ≥ 3) QTLs observed for the same phenotype in both the basal and low Ca groups and with a matched parental influence.
4. Two or more QTLs (one must be significant and the other(s) must have LOD ≥ 3) observed within the same condition (basal, low Ca or RCR) or within the low Ca group and RCR with a matched parental influence.

We gave a score of 1 for each criterion that was met. These criteria are not mutually exclusive, e.g. a single locus may earn a score of two if it has an LOD > 5 and it is a significant RCR QTL. The loci with the score of 2 or greater were considered as *high-priority* loci that were then examined with in-depth bioinformatics analysis.

4.3.6.2 Ranking Criteria for Loci from L5 and the Distal Femur

Because we previously conducted a genetic mapping experiment on Tb phenotypes from the distal femur of the same set of animals, we aggregated the information of loci from both skeletal sites and screened for overlapping loci that contain at least 1 significant phenotype from either bone site. We then excluded the loci that have already been characterized in the previous study (i.e. considered as high-priority list for femur, see Chapter 3) as well as any loci that we already considered as high-priority loci for L5 vertebra from the previous step. The remaining loci were scored based on the following criteria:

1. Contains QTLs that mapped to Tb phenotypes from a similar diet group (basal or low Ca), from the analysis of RCR or from the low diet group and the RCR.
2. Contains at least 1 significant QTL from each bone site
3. Contains QTLs (significant or putative) that mapped to the same Tb phenotype from both sites.
4. Contains 2 or more significant QTLs in at least one bone site.
5. Parental line that influenced the QTLs from each site matched.

We gave a score of 1 for each criterion that was met. These criteria are not mutually exclusive, e.g. a single locus may earn a score of two if it has at least 1 significant QTL from each bone site and those QTLs controlled the same phenotypes in both sites. The loci with the score of 4 or greater were considered as high-priority loci that were then followed-up with bioinformatics analysis.

4.3.7 Bioinformatic Characterization of Loci

For each locus, single nucleotide polymorphisms (SNPs) between B6 and DBA mice were obtained using the Mouse Phenome Database (MPD, https://phenome.jax.org/snp/retrievals)[38]

with the Sanger4 data set that contains data on >89 million SNP and indels from 37 inbred strains of mice.[39] MPD annotations (NCBI dbSNP 138) were used to categorize polymorphisms by gene attribute: i.e. intronic and non-coding, insertions/deletions, mRNA untranslated region (5'and 3' UTR), and exon-associated (i.e. synonymous and non-synonymous codons, stop codons, splice sites, or frameshift mutations). Genes with SNPs that lead to gain or loss of a stop codon, cause a frameshift, and or alter a splice site were automatically considered candidate genes. Effects of non-synonymous amino acid changes were examined for potential functional effects using PROVEAN v1.1 (score < -2.5)[40] and SIFT v4.0.3 (score < 0.05).[41] Genes with deleterious/damaging SNPs identified by at least one tool were considered as candidate genes.

4.3.8 Expression QTL Mapping

Local (*cis*) eQTL analysis was conducted *in silico* using publicly available microarray data from the femur mRNA of the BXD panel (GN accession: GN414) that is available at the GeneNetwork (www.genenetwork.org/webqtl/main.py). We chose to use the femur mRNA data set because that is the only available data set for the BXD RI panel. Because our group have previously reported that there are site-specific genetic regulation as well as GxD interactions on Tb phenotypes in the distal femur and the L5 vertebrae,[21] we accepted that by using this data set we only capture cis eQTL that have consistent effects on both the femur and L5 vertebrae. Searches were conducted on the 1.5-LOD support interval of each locus. We defined the LRS threshold as > 9 (~ LOD = 2) to capture both significant and putative eQTL. Of these eQTL, we examined the correlation between the abundance of a gene transcript within the cis eQTL and BS-corrected line means of each phenotype mapped to the locus. Genes whose expression level correlated with our phenotypes (Pearson correlations $r > 0.3$, $p < 0.10$) are considered as candidate genes.

4.3.9 Annotation of Potential Candidate Genes

We identified the final list of candidate genes that are likely to have function altering polymorphisms influencing Tb phenotypes by searching the literature and public databases for information that links the candidate genes to bone biology: i.e. those with a significant association with bone phenotypes in GWAS, those shown to have a functional role in bone using KO mice, and those with evidence from animal or cell studies identifying mechanistic role for the candidate gene in bone cell biology or other pathways known to influence bone phenotypes. Associations between gene mutations in candidate genes and bone phenotypes, for candidate gene expression in limbs or skeletons at different stages of mouse embryo, and for functional annotation of the candidate genes using gene ontology (GO) terms was conducted using the Mouse Genome Informatics database (MGI, www.informatics.jax.org). Tissue expression patterns for each candidate gene were determined in mouse tissues and cell lines using BioGPS (http://biogps.org/). Changes in bone phenotypes resulting from candidate gene deletion were assessed using knockout (KO) mice data from the International Mouse Phenotyping Consortium (IMPC) and the International Mouse Strain Resource IMSR) databases. Finally, information regarding functional association of the candidate genes to biological pathways relevant to bone metabolism was obtained from Coremine (https://www.coremine.com/medical/).

4.4 Results

4.4.1 The Effect of Genetics, Dietary Ca Intake (D), GXD Interactions on Tb Phenotypes and Their Heritability

We observed variation in BW and FL among the 51 BXD RI lines so Tb phenotypes were examined for the effect of body size (BS) and adjustments were made as necessary (see Materials and Methods, **Supplemental Table S4.2**). BS-corrected line means for all phenotypes on each diet

as well as RCR are available in **Supplemental Table S4.3**. For each analysis (basal Ca, low Ca and RCR), there were significant relationships among all Tb phenotypes ($p < 0.05$, **Supplemental Table S4.4**) except for Tb.Th which is poorly correlated with Conn.D, Tb.TMD which was poorly correlated with Tb.N, Tb.Sp and Conn.D (only for RCR), and Tb.N which was not correlated with Tb.Th in the low Ca diet group.

For mice fed the basal Ca diet, h^2 ranged from 0.36-0.63 (lowest in Tb.TMD and highest in Tb.N and Conn.D) (all heritability values are given in **Supplemental Table S4.6**). Mice received the low Ca diet had a similar range of h^2 (0.32 at the lowest for Tb.TMD and 0.63 at the highest for Conn.D). Dietary Ca intake had a significant, independent effect on all Tb phenotypes ($p < 0.05$). There was significant variation across the lines for all of the Tb phenotypes in the basal diet group and for the RCR (**Figure 4.1** and **Supplemental Table S4.5**). In addition, the variability for the basal phenotypes were different from the variability of the RCR phenotypes. Using the two-way ANOVA, we found that Conn.D and Tb.Th were influenced by GXD interactions ($p = 0.026$ and 0.067, respectively). The RCR parameters of all phenotypes were significantly affected by genetics ($p < 0.005$) and had their heritability estimates range from 0.23-0.44.

4.4.2 Genetic Mapping

Our genetic mapping analysis of the phenotypes from mice fed the basal Ca diet, the low Ca diet and for the RCR phenotypes revealed a total of 57 loci (**Figure 4.2, Supplemental Fig. S4.1-4.3**, **Supplemental Table S4.7**). This includes 16 significant loci and 7 putative loci in the basal diet group phenotypes, 10 significant loci and 17 putative loci in the low Ca diet group phenotypes, and 12 significant and 10 putative loci for the RCR phenotypes. Some of loci from each analysis overlapped and the loci that contain at least one significant QTL are summarized in **Figure 4.3**.

We prioritized these loci for in-depth bioinformatics analysis and narrowed our interest from 57 to 9 loci. Four of the 9 loci were identified as QTLs for multiple basal phenotypes; three of these loci contained at least two QTLs with LOD > 4.5: S-2d, S-12b, and S-15a. The first locus, S-2d, contains QTLs controlling Tb.Sp, BV/TV, and SMI (**Supplemental Table S4.7**). The effect of this locus on Tb.Sp and SMI was influenced by the B6 alleles. The second locus, S-12b, has significant QTLs controlling Conn.D and Tb.N (LOD > 7) and a putative QTL for Tb.TMD (LOD=3.17). The effect of this locus on Conn.D and Tb.N was driven by the DBA alleles. The third locus, S-15a, contains significant QTLs controlling BV/TV and Tb.N, and a putative QTL controlling Tb.Th (LOD=2.16). The effect of this locus on BV/TV, Tb.N and Tb.Th is positively influenced by the B6 alleles. The fourth locus, S-7a, has significant QTL controlling BV/TV and Tb.Th, and putative loci that QTLs for Tb.Sp and Conn.D (LOD=3.08 and 3.07, respectively). The effect of this locus on BV/TV, Tb.Th, and Conn.D was positively influenced by the DBA alleles.

The loci S-2d, S-7a and S-12b mapped to QTLs for both basal and low Ca diet phenotypes. For example, S-12b contains robust low Ca diet QTLs (LOD > 9) for two phenotypes that also appeared in the analysis of basal diet group: Conn.D and Tb.N. The effect of this locus for Conn.D, Tb.N was driven by the DBA alleles in both diet groups. Thus, when a locus appears in both diet groups, this provides independent experimental confirmation of the validity of a genetic effect at that locus that is independent of the dietary environment of low calcium.

Several of the QTLs controlled phenotypes in either the basal diet group or the low Ca diet group only, or exclusively in the RCR phenotypes. This demonstrate that some genetic effects are sensitive to the dietary environment. Two of the high-priority loci we selected contain QTLs that control the RCR phenotypes: S-7b and S-20b. The first locus, S-7b, has two significant QTLs controlling the RCR of BV/TV and Tb.Th (LOD = 9.1 and 6.2 respectively, **Supplemental Table**

S4.7) and a putative QTL for SMI RCR (LOD = 2.26). The effect of this locus was positively influenced by the B6 alleles. S-20b (in chromosome (chr) X) contains QTLs for two RCR phenotypes including Tb.N and Conn.D. (LOD > 5).

In addition to 6 high-priority loci selected as the most robust (**Supplemental Table S4.7**), we selected 3 additional high-priority L5 loci that co-located with femur loci we have previously identified (see Materials and Methods). Initially, we identified 26 overlapping loci that contains at least 1 significant QTL from the combined list of all 57 L5 loci and 56 femur loci (**Supplemental Table S4.8)**. Three of the loci had mismatched effects between the two sites (i.e. the locus in L5 controlled Tb in the basal diet group whereas the locus in femur controlled RCR phenotype); thus, were excluded from the list. Of the remaining 23 loci, 6 loci have already been characterized and discussed in details in Chapter 3 (S-6b, S-8, S-9a, S-9c, S-10, S-12b and S-15b that overlapped with F-6c, F-8a, F-9a, F-9d, F-10c, F-12c and F-15b, respectively) so we excluded them from this list. Of the remaining 17 loci, two loci on chr 7 (i.e. S-7a overlapped with F-7b, and S-7b overlapped F-7c) were already reported as L5 high-priority loci in previous paragraphs based on the prioritization conducted within the entire list of L5 loci; hence, we removed them from the current list. Next, we prioritized the remaining 15 loci based on the 5 ranking criteria for a combined list of loci from two skeletal sites (see Materials and Methods section 4.3.6.2) to select the most robust co-locating loci between two sites. Of the 15 loci, two loci, *S-20c* and *S-20d*, had the priority score of 5 (the highest score) and one locus, *S-5c*, had a score of 4. The first locus, S-20c (in chr X) has two significant low calcium diet QTLs controlling Tb.Th and Tb.TMD in L5 vertebra, and a significant low Ca diet QTL controlling Tb.Th in the distal femur. The effect of this locus on these phenotypes was influenced by the B6 alleles. The second locus, S-20d (also in chr X) contains two significant low Ca diet QTLs controlling BV/TV and SMI in L5 vertebra

(LOD > 5), and a significant low Ca diet QTL controlling SMI in the femur. The effect of this locus on SMI was driven by DBA alleles in both skeletal sites. The third and last locus, S-5c, contains a significant basal diet QTL for Tb.Th in L5 vertebra (LOD = 7.96, high allele = DBA) and distal femur (LOD = 4.15, DBA) as well as a putative basal diet QTL for BV/TV (LOD = 3.55, DBA) in L5 vertebra.

4.4.3 In-depth Bioinformatic Analyses of High-priority loci

The nine high-priority loci were examined in detail using bioinformatics analysis. The number of genome features, genes, functional polymorphisms and eQTL at each locus is given in **Supplemental Table 4.9**. We systematically identified candidate genes in each locus and we chose S-7b to present in detail as an example (**Figure 4.4**). This locus contains QTLs controlling three RCR phenotypes of BV/TV, Tb.Th, and SMI. The 1.5-LOD confidence interval for this locus (49.73-74.18 Mb) encompasses 175 genes and 122,253 polymorphisms. In the coding sequence, there are 17694 SNPs: 96 SNPs in 5'UTR, 456 SNPs in 3'UTR, 5 splice-donor, 16818 indels, 5 premature-stop and 99 non-synonymous polymorphisms. Of the 99 non-synonymous polymorphisms, only 17 SNPs of the 13 genes were predicted to cause deleterious amino acid substitutions (**Table 4.1** and **Figure 4.4**). Of these 13 genes, only *Atp10a*, *Peg12*, *Tjp1, Pcsk6*, *Lrrk1*, *Lins1* (aka *Lins2*), and *Mctp2* have moderate to high expression in either osteoblasts, osteoclasts or bone tissue suggesting that these genes might be involved in bone development. Thus, we considered these seven genes as candidates for this locus. In the non-coding sequences of S-7b, five cis eQTL had maximum LRS > 9: *Lins1*, *Ube3a*, *Hddc3*, 2*810402K13Rik*, and *Lrrk1*. Though all of them correlates with at least one RCR phenotypes of this locus, only *Lins1* had significant correlations with all of the three RCR phenotypes and had the highest maximum LRS (25.8) As a result, we only considered *Lins1* a candidate gene from eQTL analysis.

Following this systematic pipeline, we identified several candidate genes for each of the other 8 high-priority loci (**Table 4.1** and **4.2**). Expression data and published evidence to support their function in bone phenotypes are provided in **Supplemental Tables S4.10 and S4.11**.

4.5 Discussion

Previous studies have shown that genetics contributes to Tb mass accrual and the development of bone microarchitecture.[21,24,25] Here we demonstrate that, in male mice from the BXD RI population, vertebral Tb phenotypes have strong heritable components with heritability estimates (h^2 = 0.36-0.63) much greater than those reported before in the L5 vertebra of the female F2 population of B6 x C3H intercross (B6C3-F2) (h^2 = 0.22-0.33).[24] More importantly, this is the first study to reveal genetic loci controlling the response of L5 vertebral Tb phenotypes to low dietary Ca intake during growth. The loci controlling the RCR phenotypes differed from the loci controlling the basal phenotypes and this supports the hypothesis that there are GxD interactions affecting Tb phenotypes.

Within our high-priority loci, we observed several novel loci that controlled basal Tb phenotypes. There is very limited data on genetic regulation of vertebral Tb phenotypes in growing animals. Our findings are inconsistent with existing literature from a female B6C3-F2 population.[24,42] This is likely due to differences in the gender we studied (male vs female), the inbred lines used in our crosses (DBA vs C3H), and the strict age and environmental controls we used in our study. Therefore, our study extends genetic effects on vertebral Tb phenotypes identified in the existing literature and allows us to gain insight in genetic regulations in Tb of the vertebrae.

One of the most robust loci we identified was S-2b. This locus contains QTLs controlling BV/TV, ConnD, SMI in both diet groups, Tb.TMD and Tb.Sp in the basal diet, and Tb.N in the

low Ca diet. We found *Wisp2* and *Cd40* as strong candidates for this locus. These two candidate genes have been discussed in Chapter 3 for the high-priority femur loci F-2e that controlled Conn.D on the low Ca diet and the Conn.D RCR phenotypes. Briefly, *Wisp2* promotes differentiation and function of osteoblasts[43-45] whereas *Cd40* promotes the production of osteoprotegerin in bone marrow which in turn controls the activity of osteoclasts.[46]

Another robust locus observed in the basal diet group is F-7a (chr 7, 24.45-42.18 Mb), which controls BV/TV, Tb.Th, Tb.Sp, and Conn.D. Among several candidate genes identified from the cis eQTL analysis, two genes had transcript levels that were strongly correlated with multiple Tb phenotypes and also have published evidence suggesting its role in bone biology: *Capns1* and *Nr1h. Capns1* encodes the calpain small subunit that forms a heterodimer with the calpain large catalytic subunit to modulate the cysteine protease activity of the complex. Capns1 binds to the PTH/PTH-related peptide (PTH1R) protein-coupled receptor and through this binding, calpain can modulates calcium-dependent hydrolysis of the PTH1R and PTH-mediated receptor signaling.[47,48] In addition, 2-week-old, pre-osteoblast-specific *Capns1* knockout (KO) mice showed significant reduction of both Tb and cortical bone in the tibia, while no difference in skeletal phenotypes was observed in mature osteoblast-specific *Capns1* KO mice when compared to wild-types.[48] This line of evidence suggests the role of Capns1 in proliferation of the osteoblast lineage potentially through regulation of PTH signaling.[48] We detected an eQTL of this gene (max LRS = 9.5) and the transcript level of *Capns1* showed a significant, negative correlation with Conn.D and BV/TV (r^2= -0.476 and -0.465, respectively with $p < 0.001$) as well as a positive correlation with Tb.Sp (r^2= -0.458). We hypothesize that the B6 allele will cause the reduction of *Capns1* transcript level. This will reduce the PTH signaling and possibly lead to an increase in the proliferation of pre-osteoblasts. Over the long term, this in turn will lead to greater Conn.D, BV/TV, and lower Tb.Sp

in vertebral Tb. Since the effect of this gene in bone biology is very sparse, future functional studies that involve PTH signaling as well as *in vivo* experiment with *Capns1* KO mice will be needed to confirm our hypothesis.

