ABSTRACT

Amino Acid Transporters Regulate Bone Formation

by

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Abstract

Bone development and homeostasis are governed by a number of developmental signals, transcription factors and cellular metabolism. This process is also dependent on the orchestration of multiple cell types including osteoblasts, chondrocytes, skeletal stem cells and osteoclasts. Osteoblasts are the principal bone forming cells responsible for producing and secreting the type I collagen rich extracellular bone matrix. Protein synthesis is an energetically and biosynthetically demanding process. This requires copious amounts of ATP and amino acids amongst other metabolites. However, the precise mechanisms and systems that osteoblasts utilize to meet these synthetic demands are poorly understood. Previous studies have shown amino acid consumption is increased in osteoblasts during differentiation. This process is regulated by transcription factors ATF4 and FOXO. Additionally, osteogenic signals like WNT and PTH can stimulate amino acid uptake. For example, WNT signaling can rapidly stimulate glutamine uptake and metabolism required for osteoblast differentiation. Unfortunately, transporters mediating glutamine uptake in osteoblasts are unknown. Moreover, the mechanism by which WNT stimulates increased glutamine consumption is also unknown. We identified two amino acid transporters, Slc7a7 and Slc1a5, as the primary glutamine transporters in response to WNT. Slc7a7 is responsible for the rapid WNT-induced glutamine uptake via the β-catenin dependent pathway. Conversely, Slc1a5
sustains basal glutamine uptake, which is regulated by ATF4 downstream of the mTORC1 pathway. In summary, these data demonstrate the biphasic role of WNT signaling in regulating glutamine consumption, by two amino acid transporters Slc7a7 and Slc1a5, during osteoblast differentiation.

While we have shown the importance of glutamine in bone cells, the role of other amino acids is not clear. Proline has long been considered as a critical amino acid due to its enrichment in collagens. Furthermore, PTH stimulates proline consumption in osteoblasts. The transport of proline is characterized by its dependency on sodium and sensitivity to MEAIB. However, the precise transport system responsible for proline import is not known. Here we identified the amino acid transporter Slc38a2, which encodes SNAT2, as the primary proline transporter in osteoblasts. Deletion of Slc38a2 results in defects in both intramembranous and endochondral ossifications. The phenotype is associated with defective osteoblast differentiation highlighted by reduction of proline enriched proteins (e.g. RUNX2, OSX and COL1A1). Slc38a2 provides proline to support osteoblast differentiation through two mechanisms. First, majority of proline is directly incorporated into proteins and does not contribute to amino acid biosynthesis. Second, proline oxidation regulates bioenergetics required for osteoblast differentiation. These findings highlight the multifaceted functions of proline, which is provided by Slc38a2, in osteoblast differentiation and bone formation. Collectively, my work demonstrates the critical role of amino acid transporters in osteoblast differentiation and
provides novel insights in their potential applications in treatments of bone diseases like osteoporosis and bone fracture.
Dedication

My dissertation is dedicated to my paternal grandfather Chunshi Shen (沈椿仕) and my maternal grandfather Changgen Yao (姚昌根), both of who have been lifetime role models to me.
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Chapter 1. Introduction

1.1 Overview

The skeletal system functions as a physical scaffold to support and protect the body and also enables locomotion. Skeletal development and homeostasis depend on exquisitely concerted actions of developmental signals, transcriptional regulation and cellular metabolism in different bone cells. Mistakes occurring in any step of this intricate orchestration result in skeletal abnormalities. Genetic mutations in key components and regulators of bone formation leads to developmental skeletal disorder. For example, patients with mutations in collagens develop osteogenesis imperfecta characterized by fragile bones (Marini et al., 2017). Moreover, a delicate balance between bone formation and bone resorption is also required for the homeostasis of the healthy bone. Osteoblasts, the chief bone-making cells, are responsible for bone formation. Conversely, osteoclasts can resorb bone matrix. With ageing or pathological conditions, increased osteoclast-dependent bone resorption and decreased osteoblast-dependent bone formation result in osteopenia or osteoporosis. Osteoporosis affects millions of people and causes heavy economic burden in the US. Recent treatments mainly target bone resorption but few focus on bone formation (Compston et al., 2019). However, targeting bone resorption can only slow down bone loss because it has no significant bone anabolic effect (Pazianas et al., 2014). In order to combat osteoporosis, it is important to stimulate bone anabolism for better bone quality. To boost osteoblast activity and bone formation, it is essential to
understand how osteoblasts differentiate and how skeleton is formed. The goal is to understand the developmental processes that form bone in order to rationally apply the knowledge to treat the diseases. Over the past several decades, extensive studies have been dedicated to transcriptional regulation and endocrine or paracrine signaling pathways in osteoblast differentiation and bone formation. Recent studies have shifted attentions to elucidate the cellular metabolism of osteoblasts and bone formation including glucose, fatty acid and amino acid metabolism. Here, I will review the processes of osteoblast differentiation and bone formation, and also highlight what is known about the cellular metabolism in osteoblasts and other bone cells.

1.2 Skeletal development

Skeletal development occurs through two distinct processes: intramembranous and endochondral ossification. Both bone-forming processes begin with the condensation of mesenchymal progenitors that can differentiate into osteoblasts or chondrocytes (Couly et al., 1993; Olsen et al., 2000). This step is initiated at around embryonic day 10.5 (E10.5) in mice. Mesenchymal progenitors originating from neural crest give rise to skeletal elements in the craniofacial regions. In some craniofacial bones, mesenchymal progenitors directly differentiate into osteoblasts, which forms bones through intramembranous ossification. On the contrary, mesoderm-derived mesenchymal progenitor cells form axial and appendicular skeleton. In these bones, mesenchymal progenitors first differentiate
into chondrocytes and lay down a cartilaginous template, which is gradually replaced by bone. This process is known as endochondral ossification.

### 1.2.1 Intramembranous ossification

During intramembranous ossification, mesenchymal progenitors migrate from neural crest into craniofacial region and directly differentiate into osteoblasts, laying down bone matrix (Kozhemyakina et al., 2015; Takarada et al., 2016). Osteoblasts are chief bone-making cells, responsible for producing and secreting extracellular matrix (ECM). Bone matrix is mainly made of type I collagen (COL1), known as osteoid. Osteoid is mineralized via the accumulation of calcium phosphate in the form of hydroxyapatite. This process is regulated by multiple enzymes associated with phosphate production and transport including alkaline phosphatase (AKP2), ectonucleotide pyrophosphatase 1 (ENPP1) and Ankylosis (ANK) (Harmey et al., 2004). Osteoblast differentiation can be categorized into five stages: condensation, commitment, specification, differentiation and entombment (Karner and Long, 2017; Long, 2012) (Fig. 1). Mesenchymal progenitors first undergo condensation (Akiyama‡ et al., 2005). Mesenchymal progenitors in condensed mesenchyme express master transcription factor RUNX2. These progenitors are committed to the osteoblast lineage fate. Loss of RUNX2 results in failure in bone formation (Komori et al., 1997; Otto et al., 1997). Human or mice with mutations in RUNX2 develop cleidocranial dysplasia, a skeletal defect that affects bone and tooth development.
Figure 1 Osteoblast differentiation.

Osteoblasts are derived from mesenchymal progenitors. Progenitors start expressing Runx2, committed to osteoblast lineage. Committed progenitors are further specified, characterized by the expression of Sp7. They are then differentiated into mature osteoblasts, expressing terminal markers like Atf4, Ibsp and Bglap. Mature osteoblasts secrete matrix and reside on the surface of bone. Some are further into osteocytes entombed in the matrix.

(Ducy et al., 1997; Mundlos et al., 1997). RUNX2 induces the expression of osteogenic genes like osteopontin (OPN, encoded by Spp1), integrin-binding sialoprotein (IBSP, encoded by Ibsp), osteocalcin (OCN, encoded by Bglap) and COL1A1 (Ducy et al., 1997; Kern et al., 2001). RUNX2 directly regulates the downstream target gene OSX, another master transcription factor, which leads to the specification of osteoblasts (Nishio et al., 2006). Absence of OSX in the condensed mesenchyme prevents osteoblast differentiation, resulting in failure of bone development (Nakashima et al., 2002). OSX binds to the promoter of Col1a1 and activates its transcription. This facilitates osteoblasts to deposit Type I collagen (COL1) to ECM. Loss of OSX also abolishes the expression of osteogenic markers Ibsp and Bglap, suggesting that OSX regulates the expression these genes (Nakashima et al., 2002). Subsequently, specified preosteoblasts are differentiated to mature osteoblasts, featured by an array of markers like OCN, OPN and IBSP. (Bianco et
At this stage, osteoblasts are characterized by the transcription factor activating transcription factor 4 (ATF4). Mutations in ATF4 leads to DuCoffin-Lowry syndrome in humans, which is associated with skeletal abnormalities including shortened long bones and delayed closure of skull suture (Yang et al., 2004). Similarly, mice lacking ATF4 exhibit delayed skeletal development (Elefteriou et al., 2006; Yang et al., 2004). ATF4 has been identified with two major functions in osteoblasts so far. First, ATF4 forms a complex with RUNX2. This complex of transcription factors directly binds to Bglap promoter, synergistically activating transcription of the gene (Xiao et al., 2005). Furthermore, ATF4 interacts with forkhead box O (FOXO) family proteins to promote amino acid uptake for protein synthesis in osteoblasts (Elefteriou et al., 2006; Rached et al., 2010). In the next step, most osteoblasts undergo apoptosis and a small proportion of osteoblasts are entombed in the matrix and become osteocytes. This percentage varies from 10% to 30% based on different reports and estimations summarized by Franz-Odendaal et al. (2006).

1.2.2 Endochondral ossification

Mesenchymal progenitors originating from lateral mesoderm form appendicular skeletons while the ones from paraxial mesoderm form vertebral skeleton. During endochondral ossification, mesenchymal progenitors first differentiate into chondrocytes, forming a cartilaginous primordium, which is gradually replaced by the bone (Kozhemyakina et al., 2015; Long and Ornitz, 2013). Different transcription factors and
developmental signals regulate sequential steps of chondrocyte differentiation during endochondral ossification: condensation, differentiation, hypertrophy and apoptosis or transdifferentiation (Fig. 2). In the first step, mesenchymal progenitors undergo condensation at around E11.5 in mice. In the middle of condensed mesenchyme, mesenchymal progenitors differentiate into chondrocytes. Conversely, mesenchymal progenitors at the outermost layer of the condensation, which is known as perichondrium, remain undifferentiated. Perichondrial progenitors eventually give rise to osteoblasts during endochondral ossification. Progenitors in the condensed mesenchyme express transcription factor SOX9, which is required for chondrogenesis. Loss of SOX9 expression in mesenchymal progenitors abolishes further steps of chondrogenesis, leading to complete failure of limb formation (Akiyama et al., 2002). SOX9 induces expression of SOX5 and SOX6. These three SOX proteins interact and form a trio of transcription factors. The SOX trio activates the expression of a number of cartilaginous matrix proteins like type II collagen (COL2) and aggrecan (ACAN) (de Crombrugghe et al., 2000; Han and Lefebvre, 2008; Lefebvre et al., 1998). Early chondrocytes also proliferate rapidly. This drives the longitudinal growth of skeletal elements. After exiting the cell cycle, chondrocytes undergo hypertrophy, expressing and secreting type X collagen (COLX) at around E13.5. Terminal chondrocytes express matrix metalloproteinase 13 (MMP13) and most of them undergo apoptosis (Inada et al., 2004). At this stage, blood vessels invade the cartilage primordium, which is gradually degraded to form marrow cavity. At around
During endochondral ossification, SOX9+ mesenchymal progenitor cells undergo condensation and differentiated to COL2A1+ chondrocytes, laying down a cartilaginous template. In the middle of the template, chondrocytes undergo maturation and hypertrophy, starting the expression of COL10A1. The template is surrounded by a layer of undifferentiated mesenchymal progenitors named perichondrium, which is the primary source for osteoblasts. At 15.5, hypertrophic chondrocytes start expressing terminal markers MMP13, some undergo apoptosis, gradually replaced by the bone, while others dedifferentiate to mesenchymal progenitors or transdifferentiate into osteoblasts. At the same time, vessels invade the template, bringing osteoblast progenitor cells from perichondrium. Osteoblast progenitors differentiate and remodel the template.
E15.5, perichondrial cells migrate into the marrow cavity via invading blood vessels and differentiate into osteoblasts, which forms the bone. Relying on the advancement of lineage tracing techniques, recent studies also demonstrate that terminal chondrocytes undergo either dedifferentiation to mesenchymal progenitors or transdifferentiate to osteoblasts (Mizuhashi et al., 2018; Ono et al., 2014; Yang et al., 2014).

Endochondral ossification is regulated by a series of developmental signals. For example, bone morphogenetic protein (BMP) is required for the condensation of mesenchymal progenitors and early chondrogenesis. Deletions of BMP receptors BMPR1A and BMPR1B result in loss of majority of skeletal elements formed via endochondral ossification (Yoon et al., 2005). Conversely, WNT/β-catenin signaling inhibits chondrocyte differentiation. Overexpression of β-catenin in mesenchymal progenitors leads to total failure of limb formation (Hill et al., 2005). Parathyroid hormone-related peptide (PTHrP), expressed in proliferative chondrocytes, regulates chondrocyte maturation via its receptor PTHR1, which is highly expressed in prehypertrophic chondrocytes (Karaplis et al., 1994). Inactivation of PTHrP or PTHR1 results in limb dwarfism due to premature chondrocyte hypertrophy (Lanske et al., 1996). Indian hedgehog (IHH) coordinates with PTHrP, regulating chondrocyte maturation, forming a negative feedback loop (Kobayashi et al., 2002). IHH, expressed in prehypertrophic chondrocytes, antagonizes GLI3 repressor function on PTHrP and chondrocyte proliferation (Hilton et al., 2005). In turn, PTHrP suppresses IHH-mediated
chondrocyte hypertrophy. IHH also signals to perichondrium, which is critical for osteoblast differentiation. Inactivation of IHH prevents mesenchymal progenitors from expressing master transcription factors RUNX2 or Osterix (OSX, encoded by Sp7) (Hilton et al., 2005; Long et al., 2004).