Another interesting candidate gene for the locus S-7a is *Nr1h2* that is also known as *LXRβ* (liver X receptor beta). The LXR family consists of LXRα and LXRβ and is well-known for its regulation of cholesterol homeostasis in cells.[49] Moreover, LXRβ is also highly expressed in bones, differentiating osteoblast-like MC3T3-E1 cells, and osteoclast-like RAW 264.7 cells at a much higher level than LXRα.[50] Robertson et al. have demonstrated that bones from 4-month-old female *LXRβ* KO mice had an increased expression of Runx2, the transcription factor necessary for osteoblast differentiation, with a trend towards elevated expression of osteocalcin and alkaline phosphatase.[50] Though these mice had no alteration in either cortical and trabecular BMD compared to the wild-type mice, *LXRβ* KO mice had a significant decrease in the number of osteoclasts in the trabecular compartment. We observed a cis eQTL that was significantly, negatively correlated with Conn.D and BV/TV in both diet groups ($r = -0.366$ to -0.436, $p<0.001$). We hypothesize a reduction of *LXRβ* mRNA will promote osteoblast activity and decrease the production of mature osteoclasts. This will result in a greater Conn.D and BV/TV that we observed in the L5 vertebra.

In addition to the robust loci S-2d and S-7a, S-15a (chr 15, 22.26-39.09 Mb) has a strong effect on BV/TV (LOD=6.11) and Tb.N (LOD=4.66) in the basal Ca diet group. We identified one potentially deleterious, coding variant in *Dnah5* and a cis eQTL in *Sema5a*. *Dnah5* encodes a dynein protein, which is part of a microtubule-associated motor protein complex consisting of heavy, light, and intermediate chains. Dnah5 gene deletion in B6 mice had normal BMD and BMC (https://www.mousephenotype.org/data/genes/MGI:107718) but showed mandibular hyperplasia

and enlarged cranium (http://www.informatics.jax.org/diseasePortal/genoCluster/view/2501) suggesting the gene KO favors bone formation. We hypothesize that the DBA variant affecting this gene may disrupt osteoclast function. However, direct functional studies examining the role of this gene in osteoclasts are warranted. The second candidate gene *Sema5a* encodes semaphorin 5A, a member of semaphorin protein family that have major physiological roles during the development of various organs including normal bone patterning (regulated by semaphorin 3A,[51] 4D,[52,53] and 7A).[54,55] Semaphorins binds to their receptor plexins to maintain homeostasis between osteoblasts and osteoclasts. Though there is no direct evidence of a role for semaphorin 5A in bone, data from the Human Phenotype Ontology (HPO) project have linked *Sema5a* to abnormal BMD (https://www.genecards.org/cgi-bin/carddisp.pl?gene=SEMA5A). The finding from our study, the links between other semaphorins with bone, and a possible relationship between *Sema5a* to BMD have led us to hypothesize that this gene plays a role in bone homeostasis and deserves further mechanistic investigation.

Among the robust loci, we have identified both common and site-specific loci controlling Tb phenotypes in the L5 vertebra and the femur. Our group have previously conducted genetic mapping analysis of femoral Tb phenotypes of the same mouse population (as reported in Chapter 3). When we compared loci identified from the genetic mapping of Tb phenotypes in the femur and those from the L5 vertebra, we found that there are some co-localized loci, e.g. a locus on chr 12 (S-12b and F-12c both controlled multiple Tb phenotypes including Tb.N in both diet groups, see Chapter 3). This finding demonstrates polymorphisms underlying this locus control Tb phenotypes in the same manner in both bone sites. Conversely, some robust, diet-insensitive loci in the L5 vertebra have not been detected in the femur, e.g. S-2d, S-7a. This suggests that strong genetic effects can differentially influence Tb phenotypes depending on bone site. Given that the

effects of polymorphisms within this locus are bone cells, one would expect to see similar impact of the polymorphisms in both bone site. However, but that was not we found when comparing the results from this study to the femur genetic mapping. The differential effects between sites could depend on some variables that vary among sites, e.g. could be the nature of mechanical loading as well as differential developmental program of bone cells at each site.[56] This line of evidence demonstrates that there are SxG interactions affecting Tb phenotypes and this supports what our group has previously reported.[21]

Because we studied two BXD RI mouse cohorts under the same controlled environment with dietary Ca intake as the only environmental variable, the second cohort receiving the low Ca diet can be used as a replication cohort for validating the effect of diet-insensitive genetic effects. The loci that mapped to the same phenotype in both cohorts are the environmentally independent, robust genetic effects. These loci include S-2d, S-7a, S-12b, and S-15a. Though these genetic effects showed strong influence on Tb phenotypes, for some loci, we did not find strong candidate genes that are associated with bone biology from our bioinformatic analysis, e.g. *Dnah5* and *Sema5a* in S-15a. We speculated that polymorphisms in these genes modulate Tb through other pathways aside from bone cells that have yet been identified. Therefore, it highlights the potential role of these genes in bone biology that deserve future functional validations.

We examined the impact of GxD interactions on vertebral Tb phenotypes in two ways; first, we identified loci that appeared only in either the analysis of basal or low Ca diet; second, we identified loci controlling for RCR phenotypes. The former approach revealed several loci that only controlled Tb phenotypes under the low dietary Ca environment, e.g. S-20c and S-20d. The first locus, S-20c (chr X: 127.95-140-89 Mb, high allele=B6), mapped to Tb.Th and Tb.TMD (LOD>4) in the low Ca diet. We found a potentially deleterious non-synonymous polymorphisms

for *Esx1*. This gene encodes extraembryonic, spermatogenesis, homeobox 1 that plays a role in placental development and spermatogenesis. Data from the mouse Gene Expression Database showed that this gene is strongly expressed in central nervous system, limb, tail mesenchyme, and skeleton of mouse embryo day 15 (http://www.informatics.jax.org/marker/MGI:1096388); however, the expression is restricted toward placenta and testes in adult (http://biogps.org/#goto=genereport&id=13984). This led us to hypothesize that this gene may be essential for embryonic skeletogenesis. Consistent with this hypothesis, female *Esx1* KO mice showed fetal growth retardation, decreased body size (http://www.informatics.jax.org/diseasePortal/genoCluster/view/24420). Future studies examining functional roles of this gene in bone development in both male and female mice will be necessary. The second locus in the low Ca diet analysis is S-20d (chr X: 149.30-157.89 Mb, high allele=B6). This locus controlled BV/TV and SMI (LOD>5). Unfortunately, we did not find any candidate genes from the bioinformatic analysis. Because there is no deleterious coding variant and no eQTL within this locus, we speculated that polymorphisms that strongly drives the RCR phenotypes may have been within the regulatory region which have not been captured by the eQTL analysis.

The effect of some genetic loci regulating Tb phenotypes in the low dietary Ca environment were observed in both bone sites. The two loci on chr X we discussed above (S-20c and S-20d) are also significant QTLs for femoral Tb.Th (F-20b, high allele=B6) and femoral SMI (F-20c, high allele=DBA) in the low Ca diet group. Another example of loci consistently mapped to Tb phenotypes of both sites in the low Ca diet group is S-10. The locus S-10 controlled vertebral Tb.Th and Tb.TMD in the low Ca diet group. S-10 completely overlapped with F-10c that controlled several femoral phenotypes (Tb.Th, SMI, Tb.N, and Tb.Sp) in the low Ca diet group

and Tb.N RCR (see Chapter 3). Collectively, the fact that overlapping loci control a similar phenotype (e.g. Tb.Th for S-20c and F-20b) in two skeletal sites under the low dietary Ca condition confirms that under low dietary Ca condition, there are common genetic regulation on Tb phenotypes across vertebra and femur.

The second approach that we used to examine GxD interactions was to identify loci controlling RCR phenotypes. All significant RCR loci were found at different locations from loci controlling Tb phenotypes in the basal diet. Two of our high-priority loci that strongly controlled RCR phenotypes include F-7b and F-20b. The first locus, F-7b, contains significant QTLs controlling BV/TV RCR and Tb.Th RCR (LOD>6, high allele=B6), and a putative QTL for SMI RCR (LOD=2.26, high allele=DBA). This locus accounts for > 30% of the variation in each of the first two RCR phenotypes. We identified two candidate genes that have strong published evidence to support their roles in bone: *Lins1* and *Lrrk1*. *Lins1* (aka *Lins2, Lins, Wins2*) is a mouse homolog of *Drosophila* segment polarity gene *lin* that encodes an essential regulator of the wingless/Wnt signaling called lines.[57] Because this gene was first discovered in *Drosophila*, the majority of the literature examining the function of this gene was done in the fruit fly models. The protein lines have been shown to be essential for coordination among wingless, hedgehog, and notch pathways to regulate normal development of *Drosophila* wings.[58,59] In vertebrates, these three pathways are integral for bone development. This notion is supported by the observations by Benitez et al.[59] that *Lins1* involved in the regulation of Wnt signaling pathways by acting as one of the transcription regulator and upregulated the transcription of genes whose human homologues are involved in bone formation: *WNT1,*[60] *SMAD3,*[61] and *EN1/2*.[62] Along the same line, data from Mouse ENCODE showed that *Lins1* has the highest expression in limb of mouse embryo Day 14.5.[63] Because we observed three potentially deleterious non-synonymous coding variant and

one eQTL (LRS=25.8, r for SMI-RCR, Tb.Th-RCR and BV/TV-RCR= 0.663, -0.532, and -0.335, respectively), we believe that reduced function of *Lins1* caused by point mutations (from B6 alleles to DBA alleles) will weaken the interaction of Lins1 and β-catenin resulting in reduced β-catenin mediated transcriptional activation of genes that are important for the skeletal development and this impact is only discernable under the dietary Ca restriction. Consequently, Tb mass accrual and the development of Tb microarchitecture is impaired. Consistent with the hypothesis, under normal growing condition, *Lins1* KO mice did not show significant decrease in BMC or BMD compared to wild types (https://www.mousephenotype.org/data/genes/MGI:1919885). This gene has a promising role in bone development and future research should investigate functional roles of *Lins1* in *in vitro* and mouse models under the low dietary Ca condition.

Another candidate gene in F-7b is *Lrrk1*. This gene is highly expressed in late stage osteoclasts[64] and *Lrrk1* KO mice showed severe osteopetrosis in the metaphysis of the long bones and vertebrae bones.[65,66] Xing et al. showed that *Lrrk1* KO mice had the same number of mature osteoclasts as the wild types, but these osteoclasts cannot form peripheral sealing zones and ruffled borders; hence, had reduced bone resorption.[64,65] Lrrk1 regulates osteoclast function by directly modulating phosphorylation and activation of small GTPase RAC1 and CD42.[67] These small GTPases play crucial roles in osteoclast differentiation, function and survival.[68] In humans, mutation of this gene in humans causes osteosclerotic metaphyseal dysplasia which is a unique form of autosomal recessive osteopetrosis characterized by severe osteosclerosis localized to the bone ends.[64] We observed a deleterious coding variant and an eQTL (LRS=11.8, r for Tb.Th-RCR=0.447) of this gene in F-7b. This leads us to hypothesize that mutations of this gene will lead to reduced functional mature osteoclasts. As a result, under low dietary Ca condition, individuals with these mutation will have compromised Tb bone mass and thickness. Though the functional

role of this gene has been confirmed in *in vitro* studies, KO mouse model as well as in humans, our study suggest that this gene might be protective to Tb in the low dietary Ca environment. Future KO mouse study with the low Ca diet will help confirm its function in this specific situation.

In addition to S-7b, we identified S-20b as another strong locus controlling Conn.D RCR (LOD=6.27) and Tb.N RCR (LOD=3.91). The genetic effect of this locus accounts for >20% of the variation of each RCR phenotype. We found a deleterious non-synonymous polymorphism of *Mamld1* in this locus. *Mamld1* encodes transcriptional co-activator mastermind-like domain containing 1. Female *Mamld1* KO mice had significant decreases in BMC/body weight and tibia length compared to wild types (https://www.mousephenotype.org/data/genes/MGI:3045303). Previous studies showed that *Mamld1* mutations are linked to sexual development disorders (i.e., hypospadias[69] and gonadal dysgenesis[70] This combined evidence suggest that the effect on Tb phenotypes observed in this study can be the consequence of abnormality of sex hormone production. We hypothesize that under low Ca stress, mice with *Mamld1* mutation lose the ability to retain in Conn.D and Tb.N in the L5 vertebra but the underlying molecular mechanisms have not been elucidated. S-7b and S-20b well exemplified the impact of diet-sensitive loci that regulate vertebral Tb phenotypes independent of genetic factors controlling the phenotypes in the basal Ca diet.

In comparison to the RCR loci from the genetic mapping of the femur, we observed two diverging patterns of genetic effects among two bone sites, i.e. those that are common and the others that are site-specific RCR loci (see **Supplemental Fig. S4.4**). The RCR loci that are common between two sites indicate their robust effects that are independent of site or other variables between the two sites. In contrast, the site-specific effect of RCR loci suggests that the influence of these loci on the RCR phenotypes are dependent on site or underlying factors that

differs between the two bone sites, e.g. mechanical loading. The unknown factors that contribute to these site-specific differences deserve further research. Collectively, these observations corroborate what our group previously published[21] that there are differential effects of GxD interactions (or SxGxD interactions) on Tb phenotypes in the femur and the L5 vertebra.

Our study has the following strengths. First, we used the BXD RI population which allowed us to leverage the fixed genetic nature of each line to test the GxD interactions on Tb phenotypes is unethical and infeasible in human studies to discover genetics underlying normal variation in vertebral Tb phenotypes during murine growth period. Second, we strictly controlled all of environmental factor including the use of well-define diets. As a result, we extended the literature by distinguishing genetic loci controlling Tb phenotypes that are strong and robust across our diet groups from those that are sensitive to dietary Ca intake level. In addition, we demonstrated that there are diet-sensitive genetic loci that specifically influence the development of Tb phenotype only in the L5 vertebrae. Nonetheless, our study has certain limitations. First, our study was designed to capture genetic modulation through bone cells that results in Tb phenotypes. Because bone is not exclusively regulated by bone cells, but rather by crosstalk of multiple tissues (e.g. kidney, intestine, muscle), our findings are not fully comprehensive. Second, we used the femur mRNA data set for our eQTL analysis with the assumption that the activity of bone cells controlled by genetics are similar between the L5 vertebra and the femur. We are limited to this assumption because the femur data set is the only bone-related data set available for the BXD RI population. However, as our group reported that there is site-specific genetic regulation on Tb phenotypes, it is critical to point out that our finding did not capture any eQTL that may be specific to the L5 vertebrae. Third, we did not find candidate genes for a few of genetic loci we identified. This does not minimize the effect of those loci but emphasize the lack of existing resource for gene

identification. Because we did not observe any deleterious coding variants in those loci, we speculated that the bottleneck is to identify and predict polymorphic putative regulatory regions for genes that might play a role in bone development. Lastly, our study only used male mice and therefore, might have not captured genetic effects that are influenced by female sex hormones.

4.6 Conclusion

This study is the first to use the BXD RI panel to identify genetic polymorphisms controlling Tb phenotypes in the L5 vertebra of growing male mice as well as to identify the loci controlling the response of vertebral Tb phenotypes to a low dietary Ca environment. This research has confirmed our earlier observations revealing GxD interactions on vertebral Tb phenotypes [(21)] and they have extended them by uncovering novel genetic loci underlying these GxD effects on L5 vertebral Tb phenotypes. Through bioinformatics analysis, we have identified a number of promising candidate genes that may play crucial roles in Tb homeostasis. Our findings provide a strong foundational evidence for furthers mechanistic research relating these candidate genes to bone biology. Finally, we established the existence of bone site-specific genetic influences on Tb phenotypes, including those that are sensitive to the dietary environment.

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4.8 Author's Roles

Study design: JCF. Study conduct: JCF, Bone analysis: PRF and KC, Data management and statistical analysis: KC, Data interpretation: JCF, PRF, and KC. Drafting manuscript: JCF and KC. Revising and approving final version of manuscript: JCF, PRF and KC. JCF takes responsibility for the integrity of the data analysis.

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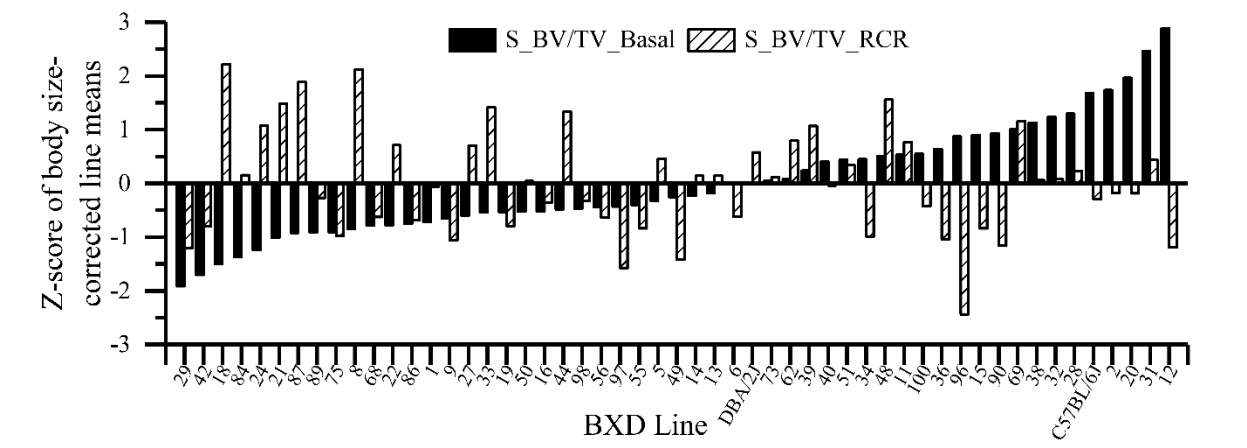

Figure 4.1 *Z-scores of body size corrected trabecular bone volume fraction (BV/TV) from the basal Ca diet and Tb.N RCR values from 51 BXD lines.* Lines are ordered for smallest to largest for the BV/TV basal phenotype. Values for the parental lines DBA/2J and C57BL/6J are included for reference.

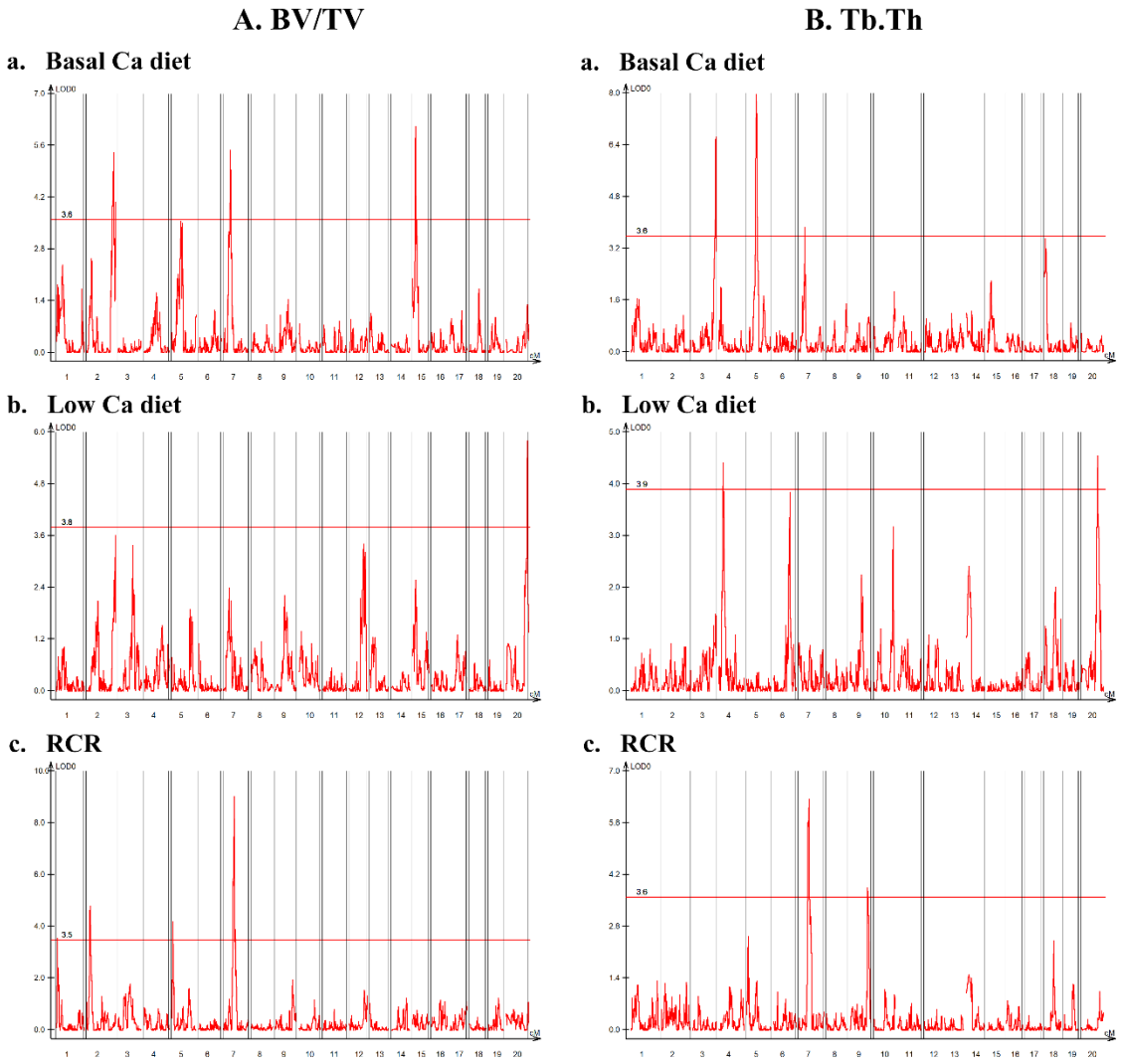

Figure 4.2 *Composite interval maps for vertebral BV/TV (A) and Tb.Th (B).* For each phenotype, the maps are presented in the vertical order of basal (a), low Ca (b), and RCR (c) phenotypes

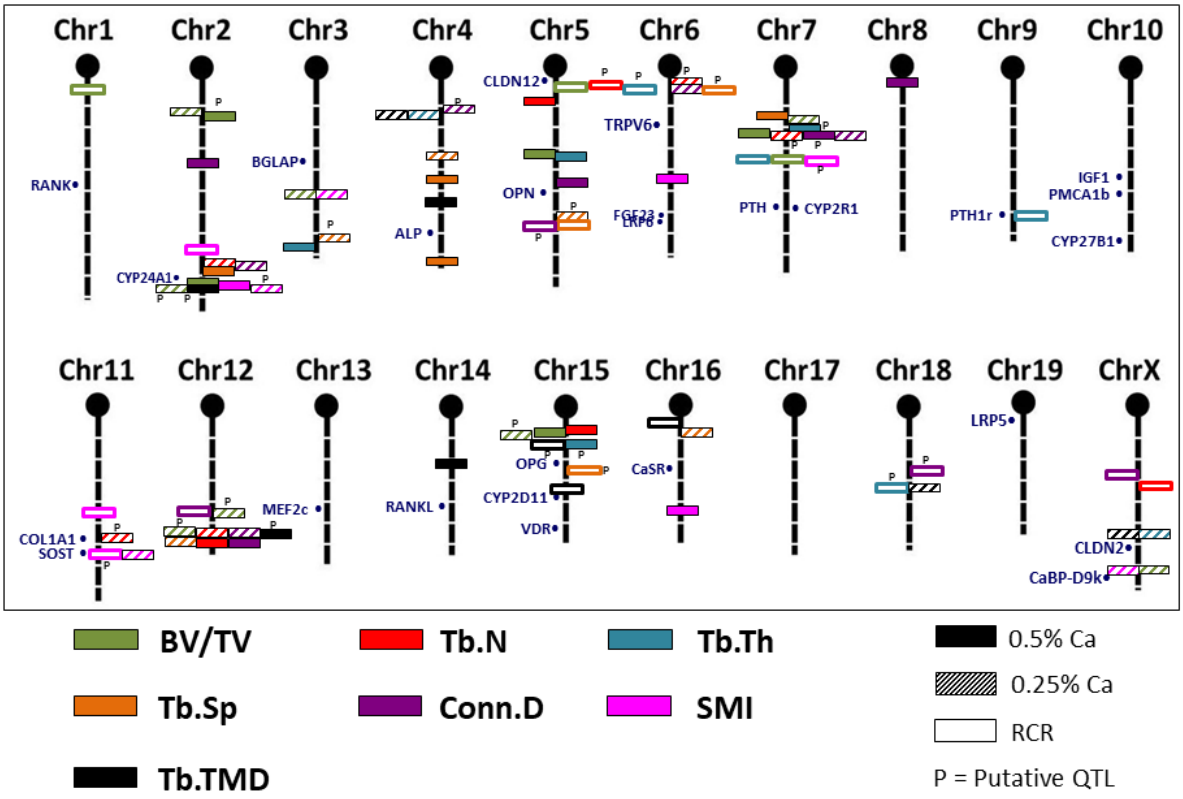

Figure 4.3 *A whole genome summary of QTL identified for femoral Tb phenotypes from 51 BXD mouse lines*. Only loci that has at least one significant QTL are shown in this map (36 loci out of 57). Trabecular bone phenotypes are coded by color and for diet group used. RCR = response to dietary Ca restriction. P = putative QTL.

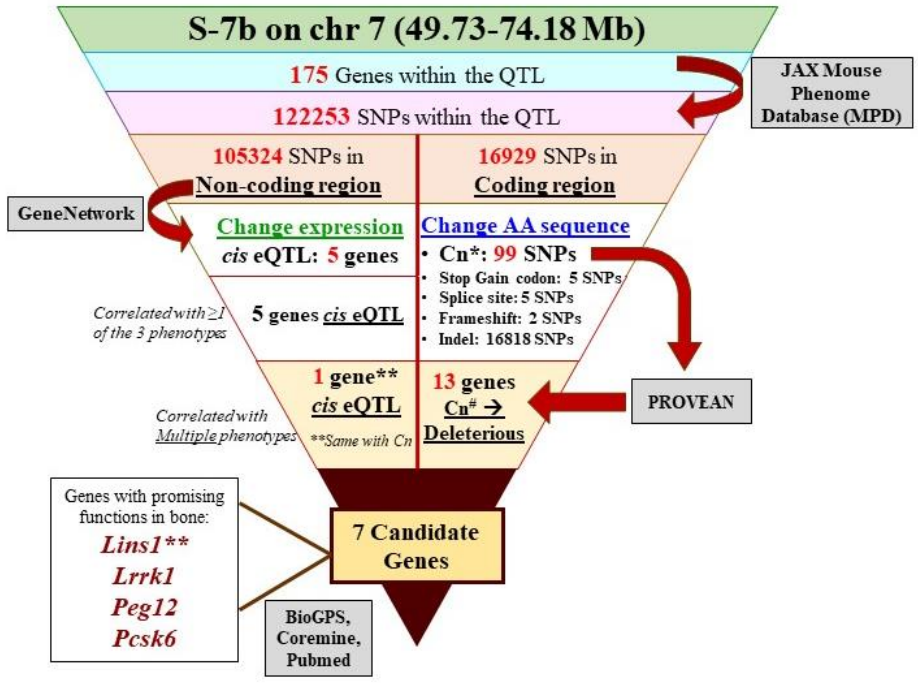

Figure 4.4 *Systematic identification of candidate genes in a representative high-priority locus S-7b.* This locus controls three RCR phenotypes (BV/TV, Tb.Th, and SMI). SNPs = single nucleotide polymorphisms. eQTL= Expression quantitative trait loci. * Polymorphisms for non-synonymous amino acid substitutions. # Polymorphisms scored as potentially deleterious nonsynonymous amino acid substitutions. ** Candidate gene with both deleterious non-synonymous amino acid substitutions and an eQTL. Bioinformatic tools and websites used for identifying candidate genes are indicated in the grey boxes.

Table 4.1 Summary of Potential Functional Candidate Genes within Prioritized QTL in the L5 vertebra *

QTL Block ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Types of Consequence in Protein Coding Region[d]			
				Altered Amino Acid Sequence[e]	Premature Stop Codon	Splice Site Mutation	Frameshift
S-2d	2	162.61-178.48	Tb.Sp_Basal (B6[b], 8.85[c]), BV/TV_Basal/LowCa (DBA, 5.40/3.61), SMI_Basal/LowCa (B6, 5.52/2.57), Conn.D_LowCa (DBA, 5.05), Tb.N_LowCa (DBA, 4.17), Tb.TMD_Basal (B6, 2.73)	**Wips2**, Kcnk15, Zfp335(2), **Cd40**, Sall4, Bcas1	-	Wfdc8, LOC105244377, Prex1	-
S-5c	5	52.77-73.18	BV/TV_Basal (DBA, 3.55), Tb.Th_Basal (DBA, 7.96)	Cckar, G6pd2(2), Tmem33,	Atp10d	Txk, LOC105244755	Pgm1
S-7a	7	24.45-42.18	BV/TV_Basal/LowCa (DBA, 5.34/2.40), Tb.Th_Basal (DBA, 3.86), Tb.N_LowCa (DBA, 3.26), Tb.Sp_Basal (B6, 3.08), Conn.D_Basal/LowCa (DBA, 3.07/3.60)	Axl, Cyp2g1, Prx, Zfp84, Zfp790, Zfp27, Zfp82(2), Zfp382, Wdr62(2), Syne4, E130208F15Rik, Kirrel2, Nphs1, Prodh2(2), Proser3(2), Lin37, Zbtb32, Etv2, Haus5(2), Sbsn, Dmkn, Cd22, Hamp2, Fam187b, Lgi4, Scgb2b2, Scgb1b3, Scgb1b24, Scgb2b26(5), Scgb2b27, Lsm14a, Chst8, 4930433I11Rik, AI987944(3), Vmn2r57(6), AW146154, Vmn2r58(3), Vmn2r59	Fxyd1[StopL], Proser3[StopL]	Zfp507, Hipk4, Chst8, Ovol3, Zfp27(2), Arhgap33os, LOC105242912, Vmn2r-ps54	Fxyd1, Gapdhs, Hspb6, Lgi4, Scgb1b30, Scgb2b24, Wtip
S-7b	7	49.73-74.18	BV/TV_RCR (B6, 9.01), Tb.Th_RCR (B6, 6.24), SMI_RCR (DBA, 2.26)	Tubgcp5(2), Atp10a, Magel2, Peg12, Trpm1, Fan1, Tjp1(2), **Pcsk6**, **Lrrk1**, **Lins1**(3), Synm, Mctp2, A830073O21Rik	Klf13, E030018B13Rik, Otud7a, Slc6a5	Otud7a, A230057D06Rik LOC105242973, Tjp1	Htatip2, LOC102641467

Table 4.1 continued

QTL Block ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Types of Consequence in Protein Coding Region[d]			
				Altered Amino Acid Sequence[e]	Premature Stop Codon	Splice Site Mutation	Frameshift
S-12b	12	102.99-111.26	Conn.D_Basal/LowCa (DBA, 7.47/10.49), Tb.N_Basal/LowCa (DBA, 7.36/9.28), Tb.Sp_LowCa (B6, 8.49), BV/TV_LowCa (D, 3.21), S_TbTMD_H (B, 3.17)	Serpina1f(2), Serpina1c, Serpina3a(4), Serpina3b(6), Serpina3c, Serpina3i(3), Serpina3k(2), Serpina3m, **Serpina3n**(2), Syne3, Slc25a47, Tecpr2(2)	Serpina3i, Serpina3b^{StopL}, Serpina3f	Serpina3a, Serpina9, **Serpina3n**, Wdr25, Wars, LOC105245053, LOC105245074	Serpina3j, Serpina3m, Serpina3i
S-15a	15	22.26-39.09	BV/TV_Basal/LowCa (B6, 6.11/2.57), Tb.N_Basal (B6, 4.66), Tb.Th_Basal (B6, 2.16), Tb.TMD_RCR (B6, 3.36)	Dnah5	-	-	-
S-20b	X	50.73-71.53	Conn.D_RCR (B6, 6.27), Tb.N_RCR (B6, 3.91)	Zfp36l3, Vgll1(2), **Mamld1**	-	-	-
S-20c	X	127.92-140.89	Tb.Th_LowCa (B6, 4.55), Tb.TMD_LowCa (B6, 4.02)	Kir3dl1(3), Tmsb15b1, Tmsb15b1, Tmsb15l, Tmsb15b2, Esx1	Kir3dl1	-	Kir3dl1
S-20d	X	149.30-157.89	BV/TV_LowCa (B6, 5.80) SMI_LowCa (DBA, 5.11)	-	-	-	-

* This table does not include polymorphisms found in predicted genes. Abbreviation: Chr = chromosome; Mb = megabase; BV/TV = bone volume fraction; Tb.N = trabecular number (mm-1); Tb.Th = trabecular thickness (mm); Tb.Sp = trabecular separation (mm); Conn.D = connectivity density (1/mm^3); SMI = structure model index; Tb.TMD = Trabecular tissue mineral density (mg HA/cm3).