1.3 WNT signaling in bone development

WNTs, a family of secreted glycoproteins, are able to activate multiple intracellular signaling cascades, which can be categorized into canonical β-catenin-dependent and noncanonical β-catenin-independent pathways. In canonical WNT signaling, a WNT ligand first binds to transmembrane G protein coupled receptor frizzled (FZD) and coreceptor, lipoprotein related receptor (LRP5/6) (He et al., 2004) (Fig. 3). Dickkopf (DKK1 and DKK2) and Sclerostin (SOST) interfere with the interaction between WNT ligands and coreceptors LRP5/6, functioning as antagonists for WNT signaling (Mao et al., 2001; Semënov et al., 2005). Without WNT ligand binding, glycogen synthase kinase 3 (GSK3) and casein kinase-1 (CK1) bind to the destruction complex, consisting of adenomatous polyposis coli (APC) and the scaffolding protein Axin (Taelman et al., 2010). β-catenin, subsequently phosphorylated, is exposed to E3 ubiquitin ligase and thereby ubiquitinated. Ubiquitinated β-catenin eventually undergoes proteasomal degradation. In the presence of WNT, FZD receptor recruits disheveled (DSH) to membrane, which provides a platform for LRP5/6 to bind with GSK3β and Axin. This complex is then
Figure 3. WNT signaling pathway.

WNT signaling pathway can be categorized as canonical and noncanonical signaling pathway. Canonical WNT signaling is mediated by β-catenin, whereas noncanonical WNT signaling is independent of β-catenin. One of the noncanonical WNT signaling is WNT-mTORC1 axis. Via ribosomal S6 kinase and phosphorylation of 4EBP1, WNT-mTORC1 regulates protein translation. Transcription factor ATF4 is also a downstream effector of WNT-mTORC1 pathway (Park et al., 2017).
endocytosed, preventing its interaction with and phosphorylation of cytosolic β-catenin. It ultimately results in β-catenin stabilization. Stabilized β-catenin can then translocate to the nucleus. β-catenin interacts with Lymphoid-enhancing factor (LEF) and T cell factor (TCF) family, which induces the expression of downstream target genes including LeF1, Tcf7 and Nkd2 (Behrens et al., 1996; Hovanes et al., 2001; Roose et al., 1999; Zeng et al., 2000). In noncanonical WNT signaling cascades independent of β-catenin, FZD receptor functions as G-protein coupled receptor. This initiates a number of intracellular cascades including Rho and Rac (GTPases), c-Jun N-terminal kinase (JNK), phospholipase-C/protein kinase C (PKC), calcium calmodulin dependent kinase 2 (CaMK2), Phosphoinositide 3-kinase (PI3K)/AKT, and the serine threonine kinase mechanistic target of rapamycin (mTOR) (Chen et al., 2005; Inoki et al., 2006; Sheldahl et al., 1999; Sheldahl et al., 2003; Yamanaka et al., 2002). WNT inhibits TSC2, which in turn promotes Rheb to bind and activate mTORC1(Inoki et al., 2006) (Fig. 3). mTORC1 complex consists of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8), proline-rich Akt substrate of 40kDa (PRAS40) and DEPTOR (Hara et al., 2002; Kim et al., 2002; Kim and Guan, 2019). Activated mTORC1 phosphorylates p70 S6 kinase (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Saxton and Sabatini, 2017). This promotes 5′ cap-dependent mRNA translation, leading to increased protein synthesis. WNT signaling can also activate mTORC2, which controls the downstream targets including AKT (Chen and Long, 2018).
WNT signaling is a critical regulator of osteoblast differentiation and bone formation (Bennett et al., 2005; Day et al., 2005; Karner and Long, 2017; Rodda and McMahon, 2006; Tu et al., 2007). Loss of LRP5, the receptor for WNT ligands, leads to osteopenia, whereas constitutively active mutations in LRP5 render mice with high-bone mass phenotype (Babij et al., 2003; Boyden et al., 2002; Gong et al., 2001). Patients with these mutations in LRP5 also develop osteosclerosis, characterized by elevated bone density. Similarly, loss of function mutations in WNT antagonist SOST also leads to diseases like sclerosteosis and van Buchems disease featured by high bone mass phenotype (Brunkow et al., 2001). Canonical WNT signaling pathway, specifically β-catenin, is critical for osteoblast specification and differentiation as genetic deletion of β-catenin completely abolishes osteoblast differentiation (Chen and Long, 2013; Gaur et al., 2005). Particularly, loss of β-catenin prevents differentiation from RUNX2+ committed osteoblast progenitor to RUNX2+, OSX+ specified preosteoblasts, and from preosteoblasts to differentiated mature osteoblasts (Day et al., 2005; Hu et al., 2005). Indeed, loss of β-catenin redirects progenitors to adopt chondrogenic fate at the expense of osteoblast differentiation during bone development (Day et al., 2005). In addition, deletion of β-catenin in bone marrow derived preosteoblasts results in significantly increased adiposity instead of osteoblast differentiation during bone homeostasis (Song et al., 2012). β-catenin directly targets several osteoblast differentiation associated genes, including matrix mineralization regulator Akp2, master transcription factor Runx2 (Gaur et al., 2005). β-
catenin is also required for the expression of RANKL decoy receptor Opg in osteoblasts, indirectly regulating osteoclast formation (Glass et al., 2005). Additionally, noncanonical β-catenin-independent WNT pathway also plays indispensable roles in osteoblast differentiation. For example, WNT7b regulates osteoblast differentiation via PKCδ (Tu et al., 2007). WNT7b and WNT3A also can activate mTORC1, which increases protein synthesis for osteoblast differentiation (Chen and Long, 2015; Chen et al., 2014; Karner et al., 2015). In addition, WNT-mTORC2 signaling is also required for bone formation through its regulation of glycolysis (Chen et al., 2015; Esen et al., 2013). For metabolic regulations, WNT requires LRP5 but is independent of β-catenin. Instead, WNT-LRP5 activates mTORC2-AKT via RAC1, which reprograms glucose metabolism, contributing in part to bone formation. Additionally, WNT also suppresses glucose entry into TCA cycle in ST2 cells, a bone marrow derived cell line (Karner et al., 2016). This reprogramming of glucose metabolism reduces acetyl-CoA and citrate levels, which decreases histone acetylation. Subsequently, the expression of adipogenic genes like Pparg and Cebpa and chondrogenic genes like Sox5 and Sox9 is suppressed.