[a] 1.5-LOD confidence intervals in Mb (Build GRCm38/mm10). [b] Parental influence: B6 = C57BL/6J line, DBA = DBA/2J line

[c] Phenotype-specific LOD score for each locus. [d] In parenthesis, number of polymorphisms in that gene if different from 1.

[e] Genes with polymorphisms scored as potentially deleterious nonsynonymous amino acid substitutions. ^{StopL} Genes with polymorphisms cause a lost stop codon.

Bolded gene names are the gene with published evidence for potential functions in bone.

Table 4.2 Summary of Genes with cis eQTL within Prioritized Loci that are Correlated to Vertebral Trabecular Bone Phenotypes [ϕ]

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
S-2d	2	162.61-178.48	Tb.Sp_Basal (B6[b], 8.85[c]), BV/TV_Basal/LowCa (DBA, 5.40/3.61), SMI_Basal/LowCa (B6, 5.52/2.57), Conn.D_LowCa (DBA, 5.05), Tb.N_LowCa (DBA, 4.17), Tb.TMD_Basal (B6, 2.73)	Zfp313 (14.4)	SMI: r = -0.37[#] BV/TV: r = 0.36[#]		
S-5c	5	52.77-73.18	BV/TV_Basal (DBA, 3.55), Tb.Th_Basal (DBA, 7.96)	Atp10d (14.1)	Tb.Th: r = 0.34[#]		
				0610040J01Rik (10.2)	BV/TV: r = -0.42*		
				Cep135 (9.3)	Tb.Th: r = 0.34[#]		
S-7a	7	24.45-42.18	BV/TV_Basal/LowCa (DBA, 5.34/2.40), Tb.Th_Basal (DBA, 3.86), Tb.N_LowCa (DBA, 3.26), Tb.Sp_Basal (B6, 3.08), Conn.D_Basal/LowCa (DBA, 3.07/3.60)	Sbsn (32.8)	Tb.Th: r = 0.35[#] BV/TV: r = 0.34[#]		
				Lin37 (31.0)		Conn.D: r = 0.37* Tb.N: r = 0.41*	
				Gpi1 (25.7)	Conn.D: r = -0.31[#] BV/TV: r = -0.42* Tb.Th: r = -0.33[#] Tb.Sp: r = 0.32[#]	Conn.D: r = -0.39* BV/TV: r = -0.37* Tb.N: r = -0.34[#]	
				Zfp27 (25.3)	BV/TV: r = -0.62* Tb.Th: r = -0.71* Tb.Sp: r = 0.35[#]	BV/TV: r = -0.41*	

Table 4.2 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
S-7a (Cont')	7	24.45-42.18	BV/TV_Basal/LowCa (DBA, 5.34/2.40), Tb.Th_Basal (DBA, 3.86), Tb.N_LowCa (DBA, 3.26), Tb.Sp_Basal (B6, 3.08), Conn.D_Basal/LowCa (DBA, 3.07/3.60)	Tbcb (24.2)	BV/TV: r = -0.38* Tb.Th: r = -0.56*		
				ILM102450041 (22.5)	Conn.D: r = -0.38* BV/TV: r = -0.49* Tb.Th: r = -0.34[#] Tb.Sp: r = 0.46*	Conn.D: r = -0.34[#] BV/TV: r = -0.43* Tb.N: r = -0.39*	
				Nr1h2 (21.5)	Conn.D: r = -0.37* BV/TV: r = -0.44* Tb.Sp: r = 0.37*	Conn.D: r = -0.42* BV/TV: r = -0.42* Tb.N: r = -0.39*	
				LOC243902 (21.4)	BV/TV: r = -0.47* Tb.Th: r = -0.41*	BV/TV: r = -0.37* Tb.N: r = -0.37[#] Conn.D: r = 0.37*	
				Zfp84 (17.7)	BV/TV: r = -0.33[#] Tb.Th: r = -0.36[#]	BV/TV: r = -0.31[#] Tb.N: r = -0.35[#]	
				Atp4a (15.3)	Conn.D: r = 0.43* BV/TV: r = 0.47* Tb.Sp: r = -0.37*	Conn.D: r = 0.42* BV/TV: r = 0.37* Tb.N: r = 0.39*	
				Zfp420 (14.4)	Tb.Th: r = -0.43*		
				4930428B01Rik (13.8)	BV/TV: r = 0.34[#] Tb.Th: r = 0.33#		
				Kcnk6 (12.3)	BV/TV: r = 0.34[#]	BV/TV: r = 0.32[#] Conn.D: r = 0.35[#]	
				Nudt19 (10.5)	Tb.Th: r = -0.37*		
				Capns1 (9.5)	Conn.D: r = -0.48* BV/TV: r = -0.47*	Conn.D: r = -0.72* BV/TV: r = -0.56*	

Table 4.2 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
S-7a (Cont')	7	24.45-42.18			Tb.Sp: r = 0.46*	Tb.N: r = -0.63*	
				Cyp2f2 (9.4)		BV/TV: r = -0.40* Conn.D: r = -0.32# Tb.N: r = -0.37*	
S-7b	7	49.73-74.18	BV/TV_RCR (B6, 9.01), Tb.Th_RCR (B6, 6.24), SMI_RCR (DBA, 2.26)	Lins1[$] (25.8)			BV/TV: r = -0.34# Tb.Th: r = -0.53* SMI: r = 0.66*
				Ube3a (17.6)			SMI: r = -0.42*
				Hddc3 (16.1)			SMI: r = 0.33#
				2810402K13Rik (13.1)			SMI: r = 0.49*
				Lrrk1 (11.8)			Tb.Th: r = 0.48*
S-12b	12	102.99-111.26	Conn.D_Basal/LowCa (DBA, 7.47/10.49), Tb.N_Basal/LowCa (DBA, 7.36/9.28), Tb.Sp_LowCa (B6, 8.49), BV/TV_LowCa (D, 3.21), S_TbTMD_H (B, 3.17)	Klc1 (21.8)	Conn.D: r = -0.40* Tb.N: r = -0.38*	Conn.D: r = -0.34# Tb.N: r = -0.31#	
				Apopt1 (20.1)	Conn.D: r = -0.37* Tb.N: r = -0.32#	Conn.D: r = -0.41* Tb.N: r = -0.36# BV/TV: r = -0.32# Tb.Sp: r = 0.35#	
				1810056I18Rik (19.4)	Conn.D: r = -0.34# Tb.N: r = -0.32#	Conn.D: r = -0.33# Tb.Sp: r = 0.33#	
				Rian (14.6)	Conn.D: r = -0.42* Tb.N: r = -0.32#	Conn.D: r = -0.42* Tb.N: r = -0.33#	

Table 4.2 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
S-12b (Con't)	12	102.99-111.26				BV/TV: r = -0.33[#] Tb.Sp: r = 0.37*	
				Cdc42bpb (14.3)	Conn.D: r = -0.33[#]	Conn.D: r = -0.48* Tb.N: r = -0.44* Tb.Sp: r = 0.44*	
				Igh-V (12.6)	Conn.D: r = 0.59* Tb.N: r = 0.63*	Conn.D: r = 0.48* Tb.N: r = 0.55* BV/TV: r = 0.56* Tb.Sp: r = -0.54*	
				Atxn3 (10.2)	Conn.D: r = 0.47* Tb.N: r = 0.43*	Conn.D: r = 0.52* Tb.N: r = 0.45* BV/TV: r = 0.59* Tb.Sp: r = -0.48*	
				LOC380800 (9.7)	Tb.TMD: r = 0.38*		
				Ppp2r5c (9.5)	Conn.D: r = -0.35[#] Tb.N: r = -0.40*	Conn.D: r = -0.35[#] Tb.N: r = -0.38* Tb.Sp: r = 0.33[#]	
				Ddx24	Conn.D: r = -0.32[#] Tb.N: r = -0.35[#]	Conn.D: r = -0.36[#] Tb.N: r = -0.38* Tb.Sp: r = 0.34[#]	

Table 4.2 continued

Loci ID	Chr	1.5-LOD CI (Mb)[a]	Phenotypes associated with QTL	Candidate genes from eQTL[h] (LRS)	Phenotypes Correlated with eQTL with Correlation Coefficient		
					Basal	LowCa	RCR
S-15a	15	22.26-39.09	BV/TV_Basal/LowCa (B6, 6.11/2.57), Tb.N_Basal (B6, 4.66), Tb.Th_Basal (B6, 2.16), Tb.TMD_RCR (B6, 3.36)	Sema5a (59.2) 9430031J08Rik (43.7) 3110047M12Rik (33.4)	BV/TV: r = 0.39* Tb.N: r = 0.36# Tb.N: r = 0.31# BV/TV: r = 0.32#		Tb.TMD: r = 0.34# Tb.TMD: r = 0.34#
S-20b	X	50.73-71.53	Conn.D_RCR (B6, 6.27), Tb.N_RCR (B6, 3.91)	Rpl7a (9.4)			Tb.N: r = 0.34# Conn.D: r = 0.34#
S-20c	X	127.92-140.89	Tb.Th_LowCa (B6, 4.55), Tb.TMD_LowCa (B6, 4.02)	Mcart6 (18.3)		Tb.TMD: r = 0.39* Tb.Th: r = 0.32#	
S-20d	X	149.30-157.89	BV/TV_LowCa (B6, 5.80) SMI_LowCa (DBA, 5.11)	-			

* This table does not include polymorphisms found in predicted genes. Abbreviation: Chr = chromosome; Mb = megabase; BV/TV = bone volume fraction; Tb.N = trabecular number (mm-1); Tb.Th = trabecular thickness (mm); Tb.Sp = trabecular separation (mm); Conn.D = connectivity density (1/mm^3); SMI = structure model index; Tb.TMD = Trabecular tissue mineral density (mg HA/cm3).

[a] 1.5-LOD confidence intervals in Mb (Build GRCm38/mm10). [b] Parental influence: B6 = C57BL/6J line, DBA = DBA/2J line

[c] Phenotype-specific LOD score for each locus. Significant correlation with Tb phenotypes: *p<0.05, #p<0.10. $ This gene is also known as Lins2, Lins, Wins2.

CHAPTER 5. RESEARCH SUMMARY AND FUTURE DIRECTIONS

5.1 Research Summary

Trabecular bone (Tb) mass and microarchitecture are important determinants for mechanical properties of long bones and the vertebrae.[1] As such, the accretion of Tb mass and development of well-connecting microarchitecture early in life play such a critical role in preventing future osteoporosis and fractures. Previous literature suggests that dietary Ca deficiency[2,3] as well as genetics[4] may have site-specific effects on bone. It was critical that this assumption be tested because this knowledge will shape the way we interpret the literature as well as design future research on bone health. My dissertation aimed to identify genetic factors that underlie Tb phenotypes in the adequate Ca environment as well as those controlling the response to low dietary Ca intake at two clinically relevant skeletal sites.

In the first part of this dissertation (Chapter 2), we conducted an experiment to test the hypothesis that dietary Ca intake (D), genetics (G), and GxD interactions have differential effects between two clinically relevant sites (S), i.e. the distal femur and fifth lumbar (L5) vertebra. Because we had absolute control over both genetics and environmental factors, we chose to assess the effect of each factor in 2 different ways. First, we considered the entire mouse cohort as a genetically diverse population, i.e. similar to the genetic diversity seen in humans. We showed that low dietary Ca intake throughout growth has a strong, independent, site-specific effect on Tb mass and microarchitecture. Specifically, Ca deprivation leads to a greater relative reduction of trabecular number (Tb.N) and connectivity density (Conn.D) in the femur than L5 vertebra. Thus, by losing trabeculae and connections between trabeculae, low dietary Ca intake has a greater impact on femur than spine. Second, we accounted for genetic structure existing in our mouse cohort (11 inbred and 51 BXD RI lines) and we systematically demonstrated for the first time that

genetic background influences Tb phenotypes in a site-specific manner. Finally, we confirmed that the influence of GxD interactions is different between the two sites, i.e. site-by-gene-by-diet (SxGxD) interactions influence bone.

Chapters 3 and 4 of the dissertation, we conducted QTL mapping using the 51 BXD RI inbred lines from Chapter 2. Because we demonstrated that there is a site-specific effect of GxD interactions on Tb phenotypes, we conducted the genetic mapping analysis separately for each bone site and identified genetic loci controlling Tb phenotypes in the basal Ca diet as well in the low Ca environment for the femur (Chapter 3) and the vertebrae (Chapter 4).

In Chapter 3, we reported that approximately 50-70% of the variation in Tb phenotypes is accounted for by genetics. When we considered the impact of GxD interactions, i.e. reflected in the response to dietary Ca restriction (RCR) phenotypes, we found that the heritability estimates of Tb phenotypes were between 0.23-0.39. This suggests that genetics controlling the RCR phenotypes is weaker than the genetics controlling the basal bone phenotypes. From the genetic mapping analysis of the femur, we identified the total of 56 loci and selected 10 high-priority loci (4 robust, diet-insensitive loci and 6 diet-sensitive loci) for further characterization. By coupling genetic mapping with bioinformatic analysis, we identified potential candidate genes accounting for the variation observed in the phenotypes for both in the basal diet and those responding to the low Ca diet.

In Chapter 4, we applied the same approach used in the genetic mapping study of the femur to assess the impact of these factors on Tb phenotypes in the L5 vertebra. This is the largest BXD study to determine genetic factors controlling for vertebral Tb phenotypes. Similar to what we observed in the femur, approximately 50-70% of the variation in L5 vertebral Tb phenotypes were regulated by genetics and the genetic regulating the RCR phenotypes was weaker. From the genetic

mapping analysis of the L5 vertebra, we identified the total of 57 loci and selected 9 high-priority loci (5 robust, diet-insensitive and 4 diet-sensitive loci) for candidate gene characterization. We identified novel promising candidate genes for both the basal diet and the RCR phenotypes. In concordance with the findings reported in Chapter 2, we observed genetic loci that preferentially influence vertebral Tb phenotypes but have much weaker effect in the femur. Moreover, the loci identified from the RCR phenotypes also showed differential effect between sites, i.e. very strong in L5, but much weaker or showed no effect in the femur. This strongly supports that genetics and GxD interactions modulate Tb phenotypes in a site-specific manner.

Taken together, my dissertation research has demonstrated that there is a site-specific effect of GxD interactions on Tb phenotypes. We used genetic mapping population to identify genetic loci and candidate genes that may account for variation observed in Tb phenotypes in the femur and the L5 vertebra. We established that, in each bone site, there are robust genetic components that control Tb phenotypes independent of dietary Ca environment and there are diet-sensitive loci (i.e. the genetic effect was visible only in the phenotype(s) of mice fed low Ca diet and/or in the RCR phenotypes). More importantly, in each of these two categories, we observed both common and site-specific loci. The most promising candidate genes for each of the high-priority loci from the femur and the L5 vertebra were summarized in **Figure 5.1**. In sum, this work not only extends the current understanding of genetic regulation of Tb mass and microarchitecture, but also provides a foundation for future research to identify novel pathways and genes underlying the development of bone as well as an adaptation to Ca insufficiency. Researchers should be aware of the existence of SxGxD interactions influencing Tb phenotypes and incorporate this concept when designing future studies in bone.