WNT reprograms metabolic activities of osteoblasts in order to fulfill high demands for protein synthesis and energy. For example, WNT promotes glucose consumption and glycolysis in osteoblasts (Esen et al., 2013; Karner and Long, 2018). In addition, WNT stimulates glutamine consumption and catabolism necessary for increased
protein synthesis and osteoblast differentiation (Karner et al., 2015). However, the mechanism by which WNT stimulates glutamine uptake in osteoblasts is elusive.

1.4 Cellular metabolism in bone development

As mentioned above, bone formation is not only regulated by transcription factors but also some developmental regulatory signals. Recent studies have also focused on the role of cellular metabolism in bone development. In this section, I will review the role and regulation of glucose, fatty acid and particularly amino acid metabolisms in osteoblast differentiation and bone formation.

1.4.1 Glucose metabolism in bone development

Glucose is the most abundant monosaccharide, serving as a major energy source in mammalian cells (Karner and Long, 2018). Glucose uptake is facilitated by glucose transporters (GLUTs) and metabolized through three major pathways: glycolysis, hexosamine biosynthetic pathway (HBP) and pentose phosphate pathway (PPP). Glucose is first phosphorylated to form glucose 6-phosphate (G6P) by hexokinase (HK) (Fig 4). G6P can enter PPP, which generates ribose 5-phosphate, the precursor of nucleotides. PPP also produces reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is important for redox regulation as well biosynthesis of amino acids and fatty acids (Patra and Hay, 2014). Additionally, G6P can also be directed to HBP, which is used for the production of uridine diphosphate N-acetylglucosamine (UDPGlcNAc) (Akella et al.,
During glycolysis, glucose is converted to two pyruvates, two ATPs and two molecules of reduced nicotinamide adenine dinucleotide (NADH) (Lunt and Vander Heiden, 2011). Pyruvate can be converted to lactate by lactate dehydrogenase. NAD+ is generated in this process, which can be used to further facilitate glycolysis. Osteoblasts are highly glycolytic. Older studies demonstrate cells isolated from bones preferentially converts glucose to lactate (Borle et al., 1960; Cohn and Forscher, 1962; Peck et al., 1964). A recent study shows that more than 80% of glucose is converted to lactate in osteoblasts even in the presence of oxygen (Esen et al., 2013). This process is known as aerobic glycolysis or Warburg effect (Warburg et al., 1927). Warburg effect is commonly observed in cancer cells. Although it is an inefficient way of producing ATP, aerobic glycolysis can not only generate ATP more rapidly, but also provide more carbons for biosynthesis of nucleotides and proteins (Liberti and Locasale, 2016). However, it still remains unclear why osteoblasts employ aerobic glycolysis. Additionally, pyruvate can also be metabolized to acetyl-CoA via decarboxylation by pyruvate dehydrogenase (PDH). Acetyl-CoA enters TCA cycle via the reaction with oxaloacetate to generate citrate. TCA cycle serves multiple functions. First, the conversion of TCA intermediate succinate to fumarate transfers electrons to electron transport chain (ETC) to produce the most ATP per glucose. This process is known as oxidative phosphorylation (OXPHOS). Furthermore, carbons that exit TCA cycle contribute to lipid and amino acid biosynthesis and redox regulation through cataplerosis, a process in which TCA intermediates exit the cycle.
Figure 4. Glycolysis.

During Glycolysis, glucose is metabolized to pyruvate, a process with production of ATP and NADH. An alternative is pentose phosphate pathway, forming NADPH and nucleotide precursor ribose 5-phosphate. HK – Hexokinase; PGI – Phosphoglucone Isomerase. PFK – Phosphofructokinase; GAPDH – Glyceraldehyde 3-phosphate Dehydrogenase; PGK – Phosphoglycerate Kinase; PGAM – Phosphoglycerate Mutase; ENO – Enolase; PKM – Pyruvate Kinase, Muscle; LDH – Lactate Dehydrogenase; PDH – Pyruvate Dehydrogenase.
(Wellen et al., 2009). For example, TCA intermediate malate, shuttled out of mitochondria, can be converted to pyruvate along with production of NADPH (DeBerardinis et al., 2007). Another TCA cycle intermediate oxaloacetate can be metabolized to aspartate, which is an important precursor of nucleotides (Son et al., 2013). In summary, glucose is not only a major source of ATP production but also plays a critical role in metabolic homeostasis, biosynthetic reactions and cellular functions.

Osteoblasts actively consume and metabolize glucose (Karner and Long, 2018). GLUT1 is the highest expressed glucose transporter in osteoblasts (Wei et al., 2015; Zoidis et al., 2011). Glucose uptake by GLUT1 regulates stability of the transcription factor RUNX2 by inhibition of ubiquitin ligase SMURF1 via phosphorylation of AMPK (Wei et al., 2015). In turn, RUNX2 also promotes GLUT1 expression. This forms a positive feedback loop between RUNX2 and glucose uptake by GLUT1, critical for osteoblast differentiation (Wei et al., 2015). In addition, GLUT1-mediated glucose consumption enhances mTORC1 activity by inhibiting AMPK. GLUT1-AMPK-mTORC1 signaling pathway is required for protein synthesis in osteoblasts. A recent study suggests that glycolysis provides around 80% of ATP in mature osteoblasts (Lee et al., 2020). However, osteoblasts still have active mitochondrial activity and OXPHOS, suggesting that osteoblasts may rely on other nutrients for sufficient mitochondrial respiration, for example, fatty acids and amino acids (Komarova et al., 2000). Glucose metabolism is regulated by numerous factors including parathyroid hormone (PTH) and WNT (Chen et
al., 2015; Esen et al., 2013; Esen et al., 2015; Zoidis et al., 2011). PTH promotes aerobic glycolysis by increasing glucose uptake and lactate production through IGF-mTORC2 axis (Esen et al., 2015). WNT signaling also regulates glucose metabolism by rapidly stimulating glucose consumption and protein expression of HK2, the rate-limiting enzyme of glycolysis, within 6 hours (Esen et al., 2013). This is followed by a series of upregulation of glycolytic enzymes including PFK1, LDHA and PDK1.

Similarly, chondrocytes also rely on GLUT1 for most glucose uptake, which is stimulated by BMP signaling via the downstream cascade of mTORC1-HIF1α (Lee et al., 2018). HIF signaling by itself also regulates glucose metabolism in chondrocytes. The absence of blood vessels in cartilage makes it a hypoxic environment, stabilizing HIF-1α. HIF-1α increases SOX9 expression, which promotes chondrogenesis and regulates glycolytic enzymes for anaerobic glycolysis (Amarilio et al., 2007; Stegen et al., 2019). Accumulation of HIF1α signaling by conditionally inactivating HIF prolyl hydroxylase 2 leads to increased energy deficit due to decreased glucose oxidation (Stegen et al., 2019).

### 1.4.2 Fatty acid metabolism in bone development

Fatty acids are another important carbon and energy source in cells. Cells synthesize or acquire fatty acids by transporters or as lipoproteins (Bartelt et al., 2017; Niemeier et al., 2008). Upon entering the cells, fatty acids can be metabolized in mitochondria by β-oxidation, by which two carbons are sequentially removed as acetyl-CoA. The translocation of fatty acids into the inner mitochondria matrix depends on two
carnitine palmitoyltransferases (CPT) (Fig. 5). CPT1 is responsible for the transferal of acyl group from fatty acyl-CoA to carnitine (Houten et al., 2016). Acyl-carnitine, shuttled into inner mitochondria in exchange of carnitine, is reversed to Acyl-CoA by CPT2. Complete β-oxidation yields more ATP than glucose by weight.

Fatty acids are actively taken up by osteoblasts and estimated to provide approximately 40%-80% of the energy provided by glucose (Adamek et al., 1987; Kim et al., 2017; Niemeier et al., 2008; van Gastel et al., 2020). In contrast, as mentioned in the previous section, a recent study estimated 80% ATP is derived from aerobic glycolysis (Lee et al., 2020). The discrepancy in ATP sources may result from different techniques. Older biochemical analysis (Adamek et al., 1987) calculated ATP production by the oxidation rate of palmitate, while the recent study (Lee et al., 2020) calculated through oxygen consumption rate measured by Seahorse analysis. The relative precision of the two techniques is debatable and requires further elaborated investigation. Fatty acid β-oxidation also plays a critical role in osteoblast differentiation. One study shows that loss of CPT1a perturbs differentiation of the osteoblast lineage cell (van Gastel et al., 2020). Another study demonstrates conditional deletion of CPT2 in mature osteoblasts impairs proper bone formation in female mice (Kim et al., 2017). Altogether, fatty acid metabolism is considered as a crucial player in osteoblast differentiation.
On the contrary, fatty acid metabolism is dispensable for chondrocyte differentiation and may play an inhibitory role in chondrocyte differentiation. For example, the presence of excessive fatty acids indeed prevent skeletal stem cells from adopting a chondrogenic fate (van Gastel et al., 2020).

Figure 5. Fatty acid metabolism.

The import of fatty acid into mitochondria relies on carnitine-dependent enzymes carnitine palmitoyltransferases (CPTs). Upon entering mitochondria, fatty acids undergo β-oxidation. CACT = carnitine acylcarnitine translocase.
1.4.2 Amino acid metabolism in bone development

Mammalian cells obtain amino acids through cell membrane surface transporters, de novo synthesis, protein degradation or autophagy. Osteogenic signals like WNT and PTH signaling regulate amino acid uptake and protein synthesis in osteoblasts (Karner et al., 2015; Yee, 1988). Mature osteoblasts express transcription factor ATF4, which is critical for amino acid import during differentiation (Elefteriou et al., 2006). Amino acids are not only the building blocks and basic units of proteins but also metabolizable, functioning as important energy, carbon and nitrogen sources. Unfortunately, the role of individual amino acid in bones is understudied. Glutamine is the most extensively studied amino acid in osteoblasts. Conversely, the knowledge about proline in osteoblasts is limited. In this section, I will review what is known about glutamine and proline, and their transporters in the context of bone.

1.4.2.1 Glutamine metabolism

Glutamine is required for osteoblast mineralization (Karner et al., 2015; van Gastel and Carmeliet, 2021). Glutamine is the most abundant amino acid in the circulation system (Bergström et al., 1974; Mayers and Vander Heiden, 2015). It serves multiple functional roles in bioenergetics, biosynthesis of amino acids, nucleotides, glutathione (Altman et al., 2016; Hosios et al., 2016; Welbourne, 1979; Windmueller and Spaeth, 1974) (Fig. 6). The first step of glutamine metabolism is the deamination of glutamine catalyzed by glutaminase (GLS or GLS2) with glutamate as the product (Curthoys and Watford,
The conversion from glutamine to glutamate can also be catalyzed by other enzymes like PPAT and GFPT (Yelamanchi et al., 2016). As a carbon donor, glutamate is further deaminated to \( \alpha \)-ketoglutarate (\( \alpha \)KG) by...

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**Figure 6. Glutamine metabolism**

Glutamine serves for multiple purposes. Glutamine can be metabolized to glutamate catalyzed by glutaminase (GLS). Glutamate can contribute to glutathione synthesis. Glutamate can also be converted to \( \alpha \)-ketoglutarate (\( \alpha \)KG) through either glutamate dehydrogenase (GLUD) or aminotransferases GOT and GPT. \( \alpha \)KG enters the TCA cycle for bioenergetics and biosynthesis. Malate can be shuttled out of mitochondria and produce pyruvate and NADPH. Oxaloacetate (OAA) can be converted to aspartate for nucleotide synthesis. \( \alpha \)KG can alternatively be metabolized backwards to produce citrate, a process known as reductive carboxylation (RC).

GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase.
glutamate dehydrogenase (Moreadith and Lehninger, 1984). \( \alpha \)KG enters the TCA cycle and produces energy for cellular functions (DeBerardinis et al., 2007; Fan et al., 2013; Wang et al., 2011). \( \alpha \)KG can also function as a cofactor and regulate histone/DNA demethylation (Carey et al., 2015). Additionally, \( \alpha \)KG can be reverted back to citrate by reductive carboxylation (Ward et al., 2010). Glutamate is not only as a carbon donor, but also a nitrogen donor for the synthesis of alanine by glutamate-pyruvate transaminase (GPT) and aspartate by glutamate-oxaloacetate transaminase (GOT) (Sorbi et al., 1999; Wroblewski and Ladue, 1956). Both processes generate \( \alpha \)KG as a by-product. Glutamate is also a crucial source for glutathione, an antioxidant in charge of neutralizing reactive oxygen species (ROS) (Welbourne, 1979). This tripeptide (Glu-Cys-Gly) is formed through the condensation of glutamate and cysteine by glutamate-cysteine ligase (GCL) and followed by the addition of glycine by glutathione synthetase (GSS).