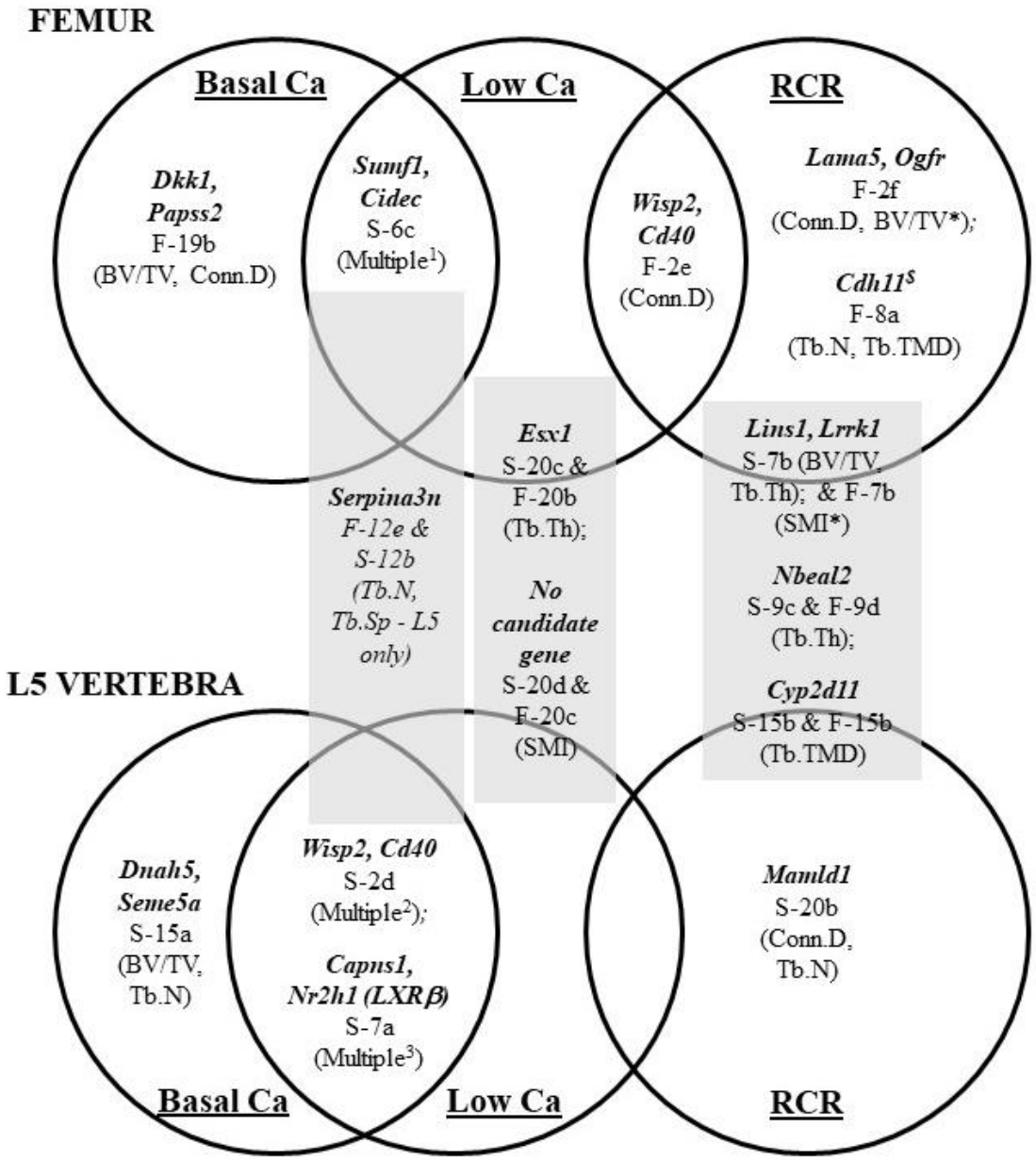

Figure 5.1 *A summary of promising candidate genes (bolded and italicized) identify from genetic mapping analyses of Tb phenotypes in the femur and the L5 vertebra of mice fed the adequate (Basal) and low Ca diet.* The response to Ca restriction (RCR) phenotypes were calculated for each Tb phenotype and used for genetic mapping analysis. Corresponding locus ID and mapped phenotypes (in parenthesis) were indicated under each candidate genes. * Phenotype mapped to a locus with a putative LOD. $ Candidate gene that was not discussed in details in the chapter. [1] Femoral BV/TV, Conn.D, Tb.N, Tb.Sp of both diet groups and SMI-Basal. [2] Vertebral Tb.Sp, BV/TV of both diet groups, SMI-Basal, Conn.D and Tb.N from LowCa. [3] Vertebral BV/TV, Conn.D from both diet groups, Tb.Th-Basal, Tb.N-LowCa (for complete details of each locus see Table 3.1 and 4.1 in Chapter 3 and 4, respectively).

5.2 Future Directions

My dissertation work confirmed the existence of GxD interactions on Tb phenotypes in the distal femur and the L5 vertebra. Through genetic mapping analysis, I have identified several genetic loci controlling Tb phenotypes in each site and those that are common as well differential among sites. Since my dissertation work is a discovery study of genetic loci and candidate gene, future work to validate and confirm these findings will be necessary. In the following section, I will divide my proposal for future directions into two subsections: (1) Short-term and (2) Long-term projects.

5.2.1 Short-term Projects

There are two major steps that are vitally important for proving the causal relationship between the genetic loci and candidate genes identified from genetic mapping analysis with Tb phenotypes. First, we need to confirm the that phenotypic variation in the Tb traits is driven by the genetic variation at the QTL, or in the specific candidate genes, we identified. Second, we need to demonstrate that the candidate genes we identified have functional roles in bone biology. Multiple loci that I reported in this dissertation are at equal importance because the confidence level of the relationship between each genetic locus and Tb phenotype(s) varies. For example, a locus on chromosome (chr) 6 (F-6c) showed a strong genetic effect on almost all Tb phenotypes both in the basal and the low Ca diet groups. Thus, by identifying this locus independently in two experimental groups, we have validated that this locus has a true genetic effect on the traits. At chr 6, future research could commence at the step of verifying functional roles in bone for the candidate genes in this locus. On the other hand, there are other novel loci that we identified that should be further validated through both steps.

5.2.1.1 Validations of the relationship between genetic loci and Tb phenotypes

To validate the relationship between genetic loci and the phenotypes we identified from QTL mapping, I have outlined several approaches:

(1) Using consomic mouse strains (chromosome substitution strain, CSs): The broadest way to confirm the influence of a QTL is to transfer the entire chromosome containing the QTL controlling a phenotype from one mouse into another other mouse line with a divergent phenotype. In Chapter 4, we identified three, strong (LOD>4.5), diet-sensitive loci on chr X (S-20b, S-20c, and S-20d). However, we only found 1 candidate gene with weak evidence relating them to bone biology for each of the first two loci and we did not find any candidate gene for the last locus. Therefore, it is critical to confirm the genetic effect of the B6 alleles in **chr X** on bone phenotypes. There is commercially available CSs with C57BL/6J (B6) that have chr X replaced with chr X from A/J mice (B6.A-ChrX, JAX Stock No: 004398, The Jackson Lab). Unfortunately, the QTL regions on Chr X are mostly not identical-by-decent between A/J and DBA/2J (DBA) mouse lines. Thus, it is not clear that this region of the A/J mouse genome has the same polymorphisms as DBA. Therefore, we will have to generate a chr X consomic strain in the B6 genetic background with the chr X from DBA line.

(2) Using congenic mouse strains: Another strain useful approach for the confirmation of QTL is the congenic mouse where a small genetic region from one line is transferred into the genome of a second line.[5,6] Fortunately, Davis et al. constructed and characterized a genome-wide panel of congenic strains derived from the donor strain DBA on the background B6.[7] Unfortunately, this panel does not have congenic strains for chr X and only some of the original strains reported in Davis's paper are available from The Jackson Laboratory. However, a locus on chr 12 that controls both femur (F-12c: 99.34-106.09 Mb) and L5 spine (S-12b:102.99-111.26 Mb, high allele=DBA) is worth validating. There is a congenic strain B6.D2.12M (JAX stock No:

005184) that contains a DBA region from 50.3 to 115.98 Mb (Build 32) inserted and this region is well cover the regions of these loci.

The use of congenic mice can be a challenge because some of the available congenic strains have large inserted regions that is hard to conclude whether the effect on the phenotype we see is due to the desired QTL regions.

(3) Using targeted gene knockout (KO) mice: The targeted deletion of gene in a mouse serves an important means to determine the biological role of genetic allele because mice and humans share about 99% of the same genes.[8] KO models were traditionally generated through constructing of gene targeting vectors and generations of homologous recombinant embryonic stems (ES) cells and the process of creating chimeric mice via embryonic stem (ES) cell injection.[9,10] Currently, there are multiple repositories for the collection, archiving, and distribution of KO mice such as the Knockout Mouse Project (KOMP, https://www.komp.org/), the Mutant Mouse Resource & Research Centers (MMRRC, https://www.mmrrc.org/), and The European Mouse Mutant Archive (EMMA, https://www.infrafrontier.eu/search). We can look into these repositories first to see if one of them contain the KO mice that we look for. Recently, Crisp-Cas9 technology enables us to obtain a targeted knockout or knockdown models at a faster rate[11] so it will be really useful in case that we need to create a model to inactivate genes that have not had available KO mice.

From our high-priority loci, I proposed three promising candidate genes that should be tested for its linkage to Tb phenotypes under the low Ca environment.

- ***Nbeal2****:* A candidate gene that is most likely responsible for the genetic effects of *F-9d* and *S-9c* (chr9: 27.26-42.54 Mb) that controlled the RCR phenotype of trabecular thickness (Tb.Th) for both sites. In this locus, there is a nonsense (gained stop codon)

mutation in *Nbeal2* that causes termination at amino acid 357 instead of amino acid 2742. It is the only gene among 4 other candidates that showed high mRNA expression in osteoclasts and bone (http://biogps.org/#goto=genereport&id=235627) and other work has shown that male KO mice have significantly decreased BMC and BMD (https://www.mousephenotype.org/data/genes/MGI:2448554). However, there were no Tb measurements tested in this earlier assessment of the Nbeal2 KO mice. Nonetheless, this line of evidence suggests that Nbeal2 may be involved in bone biology. In Chapter 3, we hypothesized that *Nbeal2* promotes osteoclast activity and that the truncated Nbeal2 protein will decrease osteoclast activity, which will in turn increase the ability to retain Tb.Th under the low Ca treatment. The *Nbeal2* KO mouse model (C57BL/6N-A^{tm1Brd} Nbeal2$^{tm1a(EUCOMM)Wtsi/WtsiCnbc}$) is commercially available for purchase through The European Mouse Mutant Archive (EMMA ID: 06100). We can design a two-by-two (2x2) factorial experiments where we test the KO versus B6 wild-type (WT) male mice fed the controlled basal versus the low Ca diets (similar to the diets we used in our genetic mapping studies) from 4-12 weeks old and measure the Tb phenotype in the femur as well as L5 vertebra.

(a) ***Wisp2***: This is the candidate gene that showed a strong relationship to bone phenotypes in the loci on chr 2 (*F-2e*: 159.79-165.96 Mb, femur Conn.D-LowCa and –RCR; *S-2d*: 162.61-178.48 Mb, L5 vertebral BV/TV and SMI in both diet group, Tb.Sp-Basal, Tb.N-LowCa, and Conn.D-LowCa). Interestingly, the locus in the femur has been shown to control Tb phenotypes only in response to the low Ca intake whereas the locus identified in the L5 vertebra mapping has been shown to control Tb phenotypes independent of dietary condition. Therefore, we should further investigate

the linkage of this gene with the phenotypes in both bone sites by designing a 2x2 factorial experiment just like the study proposed for *Nbeal2* to test the hypothesis that *Wisp2* KO male mice will have lower L5 vertebral Tb phenotypes in both diet groups, but the decrease of the femoral phenotypes will only be seen in the KO mice fed the low Ca diet. If this hypothesis is true, *Wisp2* will be the first gene that exemplify the concept of SxGxD interactions reported by our group.[12]

(b) ***Lins1***: This gene was identified as a candidate gene in the locus on chr 7 (S-7b: 49.73-74.18 Mb) that controlled BV/TV-RCR and Tb.Th–RCR in the L5 vertebra. The high values of these phenotypes were driven by B6 alleles. I found a *Lins1* KO mouse model on the C57BL/6NJ background ($Lins1^{em1(IMPC)J}$, MMRRC Stock No: 42352-JAX, The Jackson Lab). Though C57BL/6NJ mouse strain shares the majority of their genetics with C57BL/6J but it's not completely identical. It will be the most appropriate to create a new *Lins1* KO mouse model by transferring the knockout alleles to the C57BL/6J background. This could serve as a model to test the hypothesis that only under the low dietary Ca restriction, *Lins1* gene inactivation will lead to reduced Tb phenotype in the L5 vertebrae.

5.2.1.2 Identification of functional role and mechanisms by which candidate genes impact Tb

The second important step is to identify the functional role and molecular pathways by which candidate genes containing genetic variation linked to Tb phenotypes regulate bone cells. This step is mainly involved *in vitro* study in osteoblast and osteoclast cell lines. In the following paragraphs I will provide detailed examples for the most promising candidate gene *Lins1*. The paradigm presenting here can be applied for other candidate genes depending on the level of existing evidence in the literature.

Lins1 was identified as a candidate gene in the locus on chr 7 (S-7b: 49.73-74.18 Mb) that controlled BV/TV-RCR and Tb.Th-RCR in the L5 vertebra. In Chapter 4, we used existing evidence on *Lins1* from *Drosophila* to create the overall hypothesis that the DBA alleles of this gene disrupt the interaction between the lines protein (the protein product of the *Lins1 gene)* and β-catenin resulting in reduced Wnt-mediated transcription of downstream target genes including Wnt1, Smad8 and En1/2 that regulate osteoblast and bone formation. I propose three different sub-hypotheses:

Hypothesis 1: *Lins1* promotes osteoblastogenesis. We harvest L4 vertebrae from *Lins1* WT and KO mice and fix these vertebrae for immunohistochemistry. Through straining, we can measure the number of osteoblasts on the bone surface. Based on our hypothesis, we expect that although WT mice fed the low Ca diet will have a greater number of osteoblasts on the bone surface, this increase will be absent from *Lins1* KO mice.

Hypothesis 2: The β-catenin-mediated transcription activation requires Lines protein as a co-activator. We will create β-catenin luciferase reporter vector and introduce this to the osteoblasts developed by calvarial culture from WT and *Lins1* KO mice.[13] After growing these cells for a period of time, we can quantify the transcription activation level by measuring the luciferase activity in from each primary cell culture. If our hypothesis is true, we will expect to see much higher luciferase activity in proliferating WT osteoblasts than in KO osteoblasts.

Hypothesis 3: Lins1 co-activation with Wnt leads to increased transcription of *Wnt1, Smad8* and *En1/2* and this is associated with increased expression of the osteoblast phenotype. To test this hypothesis, we will use mesenchymal stem cells isolated from WT and from the *Lins1* KO mice.[13] We will culture these cells in the medium that promote osteoblast differentiation.[13] Prior to differentiation, we will expect to see reduced expression levels of these genes in proliferating

pre-osteoblast cultures from *Lins1* KO mice compared to cells from WT. After the differentiation program has been initiated, we expect to see dramatic decreases in osteoblast markers of differentiation including alkaline phosphatase, collagen type 1, and osteocalcin, in *Lins1* KO cells.

5.2.2 Long-term Projects

Rationale: Genetic predisposition of compromised bone development can increase the risk of fragility fracture later in life. GWASs uncovered several important genetic components contributed to compromised bone development. However, studying three-dimensional (3D) Tb microarchitecture in humans requires measurements from high-resolution peripheral quantitative computed tomography (HRpQCT). HRpQCT uses greater radiation as compared to DXA or pQCT and that makes Tb microarchitecture data less obtainable in humans. Another common issue in GWAS is to acquire adequate sample size to detect loci with moderate effects. In addition, human GWASs are based on the framework of linkage disequilibrium and may be confounded by genetic segregation variance. Thereby, it is likely that true associations might be hidden within those nominally significant hits and remain as false negative results.[14] However, large human cohorts with SNP measurements provides a good platform for validating the association between genetic loci (or candidate genes) and Tb phenotypes that were identified by the mouse genetic studies. In addition, current GWAS studies conducted in adolescents and young adults are exclusive to Caucasian and European ancestry. This limits the discovery of genes involved in bone development. To this end, it is important to translate the findings from the genetic mapping analysis in mouse to humans as well as to expand our understanding of genetics in bone during the developmental period. In the following section I will outline 2 main research goals with corresponding study to utilize the information from both mouse genetic studies and human GWAS cohort to further our understanding in genetics of Tb.

5.2.2.1 Use human cohorts as validation populations for candidate genes identified from mouse genetic reference population

Research Goals: To validate the results identified in mouse genetic studies using GWAS cohorts and to identify the nominally significant loci that match with polymorphisms identified in the mouse genetic studies.

Research Framework: I would like to propose that we use human GWAS data sets to validate the candidate genes I found from the mouse studies.[15] There are two important things worth noting regarding validating the candidate genes identified in mouse using human GWAS data sets. First is that most human cohorts are not controlled for dietary Ca intake. As a result, only the robust, diet-insensitive loci and candidate genes identified from my studies are qualified to be validated using this approach. Second, only one of the existing children and young cohorts directly measured Tb parameters using HRpQCT. Therefore, the most relevant phenotype we can use is trabecular volumetric BMD (Tb.vBMD) measured by pQCT. In addition to the phenotypes measured from pQCT, DXA measurements at specific Tb-rich sites including lumbar spine, femoral neck, and distal radius may also be used. The pediatric and young adult GWAS cohorts existing in the literature are summarized in **Table 5.1**.

For the analysis, mouse gene coordinates will be mapped to the human genome using the mouse comparative homology map data available at ftp://ftp.ncbi.nih.gov/pub/homology_maps/ that uses Mouse Build 37.2 and Human Build 37.2.[16] I will prioritize the effort in the analysis of the first two data sets because the phenotypes were direct measures of Tb. If we find a positive result for the candidate gene that we test, we may attempt to use the BMDCS as the second validation cohort.