Recent studies have demonstrated the critical role of glutamine in osteoblast differentiation. Of the two isozymes of glutaminase, GLS has higher expression than GLS2 in skeletal stem cells (SSC) (Karner et al., 2015; Yu et al., 2019). Removal of glutamine or inhibition of GLS prevents osteoblast differentiation (Karner et al., 2015; Stegen et al., 2020a; Yu et al., 2019). Loss of GLS in SSCs affects proliferation and leads the SSCs to adopt adipocyte fate (Yu et al., 2019). \( \alpha \)KG derived from transamination of glutamate is responsible for SSC proliferation. Glutamine metabolism is regulated by several signaling pathways. For example, Hif-1\( \alpha \) regulates glutaminase expression and production of
glutamine-derived glutathione, which promotes periosteal cell survival and bone fracture healing (Stegen et al., 2016). In differentiating osteoblasts, WNT signaling promotes glutaminase expression via mTORC1 signaling. This leads to increased flux of glutamine into TCA cycle (Karner et al., 2015). Elevated TCA activity fulfills partial energetic demands during bone formation. Interestingly, glutamine as an energy source challenges the studies that claim glucose and fatty acid as the major energy source in osteoblasts (Adamek et al., 1987; Lee et al., 2020). This is likely due to different study subjects and approaches by researchers. Karner et al. studied glutamine metabolism in ST2 cells stimulated by WNT (2015). This is different from the study by Lee et al., which focused on primary osteoblasts isolated from mouse calvaria (2020). Furthermore, increased glutamine metabolism reduces intracellular glutamine pool, triggering integrated stress response (ISR) by activation of general control nonrepressible 2 (GCN2) (Karner et al., 2015). ISR increases the expression of ATF4, which increases amino acid uptake and biosynthesis. This in turn promotes protein synthesis critical for osteoblast differentiation.

Chondrocytes also have active glutamine uptake and glutaminase activity. Similar to osteoblasts, glutamine metabolism is dispensable in energy production; rather, aspartate synthesis through transamination of glutamate mediates chondrocyte proliferation (Stegen et al., 2020b). Furthermore, acetyl-CoA synthesis from glutamate through reductive carboxylation regulates chondrogenesis epigenetically through histone acetylation (Stegen et al., 2020b). HIF-1α also promotes glutamine metabolism in
chondrocytes. Elevated glutaminase catabolism leads to accumulation of αKG that functions as a cofactor to promote hydroxylation of collagen (Stegen et al., 2019). High hydroxylation of collagens prevents cartilage matrix resorption, which impairs endochondral ossification.

### 1.4.2.2 Proline metabolism

Proline is a unique amino acid due to its pyrrolidine side chain. This five-member ring structure is critical for the stability of helix structure of proteins, especially collagens, which consists of triple fibril helices. Bone-associated collagens COL1, COL2 and COLX have proline as 19%, 18.2% and 21.3% of their total amino acids, respectively (Albaugh et al., 2017). Proline can also be hydroxylated, which increases the stability of proteins like collagens. In addition, proline can also be metabolized (Fig. 7). Pyrrolidine ring of proline also requires special enzymes to break down proline because its α-nitrogen is locked in the ring and generic amino acid enzymes like transaminases are not capable of removing the amino group from proline (Phang et al., 2008). Proline dehydrogenase (PRODH), also known as proline oxidase (POX), removes the hydrogen from the amino group in the pyrroline chain, making the ring structure prone to be opened up (Liu and Phang, 2012a). This reaction converts proline to pyrroline-5-carboxylate. PRODH is tightly associated with inner membrane of mitochondria, located in complex II of electron transport chain (ETC) (Hancock et al., 2016; Schertl et al., 2014). PRODH-mediated proline-P5C conversion is dependent on the reduction of flavin adenine dinucleotide (FAD), which
transfers free electrons to cytochrome c. Eventually, hydrogen oxidized by O\textsubscript{2} forms H\textsubscript{2}O in complex IV (Phang, 2019a). This process also generates ATP, which as mentioned above is a critical regulator of protein synthesis and RUNX2 stability through AMPK in osteoblasts. Electrons can adopt another fate, which directly reduces oxygen in complex III to form ROS (Chandel, 2010). Excessive ROS induces apoptosis in osteoblasts while low level of ROS promotes osteoblast differentiation (Deng et al., 2019; Migliario et al., 2014). Therefore, proline catabolism, mediated by PRODH, is linked to both bioenergetics and ROS production. In C. elegans, PRODH-mediated proline catabolism is required for life-extending capacity when insulin/IGF1 signaling is impaired (Zarse et al., 2012). ROS derived from proline catabolism also modulates pathogen defense in C. elegans (Tang and Pang, 2016). Proline can also be synthesized from glutamate with P5C as the intermediate. In this reaction, glutamate is first metabolized to P5C by P5C synthetase. P5C is then converted to proline catalyzed by P5C reductases (PYCR1, PYCR2 and PYCRL) using NADH or NADPH as cofactors (Valle et al., 1974). Therefore, proline anabolism mediated by PYCRs play a critical role in redox homeostasis for the balance of NAD\textsuperscript{+}/NADH and NADP\textsuperscript{+}/NADPH ratios. In addition, the generation of NADP\textsuperscript{+} from this reaction can also be used in pentose phosphate pathway for nucleotide synthesis. Loss of function of PYCR2 leads to elevated cerebral glycine synthesized by SHMT2, associated with neurodegenerative symptoms (Escande-Beillard et al., 2020). Altogether, proline oxidation to P5C and P5C reduction back to proline form a cycle, maintaining the dynamic
balance of cellular functions. Additionally, proline can also regulate histone modification in embryonic stem cells by increasing histone methylation of H3K9 and H3K36 via unknown mechanism (Comes et al., 2013).

Figure 7. Proline metabolism.

Proline can be oxidized to pyrroline-5-carboxylic acid (P5C) by proline dehydrogenase (PRODH). This reaction transfers electrons to electron transport chain (ETC) for ATP production. P5C can be converted back to proline dependent on PYCRs and oxidation of NAD(P)H. P5C downstream metabolite glutamic-γ-semialdehyde (GSA) can be converted to glutamate by P5C dehydrogenase (P5CDH). The reverse reaction is catalyzed by P5C synthetase (P5CS). Alternatively, ornithine aminotransferase (OAT) metabolizes GSA to ornithine, which enters urea cycle.
The role of proline and proline metabolism in bone is unclear due to limited studies. Studies from decades ago showed that proline was actively consumed in rat and chick bones (Adamson and Ingbar, 1967a, c; Hahn et al., 1969; Yee, 1988). Proline is one of the most abundant amino acids present in collagens, which is the major organic component of bones (Albaugh et al., 2017). Bone matrix is mainly made of COL1 and cartilage matrix is made of COL2 and COLX, all of which are proline rich (Long, 2012). The study of proline metabolism in the context of bone is even more limited. A clinical study revealed that patients with autosomal recessive mutations in Pycr2 have microcephaly and facial dysmorphism and mice lacking PYCR2 have low bone mass (Escande-Beillard et al., 2020; Nakayama et al., 2015). The mechanism underlying these phenotypes is not well-understood. Osteoblast differentiation is an energy-consuming process with high biosynthetic demand. If proline metabolism functions similarly in osteoblasts as cancer and plant cells, proline may be an omnipotent candidate to meet these needs during osteoblast differentiation.