Table 5.1 Pediatric cohorts available for genome-wide association study/ the available cohorts for pediatric bone genetics

Cohort (Ref.)	**Race**	**Age and gender**	**Sample size**	**Bone phenotypes**
The Gothenburg Osteoporosis and Obesity Determinants (GOOD)[17]	Caucasians in Sweden	Mean age: 18.9±0.6 years, males	1068 938 (for HRpQCT)	i. Tibial Tb.vBMD at 4% of bone length in the proximal direction of the distal end using pQCT ii. Tibial Tb parameters bone volume fraction (BV/TV), Tb.N, Tb.Th, and trabecular separation (Tb.Sp) using HRpQCT
The Cardiovascular Risk in Young Finns Study (YFS)[18]	Caucasian Finns	Mean age: 38±5.0 years, males and females	1558	Tibial Tb.vBMD at 5% of bone length in the proximal direction of the distal end using pQCT
The Bone Mineral Density in Childhood Study (BMDCS)[19]	23.8% African-Americans	Mean age: 5.23 years, 1022 females	2016	DXA measurements at specific Tb-rich sites including lumbar spine, femoral neck, and distal radius

From Table 5.1, it is evident that the existing cohorts of adolescents and young adults are limited to Caucasian and European descents. Several quantitative phenotypes in Asian populations have been shown to be unique and different from Caucasian and European populations.[20] The differences among different ethnic backgrounds are due to different recombination rates, allele frequencies and genetic diversity.[21] As a result, we likely miss significant part of genetic variation that controls bone phenotypes if we only study genetics of Caucasian and European populations. In the following section, I propose an initiative to recruit young individuals with different ethnicities from Asia country to construct a large Asian population for future bone genetic research.

5.2.2.2 Conduct genome-wide association studies in the Southeast Asians

Research Goals: Use young Asian populations to discover novel genetic loci for bone phenotypes in addition to those identified from Caucasian and European populations

Research Framework: Existing GWASs in bone phenotypes during growth are, like in other research areas, limited to Caucasian and European populations. Thus, there is a gap of knowledge that we can learn from genome-wide association study (GWAS) cohorts from the Asian ancestry. Table 5.1 clearly shows that there is no existing GWAS cohort in Asian adolescents and/or young adults. Majority of GWASs conducted in Asian populations are based upon the population from three large countries like China, Japan, and South Korea. These large Asian cohorts are useful for genetic research but existing cohorts from these Asian populations with bone phenotype measurements are available for only individuals from late adulthood and the elderly. Available adolescent cohorts from China,[22] Japan,[23] and Korea[24] do not include bone parameters as their phenotypes of interests. Fortunately, with the existence of the cohorts, it is likely to acquire bone phenotypes in the future. However, these large Asian countries represent the segregation of genetic pool mainly from East Asia which has been shown to have different impact on complex traits like cardiometabolic diseases as compared to the population from the South Asia (Indians).[25-27] Populations in the Southeast Asia are genetically distinct and offers a mixture of locals, Chinese and Indians. Thereby, the Southeast Asians can serve as a valuable cohort for identifying genetics that could not be achieve in other cohorts that exclusively consist of East or South Asians. Therefore, I propose that we initiate a new population cohort that specifically recruits healthy adolescents and young individuals from countries in Southeast Asia.

My long-term goal is to better understand genetics of bone during developmental years so that we can identify individuals at greater risk of bone fragility as early as possible and in turn, preventing osteoporosis. The first step is to collect data from children and adolescents in this region.

To my knowledge, the only active genetics consortium in Southeast Asia is the Singapore Population Health Studies or SPHS (http://blog.nus.edu.sg/sphs). SPHS consists of several cohorts that were initiated by the National University of Singapore with the goal to study the impact of genetics, lifestyle factors, and their interactions in chronic diseases like type 2 diabetes, cardiovascular disease (CVD) and cancer. Unfortunately, these cohorts do not include children or adolescents and do not measure bone parameters. Therefore, we cannot use these cohorts for bone GWAS study.

Another existing population cohort in Southeast Asia is the Electricity Generating Authority of Thailand (EGAT) study.[28] This cohort was first created to model the Framingham study. From its beginning in 1985, the first EGAT cohort or EGAT1 included participants only from Bangkok (Thailand's capital city) and examined factors limited to those relating to CVD. The following cohort, EGAT2, was designed to recruit individuals from rural areas who are exposed to different environmental factors from the urban EGAT1 cohort. The latest cohort EGAT3 was recruited in Bangkok in 2009. The total number of participants in the EGAT study is 9082. In the last two recruitment rounds of this cohort, the endpoints were expanded to include liver diseases, neurological problems, kidney diseases, malignancies and periodontal diseases. Unfortunately, there is no information on whether this cohort is still being followed up or whether there is a new recruitment phase happened after 2009. No bone parameters or genetics information were evaluated in all three EGAT cohorts. Therefore, there is a need to initiate a new pediatric cohort in Southeast Asia for studying genetics of bone in the future. Because this require a tremendous effort and fund, this initiative should take into consideration the long-term contribution of data that will be collected. For example, this initiative may serve as a starting point for a nation-

or region-wide survey project like the National Health and Nutrition Examination Survey or NHANES program in the U.S.

What I have proposed here is my outlook for future research that can be built upon what I have established in my dissertation research. Since my work is a discovery study, several follow-up validations and functional studies are needed. Multiple approaches including conventional genetic knockout work in mouse models, *in vitro* mechanistic experiments and big data-based analyses can be used in a complementary manner to confirm the associations between candidate genes identified from the genetic mapping analysis. These future studies hold great potential to move us forward in gaining new insights in genetics and molecular pathways critical to bone development.

5.3 References

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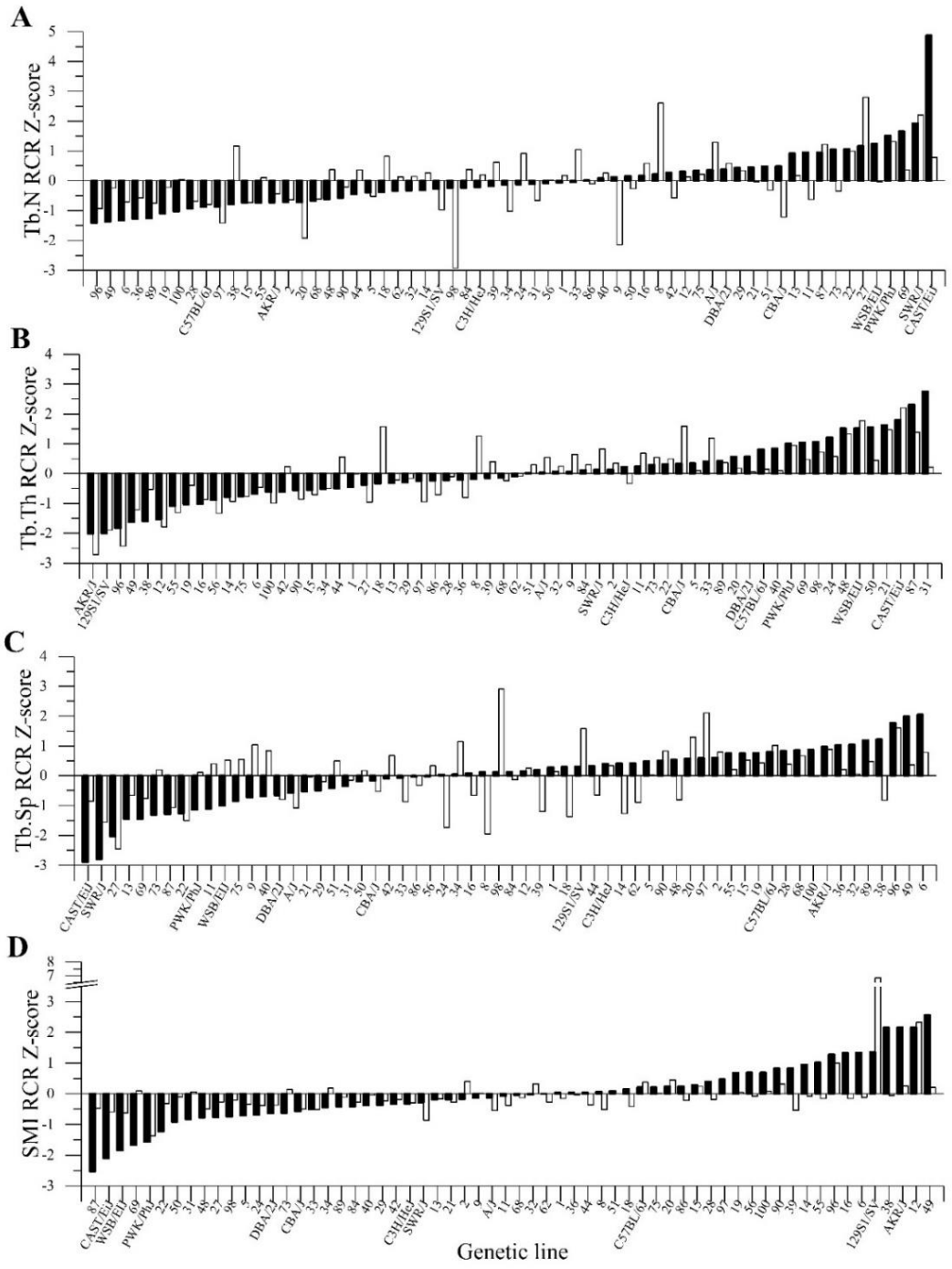
SUPPLEMENTAL DATA

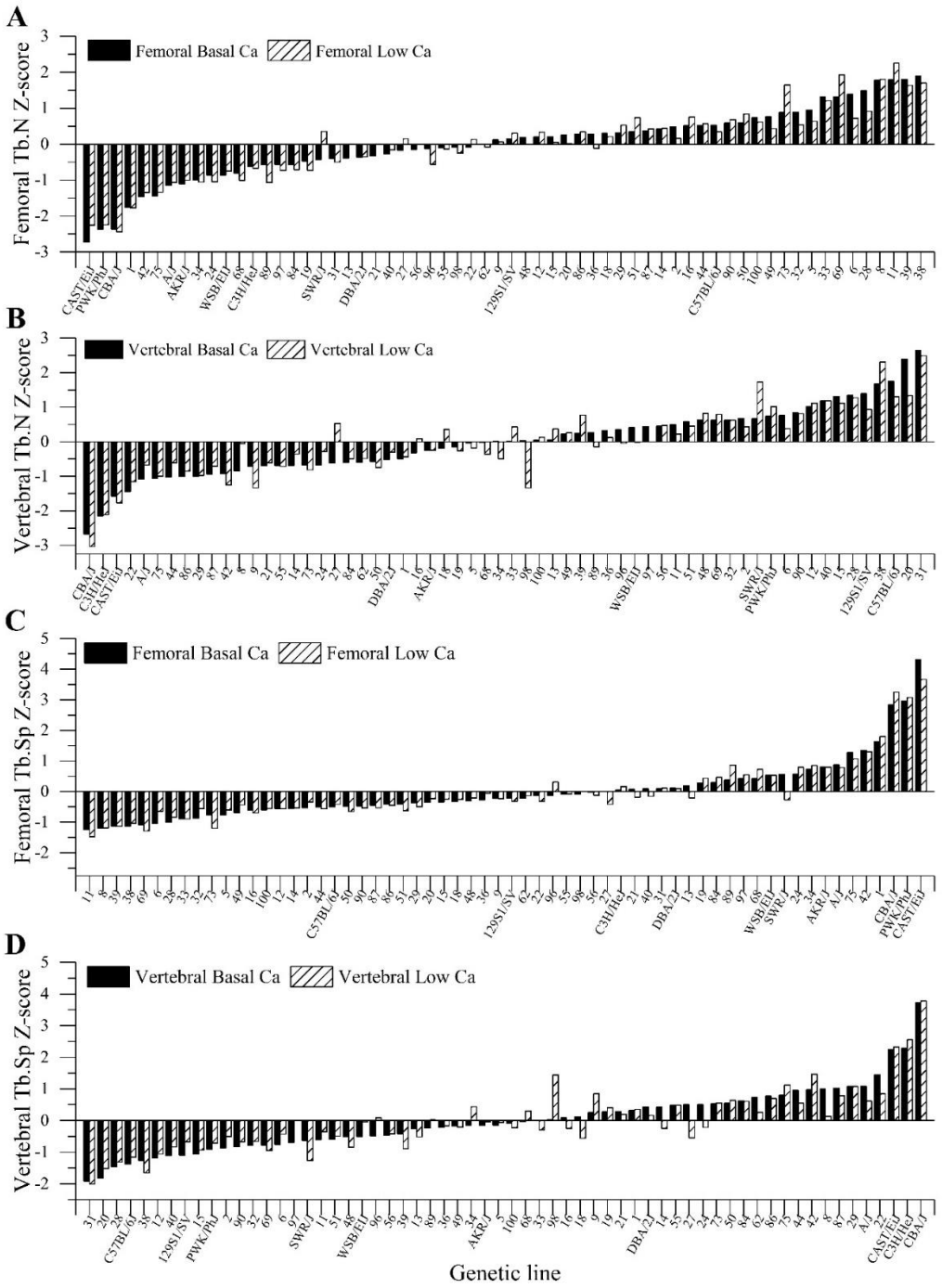
The following supplemental tables are available in the attached CD.

1. **Supplemental tables for Chapter 2**
2. **Supplemental tables for Chapter 3**
3. **Supplemental tables for Chapter 4**

Supplemental Figures for Chapter 2

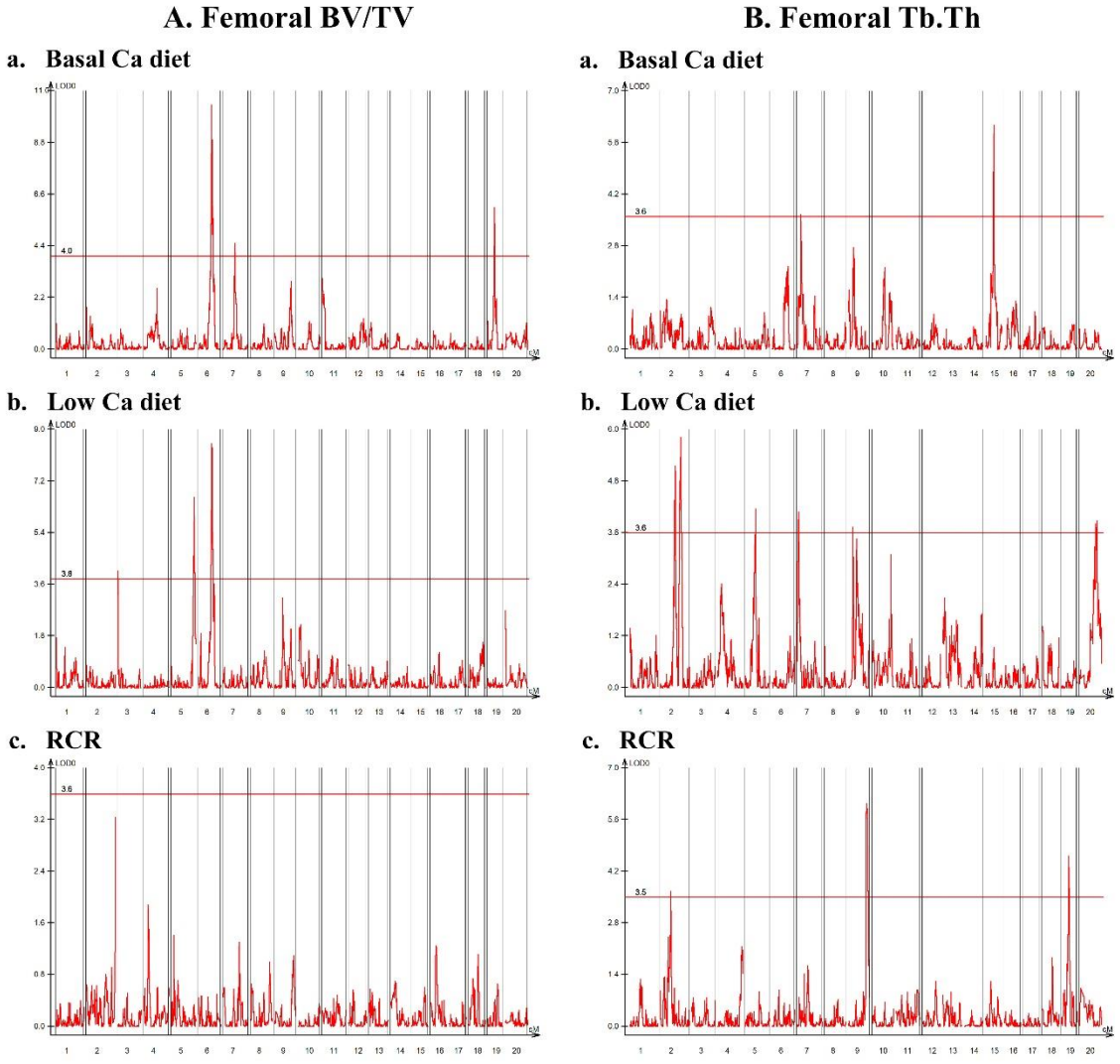


Supplemental Figure S2.1 Site-by-genotype interactions affect the response of trabecular parameters to dietary Ca restriction (RCR). (A) Tb.N RCR, (B) Tb.Th RCR, (C) Tb.Sp RCR, and (D) SMI RCR. Data are presented as Z-scores. The x-axis represents mouse lines including 51 BXD RI lines as numbers and abbreviations of the 11 inbred mouse lines. Black bars = Z-scores from the distal femur. Open bars = Z-scores from L5 vertebra.