1.4.2.3 Amino acid uptake and transporters

The import of amino acids in bones has been documented since decades ago (Adamson and Ingbar, 1967a, b, c; Finerman and Rosenberg, 1966; Hahn et al., 1969; Rosenbusch et al., 1967; Yee, 1988). Bone cells consume amino acids actively, facilitated by amino acid transporters. As mentioned above, lack of amino acids triggers ISR and subsequently activates the amino acid sensor GCN2, which in turn promotes ATF4. ATF4
upregulates the expression of amino acid transporters (Hu et al., 2020b; Karner et al., 2015). Loss of GCN2 inhibits SSC proliferation without disturbing its differentiation into osteoblast. Cells lacking GCN2 fail in promoting expressions of amino acid transporters in response to integrated stress. Amino acid transporters are membrane tethered proteins responsible for the import, export or exchange of amino acids across membrane. Most amino acids transporters belong to 11 solute carrier (SLC) families. At least 66 known amino acid transporters have been documented and studied to some extent. The current classification of amino acid transporter systems was created by Halvor Christensen, which is based on specificity of substrates and mechanism of facilitation: systems A, N, ASC, B, L, T, \( \text{x}^{-} \) and \( \gamma^{+} \) (Christensen, 1975; Kandasamy et al., 2018). Some transporters have not been categorized into these systems, for example, system IMINO transporters. Some transporters play a critical role in osteoblast differentiation. For example, osteoblast differentiation is impaired in cells overexpressed with glutamate/cystine antiporter \( \text{Slc7a11} \) (xCT) likely due to decreased glutamate-derived glutathione (Takarada-Iemata et al., 2011; Uno et al., 2011a; Uno et al., 2011b). However, the role of amino acid transporters remains elusive in osteoblasts. In this section, I will focus on glutamine and proline transporters in the context of bone.

Glutamine uptake is mediated by a diverse array of transporters categorized by multiple different systems: systems A, ASC, N, L and \( \gamma^{+} \text{L} \) (Fig. 8). These systems can be classified based on their sensitivity to pH, dependence of sodium ion, specificity against
substrates, and susceptibility to inhibitors. System L is the only glutamine transporter system that is independent of sodium. In the other systems, a sodium ion is required when the transporter facilitates the import or export of an amino acid at a time. System N, but not the other systems, is lithium tolerant, which means system N transporters can use lithium as a substitute to fulfill amino acid transport in the absence of sodium.

Figure 8. Glutamine transporters.

Glutamine transporters can be categorized into multiple systems based on their properties. System A is sodium dependent and α-methylaminoisobutyric (MEAIB) sensitive. System ASC is sodium dependent and inhibited by γ-glutamyl-p-nitroanilide (GPNA). System N is lithium tolerant. System γ+L is sodium dependent and depends on 4F2hc (encoded by Slc3a2) for stability. System L is amino acid exchanger that exports glutamine and imports branched chain amino acids (BCAA) independent of sodium.
System A transporters can be inhibited by amino acid analog, α-methylaminoisobutyric acid (MEAIB) while γ-glutamyl-p-nitroanilide (GPNA) inhibits Slc1a5 (ASCT2), the only glutamine transporter in system ASC family. Slc1a5 is the most studied glutamine transporter in the cancer field. Besides glutamine, Slc1a5 can also transport other small neutral amino acids like alanine, serine and asparagine in different cells and tissues. Glutamine uptake mediated by Slc1a5 is required for proliferation and viability of multiple cancers including non-small cell lung cancer, breast cancer and renal cell carcinoma (Bröer et al., 2016; Hassanein et al., 2015; van Geldermalsen et al., 2016). Furthermore, Slc1a5 also provides glutamine as an exchange factor for Slc7a5, which imports essential amino acids that activate mTORC1 signaling pathway (Nicklin et al., 2009). Recent studies suggest Slc1a5 has an important role in physiological conditions. Glutamine uptake in satellite cells is mediated by Slc1a5, which is required for proliferation and differentiation (Shang et al., 2020). Inhibition of Slc1a5 leads to failure in muscle regeneration. In addition, pharmacological inhibition of Slc1a5 by GPNA inhibits osteoclast differentiation (Indo et al., 2013). However, the role of Slc1a5 in osteoblasts or chondrocytes remains unknown. On the other hand, Slc7a7 (γ+LAT1), a nonclassical glutamine transporter, is very likely to be linked with osteoblast function and bone formation. Slc7a7 is mostly considered as a transporter for arginine, lysine and ornithine. Mutations in Slc7a7 has been discovered in patients diagnosed with lysinuric protein intolerance (LPI) (Ogier de Baulny et al., 2012). One of the symptoms most LPI patients
experience is delayed skeletal maturation and low bone mass (Posey et al., 2014). Since Slc7a7 can also transport glutamine, it is unclear which amino acids imported by Slc7a7 contribute to osteoblast differentiation or activity.

The properties of proline uptake have been characterized long ago using chick or rat bones (Adamson and Ingbar, 1967a; Finerman and Rosenberg, 1966; Hahn et al., 1969; Yee, 1988). Transporters for proline are sodium-dependent, pH sensitive and inhibited by MEAIB. Many systems fall under these characteristics. System IMINO transporter, Slc6a7, encoding Proline Transporter (PROT), regulates the spatial distribution of proline in synapses and is linked to neurologic disorders, despite its low expression in osteoblasts (Schulz et al., 2018). Slc38a2, which encodes sodium coupled neutral amino acid 2 (SNAT2), functions as proline transporter, required for differentiation in embryonic stem cells (Tan et al., 2011). As its name implies, Slc38a2 is capable of transporting small neutral amino acids including proline, glutamine and alanine in different cells and tissues. In cancer studies, Slc38a2 is well considered as a glutamine or alanine transporter (Bröer et al., 2016; Menchini and Chaudhry, 2019; Morotti et al., 2019). In breast cancer cells, Slc38a2 is regulated by HIF1α signaling, critical for cancer growth and resistance to cancer therapy (Morotti et al., 2019). In pancreatic ductal adenocarcinoma cells, Slc38a2 provides alanine to maintain metabolic homeostasis (Parker et al., 2020). However, the role of Slc38a2 is largely unknown in osteoblast differentiation and bone formation.
Chapter 2. Biphasic regulation of glutamine consumption by WNT during osteoblast differentiation.

Chapter 2 is modified from a manuscript with the same title published in the Journal of Cell Science in 2020. The authors include Leyao Shen (myself), Dr. Deepika Sharma, Yilin Yu, Dr. Fanxin Long and Dr. Courtney Karner. Leyao Shen and Deepika Sharma contributed equally to this publication. Both of us performed most of the experiments and wrote and revised the manuscript. Most of the work was done at Duke University under the supervision of Dr. Courtney Karner.