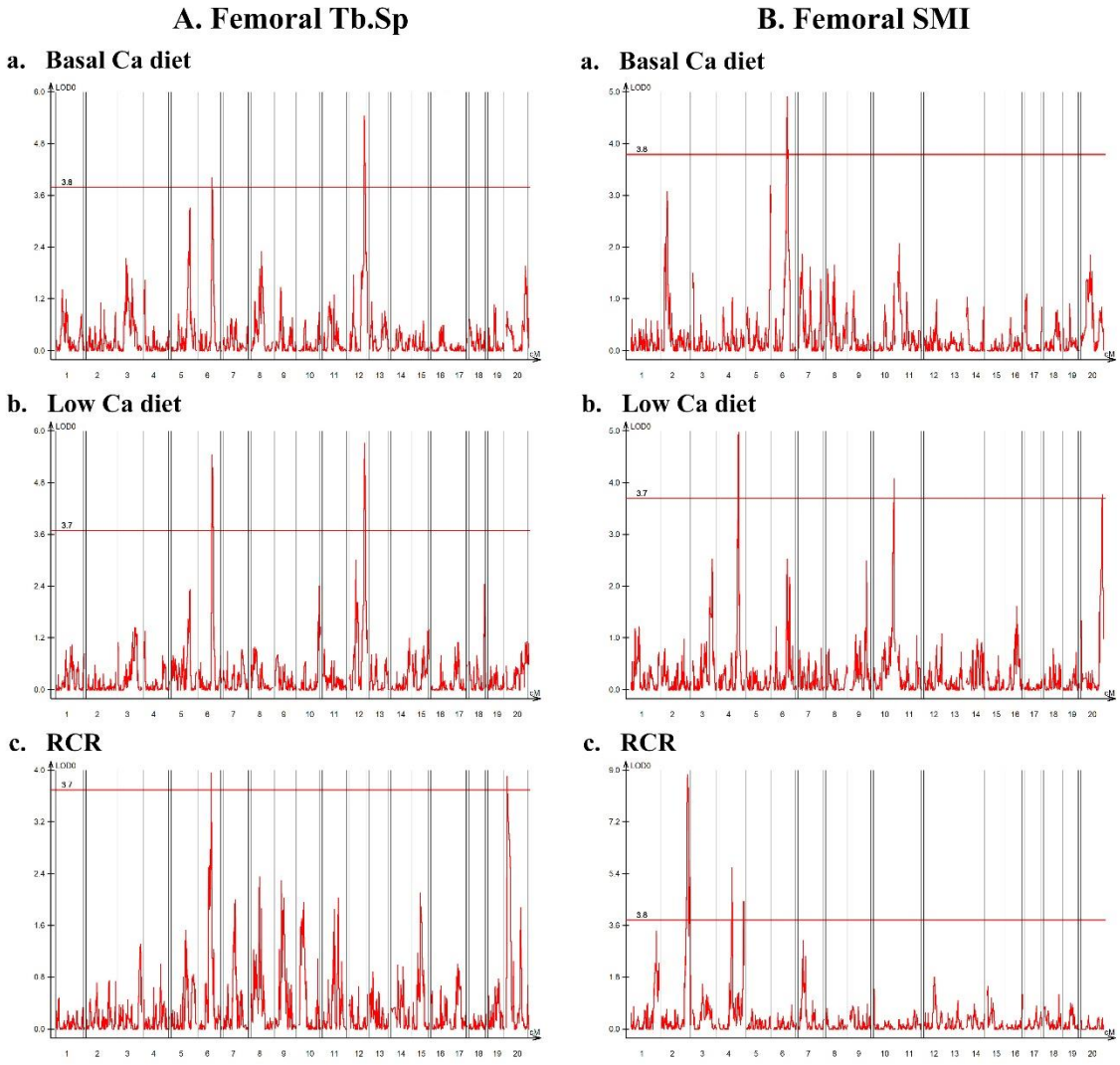


Supplemental Figure S2.2 Site-by-diet-by-genotype interactions affect trabecular parameters. (A) femoral BV/TV, (B) vertebral BV/TV, (C) femoral Tb.Th, (D) vertebral Tb.Th, (E) femoral SMI, and (F) vertebral SMI. The x-axis reports the 51 BXD RI lines as numbers and abbreviations of the 11 inbred mouse lines. Black bars = Z-scores from the basal diet group. Hatched bars = Z-scores from the low Ca diet group.

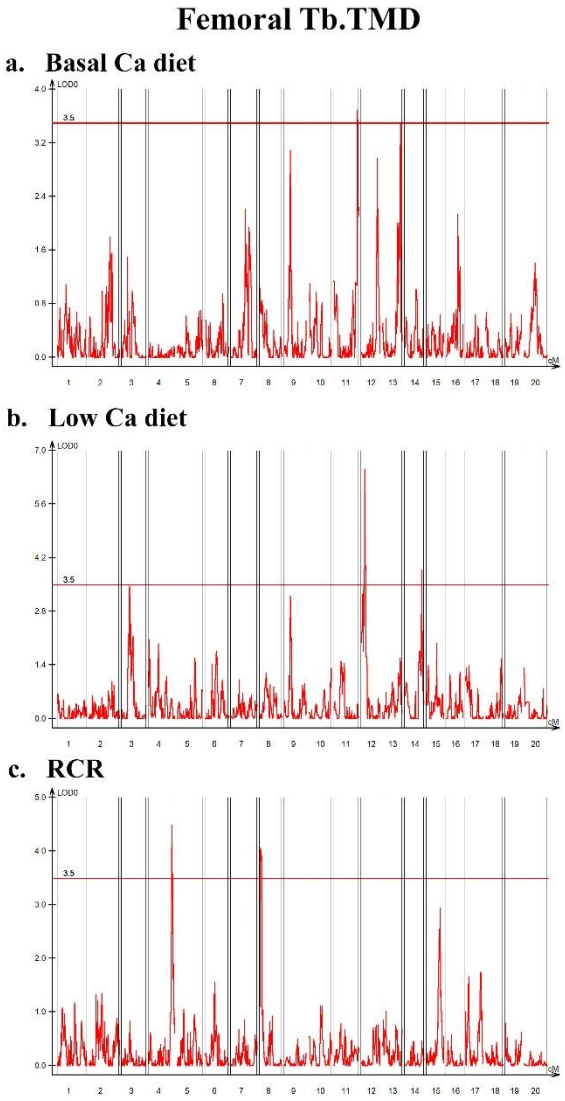
Supplemental Figures for Chapter 3



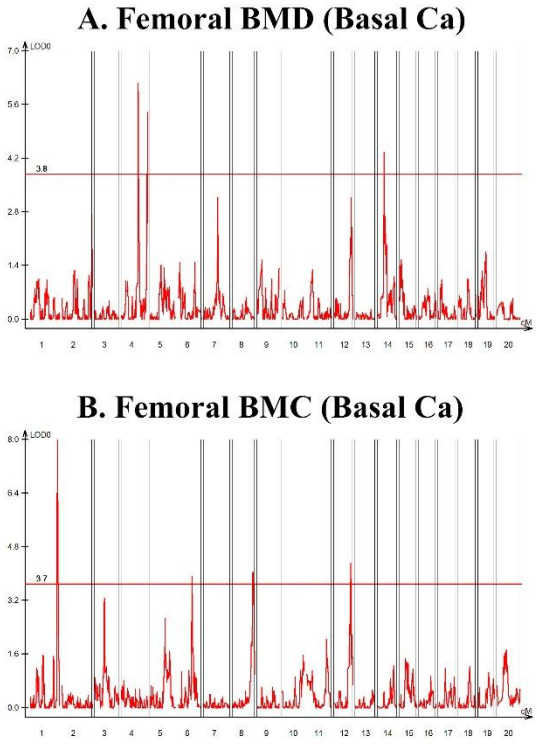
Supplemental Figure S3.1 *Composite interval maps for Femoral BV/TV (A) and Tb.Th (B).* For each phenotype, the maps from CIM of basal, low Ca, and RCR phenotypes are presented in a., b., and c., respectively



Supplemental Figure S3.2 *Composite interval maps for Femoral Tb.Sp (A) and SMI (B).* For each phenotype, the maps from CIM of basal, low Ca, and RCR phenotypes are presented in a., b., and c., respectively

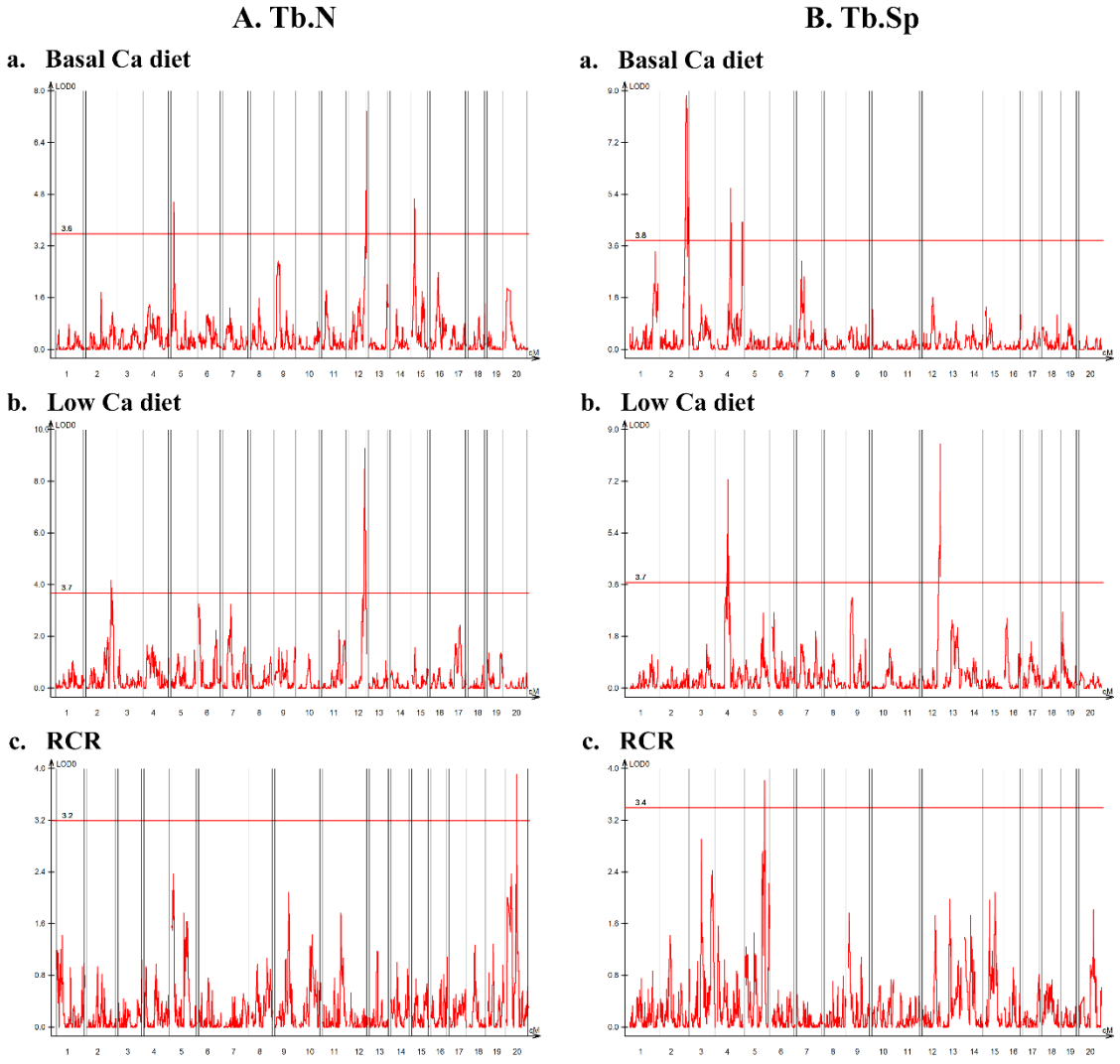


Supplemental Figure S3.3 *Composite interval maps for Femoral Tb.TMD.* For each phenotype, the maps from CIM of basal, low Ca, and RCR phenotypes are presented in a., b., and c., respectively

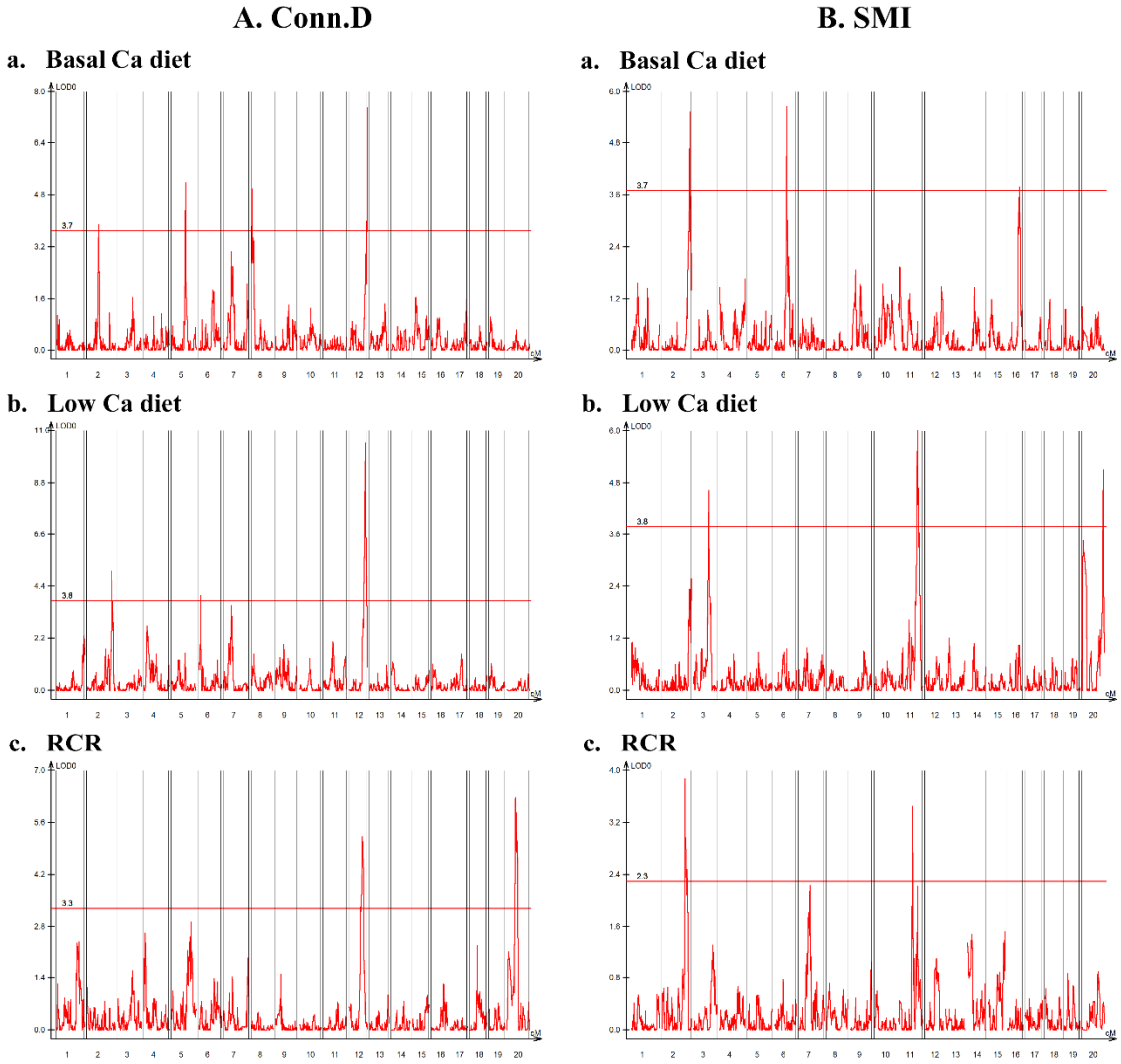


Supplemental Figure S3.4 *Composite interval maps for Femoral BMD (A) and BMC (B).* Re analysis of BMD and BMC phenotypes from the basal diet group previously reported in Reyes Fernandez et al (2016, JBMR)

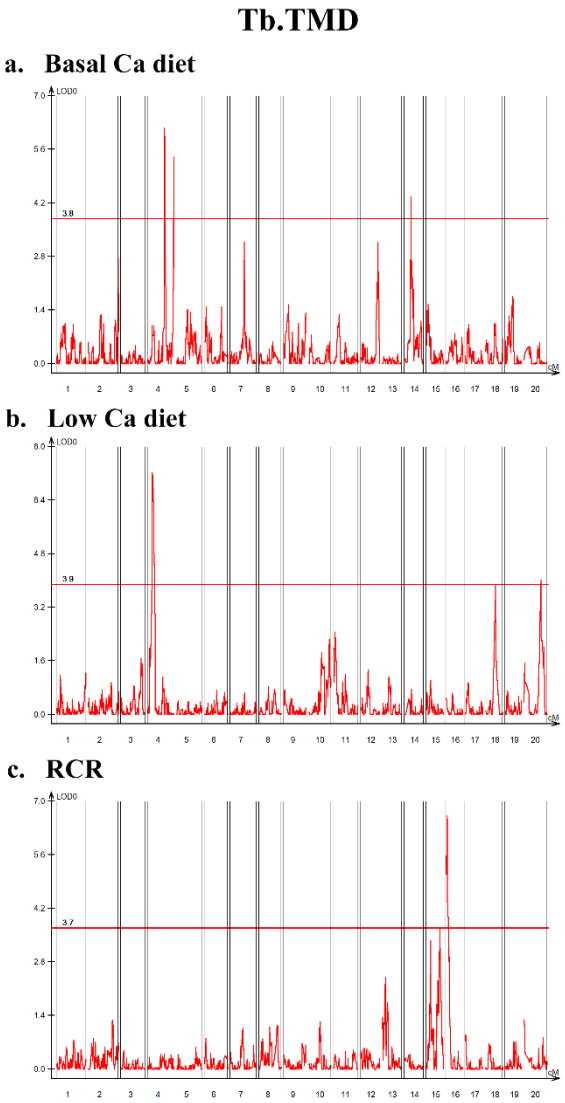
Supplemental figure for Chapter 4



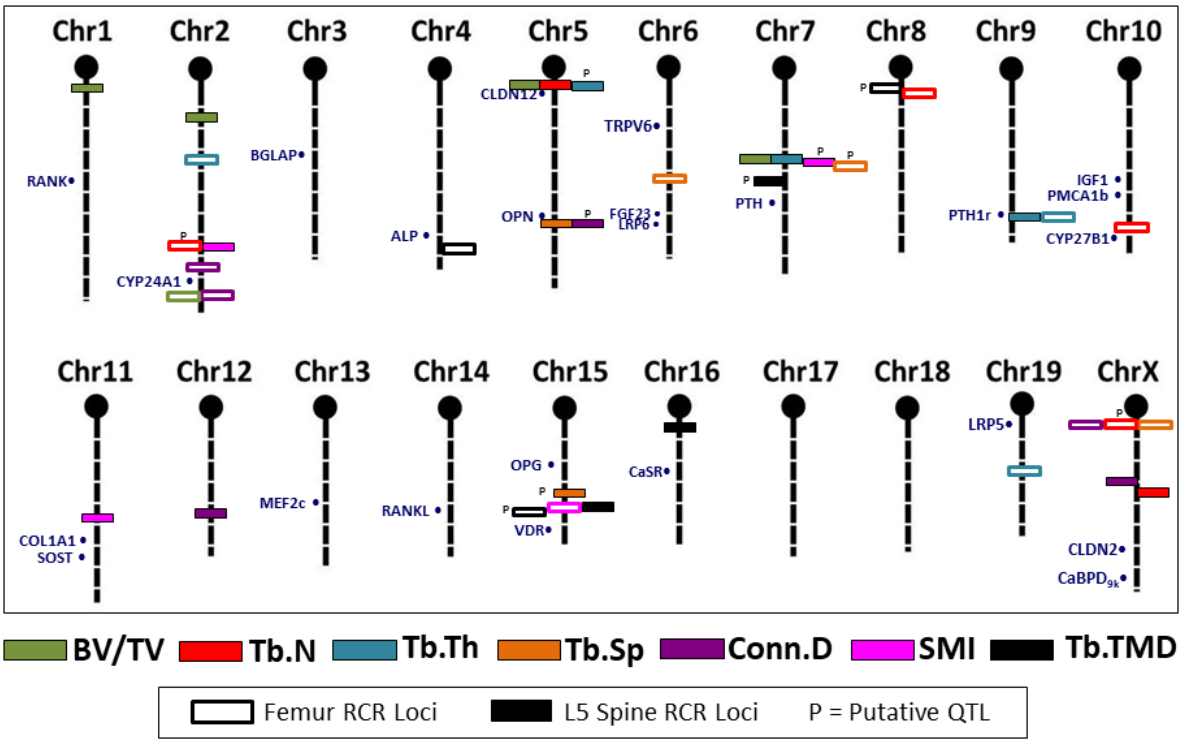
Supplemental Figure S4.1 *Composite interval maps for vertebral Tb.N (A) and Tb.Sp (B).* For each phenotype, the maps are presented in the vertical order of basal (a), low Ca (b), and RCR (c) phenotypes.



Supplemental Figure S4.2 *Composite interval maps for vertebral Conn.D (A) and SMI (B).* For each phenotype, the maps are presented in the vertical order of basal (a), low Ca (b), and RCR (c) phenotypes.



Supplemental Figure S4.3 *Composite interval maps for vertebral Tb.TMD*. The maps are presented in the vertical order of basal (a), low Ca (b), and RCR (c) phenotypes.



Supplemental Figure S4.4 *A whole genome summary of QTL identified for the RCR phenotypes of the L5 vertebra (filled box) and the distal femur (open box) from 51 BXD mouse lines.* Only loci that has at least one significant QTL are shown in this map. Trabecular bone phenotypes are coded by color. RCR = response to dietary Ca restriction. P = putative QTL.

VITA

Krittikan Chanpaisaeng

Interdepartmental Nutrition Program
Department of Nutrition Science
Purdue University

Education:

2019 Ph.D. in Biochemical and Molecular Nutrition
Interdepartmental Nutrition Program (INP), Purdue University, West Lafayette

Dissertation Title: The Site-specific Influence of Gene-by-diet (GxD) Interactions on Trabecular Bone in Male Mice
Committee: James C. Fleet (Chair), Brian P. Dilkes, Kathleen R. Hill Galant and Russell P. Main.

2015 M.S. in Biochemical and Molecular Nutrition (BMN)
Friedman School of Nutrition Science and Policy, Tufts University, Boston

2007 Bachelor of Science in Pharmacy, Chulalongkorn University, Thailand

Fellowships:

2012-2018 Royal Thai Government Scholarship for Graduate Study in the U.S.

Awards and Recognition:

2019 A winner of the Certificate of Excellence in Interdisciplinary Research Award for 2019 from the Office of Interdisciplinary Graduate Programs Spring Reception, Purdue University

2019 A winner of the Interdepartmental Graduate Program in Nutrition (INP) 2019 Graduate Student Poster Competition, Purdue University

2018 American Society of Bone and Mineral Research (ASBMR) Young Investigator

Travel Award, ASBMR Annual Meeting 2018 in Montreal, Canada

2018 Selected participant to attend the Endocrine Fellows Foundation (EFF)/ASBMR12th Fellows Forum on Metabolic Bone Diseases in Montreal, Canada.

2018 Recipient of the Certificate of Excellence in Research Award for Best presenter from the INP, the Office of Interdisciplinary Graduate Programs Spring 2018 Reception, Purdue University
Project title: Novel Genetic Loci Control L5 Spine Parameters and Their Responses to Low Calcium Intake in Male BXD Recombinant Inbred Mice.

2017 Selected participant to attend the EFF/ASBMR 11th Fellows Forum on Metabolic Bone Diseases.

2017 Selected Presenter for Nutrigenomics and Personalized Nutrition Oral Presentation Session at Experimental Biology (EB)/American Society of Nutrition (ASN) 2017 in Chicago

2017 Winner of Emerging Leaders in Nutrition Science Poster Competition Travel Award at EB/ASN 2017 in Chicago
Poster title: Femoral and L5 Spine Trabecular Bone Are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice.

2017 Recipient of Compton Graduate Student Research Methods Training or Professional Development Travel Award, School of Health and Human Science, Purdue University

2017 Finalist for the INP 2017 Graduate Student Poster Competition, Purdue University

2016 Scholarship recipient to attend the "Nutrigenetics, Nutrigenomics and Precision Nutrition" workshop from the University of North Carolina at Chapel Hill.

2016 Recipient of the Certificate of Excellence in Research Award for Best presenter from INP, OIGP Spring 2016 Reception, Purdue University
Project title: Trabecular Bone in the Distal Femur and L5 Spine are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice

2016 A recipient of Compton Graduate Student Research Methods Training or Professional Development Travel Award, School of Health and Human Science, Purdue University

Publications:

Chanpaisaeng K, Reyes-Fernandez PR and Fleet JC. Dietary Calcium Intake and Genetics have Site-specific Effects on Peak Trabecular Bone Mass and Microarchitecture in Male Mice. Bone. 2019;125:46-53. doi: 10.1016/j.bone.2019.05.011

In preparation for Journal of Bone and Mineral Research

Chanpaisaeng K, Reyes-Fernandez PR, Replogle RA and Fleet JC. Genetic Mapping in Male BXD Recombinant Inbred Mice Reveals Novel Candidate Genes Regulating Trabecular Bone Traits in the Femur and Their Responses to Dietary Ca Restriction.

Chanpaisaeng K, Reyes-Fernandez PR, Replogle RA and Fleet JC. Novel Genetic Loci Control L5 Spine Parameters and Their Responses to Low Calcium Intake in Male BXD Recombinant Inbred Mice.

Presentations

National Meetings/Conferences

Poster Presentations

2018 American Society of Bone and Mineral Research (ASBMR) Young Investigator Travel Award, ASBMR Annual Meeting 2018 in Montreal, Canada.
Title: Novel Genetic Loci Control L5 Spine Parameters and Their Responses to Low Calcium Intake in Male BXD Recombinant Inbred Mice.

2017 The Endocrine Fellow Foundation/ASBMR 11th Fellows Forum on Metabolic Bone Diseases poster session in Denver.
Title: Dietary calcium intake and genetics have site-specific effects on peak trabecular bone mass and microarchitecture in male mice.

2017 Emerging Leaders in Nutrition Science Poster Session at Experimental Biology (EB)/American Society of Nutrition (ASN) 2017 in Chicago
Title: Femoral and L5 Spine Trabecular Bone Are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice.

Oral Presentation

2017 Selected Presenter for Nutrigenomics and Personalized Nutrition Oral Presentation Session at EB/ASN 2017 in Chicago
Title: Femoral and L5 Spine Trabecular Bone Are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice.

Seminar/Poster Sessions/Competitions on Campus

Poster Presentations

2019 The Office of Interdisciplinary Graduate Programs (OIGP) Spring Reception
Title: Genetic Mapping Reveals Novel Co-Localized Genetic Loci and Candidate Genes Modulating the Response to Dietary Ca Restriction of Trabecular Bone at the Femur and Lumbar Spine in Growing Male Mice.

2019 The Interdepartmental Graduate Program in Nutrition (INP) 2019 Graduate Student Poster Competition
Title: Novel Genetic Loci Control L5 Spine Parameters and Their Responses to Low Calcium Intake in Male BXD Recombinant Inbred Mice.

2018 The OIGP Spring Reception
Title: Novel Genetic Loci Control L5 Spine Parameters and Their Responses to Low Calcium Intake in Male BXD Recombinant Inbred Mice.

2017 The INP 2017 Graduate Student Poster Session as part of Corporate Affiliates and A Day on Campus event
Title: Trabecular Bone in the Distal Femur and L5 Spine are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice

2016 The OIGP Spring Reception
Title: Trabecular Bone in the Distal Femur and L5 Spine are Differentially Influenced by Dietary Calcium Restriction and Genetics in Growing Mice

Oral Presentation

2018 The Fuqua Graduate Student Seminar Series in Nutrition Science on August 31
Title: Genetics and Diet Interact to Modulate Trabecular Bone Mass and Microarchitecture

Research Experience:

2018-2019 Provide technical support for an NIH-funded research project investigating the impact of duodenum- and colon-specific loss of vitamin D receptor on Ca absorption (PI: Prof. James C. Fleet)

Key skills include:

- Mouse colony management
- DNA extraction and genotyping
- Mouse tissue harvest (e.g. intestine, kidney, bone) and blood collection
- In situ ligated loop to test the efficiency of Ca absorption
- Uses of radioactive ^{45}Ca

2015-2019 Dissertation research project at Purdue University

- Research mentor: James C. Fleet, PhD
- Project title: The Site-specific Influence of Gene-by-diet (GxD) Interactions on Trabecular Bone in Male Mice
- Key techniques: Mouce husbandry and bone harvest, bone imaging using *ex vivo* and *in vivo* micro-computed tomography, QTL mapping. Bioinformatics and statistical analyses (SAS, R).

2014 Directed study in Vitamins and Carcinogenesis Laboratory (at HNRCA), Tufts University

- Supervisor: Jimmy Crott, PhD
- Project title: Effect of paternal B vitamin deficiency on *APOBEC -1* RNA expression and protein abundance in the liver and small intestine of mouse offspring
- Key techniques: RNA extraction, gel electrophoresis, RT-PCR, quantification of mRNA expression by spectrophotometry, and western blot.

2013 Practicum in Nutrition and Genomics Laboratory (at HNRCA), Tufts University

- Supervisors: Jose Ordovas, PhD, Caren E. Smith, DVM, MS and Chao-Qiang Lai, PhD
- Project title: Assess the relation of gastric inhibitory polypeptide receptor (*GIPR*) locus with obesity and diabetes-related traits and its interactions with dietary factors in the GOLDN population.
- Key Techniques: Gene × diet interaction study using R statistics (GWAF package) and SAS, Linkage disequilibrium analysis and identification of tag SNPs using Haploview.

2007 Research internship at Research and Development Institute, the Government Pharmaceutical Organization (GPO), Bangkok.

- Research mentor: Sanya Hokputsa, PhD
- Project title: Separation of major substances from *Borassus flabellifer* used as biomarkers for standard control of Thai herbal medicine

2006-2007 Thesis research for the bachelor's degree in pharmacy (major in Pharmacognosy).

- Research mentor: Khanit Suwanborirux, PhD
- Project title: Qualitative and quantitative analyses of Renieramycins in the egg mass of the nudibranch *Jorunna funebris* by HPLC

Service and Engagement

2016-2019 **Research mentor** for four undergraduate students

Student: Brendan Flisk, College of Science (Major in Genetics)
Project title: Principal Component Analysis of Calcium Absorption and Bone Traits from 51 BXD RI mice.

Student: Omozafe Bolanle Udegbe, Department of Biomedical Engineering
Project title: Three-dimensional Measurement of Trabecular Bone in Inbred Mice.

Student: Chandler Dykstra, Department of Nutrition Science
Project title: Measurements of Femoral Cortical Porosity in Old Mice Fed Different Dose Combinations of Calcium and Vitamin D

Student: Caitlyn Green, Department of Biological Science
Project title: Characterization of Genetic Influence on Growth Phenotypes in Mice.

2016-2019 **Purdue's Graduate School Global Ambassadors** representing Thailand
Roles and responsibilities: Serve as an advisor to the Office of Graduate Admissions working on promoting Purdue University to prospective graduate students
Website:
https://www.purdue.edu/gradschool/admissions/spotlight/students/Krittikan-Chanpaisaeng.html

2017-2018 **Social Chair**, Nutrition Science Graduate Organization, Purdue University
Roles and responsibilities: Develop strategies or programs to promote comradery and engagement amongst all INP students

Additional Skills and Trainings:

Quantitative Skills Development

2019 24th Summer Institute in Statistical Genetics, University of Washington, Seattle

- Bayesian Statistics in Genetics
- Mixed Model in Quantitative Genetics
- Advanced Quantitative Genetics

Website: https://www.biostat.washington.edu/suminst/sisg2019/modules

2017 "Functional In-silico Assessment of Genomic Regions Influencing Bone Mineral Density" workshop, ASBMR Annual Meeting

2016 "Nutrigenetics, Nutrigenomics and Precision Nutrition" workshop, University of North Carolina at Chapel Hill.

Professional development

2017 Grant and Proposal Writing Workshop, Purdue University

2011 "Critical Appraisal: Randomized Controlled Trial, Meta-Analysis and Pharmacoeconomics," the Marketing Pharmacist Group, Pharmaceutical Research and Manufacturers Association (PReMA), Thailand

2010 "Train the Trainer": How to be a good trainer (Seminar and workshop)

2010 "Scientific Literature Evaluation," the Marketing Pharmacist Group, Pharmaceutical Research and Manufacturers Association (PReMA), Thailand

2010 "Systemic Review & Meta-Analysis," the Center of Pharmaceutical Outcomes Research (CPOR), Narasuan University, Thailand

2010 "Standard Course in Clinical Trials," Faculty of Medicine, Chulalongkorn University, Thailand

Work Experience:

2009-2012 ***Pharmacovigilance and Medical Information Manager***,

Thai Otsuka Pharmaceutical Co., Ltd. (http://www.otsuka.com/en/), Thailand
Roles and responsibilities:

- Report safety information regarding pharmaceutical products received from healthcare professionals to local authority and head quarter in Japan.
- Respond to medical inquiries from healthcare professionals, consumers and internal customers
- Conduct literature review and update medical information to scientific team and sales and marketing team.

2007-2009 ***Assistant Product Manager***,

Marketing Department, SPS Medical Co., Ltd., Thailand
Roles and responsibilities:

- Product knowledge training: Warfarin, Fexofenadine, Benzbromarone (uricosuric drug), Pyridostigmine and thyroid preparations
- Product presentations to healthcare professionals
- Support detailed product information to sales and marketing team
- Marketing activities