2.1 Summary

Osteoblasts are the principal bone forming cells. As such, osteoblasts have enhanced demand for amino acids to sustain high rates of matrix synthesis associated with bone formation. The precise systems utilized by osteoblasts to meet these synthetic demands are not well understood. WNT signaling is known to rapidly stimulate glutamine uptake during osteoblast differentiation. Using a cell biology approach, we identified two amino acid transporters, Slc7a7 and Slc1a5, as the primary transporters of glutamine in response to WNT. Slc1a5 mediates the majority of glutamine uptake, whereas Slc7a7 mediates the rapid increase in glutamine uptake in response to WNT. Mechanistically, WNT signals through the canonical/β-catenin dependent pathway to rapidly induce Slc7a7 expression. Conversely, Slc1a5 expression is regulated by the transcription factor ATF4 downstream of the mTORC1 pathway. Targeting either Slc1a5
or Slc7a7 using shRNA reduced WNT induced glutamine uptake and prevented osteoblast differentiation. Collectively these data highlight the critical nature of glutamine transport for WNT induced osteoblast differentiation.

**2.2 Introduction**

Osteoblasts are the primary bone forming cell responsible for producing and secreting Type I Collagen and other proteins that comprise the bone matrix. A constant supply of amino acids is required to maintain high rates of protein and matrix synthesis associated with bone anabolism. To fulfill this demand, osteoblasts must maximize the production or acquisition of amino acids. Indeed, recent evidence links amino acid uptake and metabolism to osteoblast function (Elefteriou et al., 2006; Karner et al., 2015; Rached et al., 2010; Yu et al., 2019). However, little is known about the transporters mediating amino acid uptake, nor their regulation during bone development.

The WNT family of secreted glycoproteins are critical regulators of osteoblast differentiation (Babij et al., 2003; Bennett et al., 2005; Day et al., 2005; Gong et al., 2001; Hu et al., 2005; Rodda and McMahon, 2006; Tu et al., 2007). WNTs activate multiple intracellular signaling cascades to induce osteoblast differentiation and modulate osteoblast activity. In the canonical pathway, WNT regulates the stability of the transcriptional coactivator β-catenin, a critical regulator of osteoblast specification and differentiation (Day et al., 2005; Hu et al., 2005; MacDonald and He, 2012; Rodda and McMahon, 2006). In the absence of WNT, β-catenin is phosphorylated by the β-catenin
destruction complex and targeted for proteasomal degradation. WNT stimulation inhibits the destruction complex, resulting in β-catenin stabilization which can translocate into the nucleus and induce target gene expression (for example - Tcf7) (Angers and Moon, 2009; Clevers, 2006; Clevers and Nusse, 2012; Roose et al., 1999). WNTs can also regulate osteoblast differentiation independent of β-catenin through the serine threonine kinase mechanistic target of rapamycin (mTOR) (Chen et al., 2014; Inoki et al., 2006; Karner et al., 2015). Indeed, mTORC1 is critical for preosteoblasts to increase protein synthesis and differentiate into mature osteoblasts (Chen and Long, 2015; Fitter et al., 2017; Karner et al., 2017; Lim et al., 2016; Xian et al., 2012). We previously discovered WNT stimulates glutamine uptake and catabolism necessary for osteoblast differentiation and bone formation (Karner et al., 2016; Karner et al., 2015). Mechanistically, it is not clear how WNT stimulates glutamine uptake in osteoblasts.

Glutamine transport is facilitated by a diverse array of membrane-tethered amino acid transporters categorized into discrete transport systems based on substrate specificity, kinetics and ion and pH dependence (Pochini et al., 2014). Glutamine uptake can occur in a Na⁺ dependent or independent manner mediated by Systems ASC, A, γ(+)-L and N, or System L respectively (Biltz et al., 1983; Bode, 2001; Jacob et al., 1986; Mackenzie et al., 2003; Tamarappoo et al., 1992; Tamarappoo et al., 1997; Taylor et al., 1992). Na⁺ dependent transport is subdivided by the strict requirement of Na⁺ (System ASC, A and γ(+)-L) or the substitution of Na⁺ with Li⁺ (System N). Finally, inhibitors can
further subdivide Na\(^+\) dependent transporters with System A being sensitive to 2-(methylamino)-isobutyric acid (MeAIB) and System ASC to γ-glutamyl-p-nitroanilide (GPNA) (Esslinger et al., 2005; Freeman et al., 1999; Mackenzie et al., 2003). While a great deal is known about the functional characteristics of these transporter systems, little is known about the specific transporters mediating glutamine uptake or their regulation in osteoblasts.

Here we describe the biphasic regulation of glutamine uptake in response to WNT. WNT rapidly stimulates glutamine uptake that is sustained throughout osteoblast differentiation. Mechanistically, WNT activates β-catenin to rapidly stimulate glutamine uptake through $\text{Slc7a7}$ while mTORC1 regulates basal glutamine uptake through $\text{Slc1a5}$. These data highlight the previously unknown role for the amino acid transporters $\text{Slc7a7}$ and $\text{Slc1a5}$ and their regulation by WNT during osteoblast differentiation.

2.3 Results

2.3.1 WNT rapidly stimulates glutamine uptake during osteoblast differentiation.

We previously determined WNT stimulates glutamine uptake associated with osteoblast differentiation (Karner et al., 2015). In order to understand how WNT regulates glutamine consumption, we first evaluated the kinetics of glutamine uptake in ST2 cells, a bone marrow derived cell line that undergoes osteoblast differentiation in response to WNT (Bennett et al., 2005; Kang et al., 2007; Karner et al., 2016; Karner et al., 2015; Otsuka et al., 1999; Tu et al., 2007) (Fig. 9A). ST2 cells were treated with recombinant WNT3a
(rWNT3a) for up to 96 hours and glutamine uptake was quantified using L-(2,3,4-3H)-Glutamine. We observed a rapid and sustained increase in glutamine consumption beginning as rapidly as 6 hours after rWNT3a stimulation (Fig. 9B). Glutamine consumption continued to increase throughout the course of the experiment, even 96 hours after rWNT3a stimulation (Fig. 9B). Basal glutamine transport occurred primarily in a Na+ dependent manner that could not be rescued by lithium or inhibited by the amino acid analog MeAIB in ST2 cells (Fig. 9C). These data indicate glutamine uptake occurs primarily in a Na+ dependent manner characteristic of the amino acid transport systems System ASC and System γ(+)-L (Fig. 9D). Likewise, WNT stimulated glutamine uptake in ST2 cells was Na+ dependent and insensitive to either MeAIB or Li+ rescue (Fig. 9C). These data indicate WNT induced glutamine uptake is not the result of enhanced activity of other transport systems, rather it results from increased System ASC and/or γ(+)-L activity (Fig. 9D) (Jacob et al., 1986; Tamarappoo et al., 1997; Taylor et al., 1992).
Figure 9. WNT signaling rapidly increases sodium dependent glutamine consumption in ST2 cells.

(A) Alkaline phosphatase and von Kossa staining in ST2 cells treated with 25ng/ml rWnt3a for 72h followed by osteogenic media for 6 days. (B) Time course of radiolabeled glutamine uptake in response to rWNT3A. (C) Glutamine uptake assays performed in the presence (+) or absence (-) of sodium (Na\(^+\)), MeAIB or lithium (Li\(^+\)) in the uptake media in ST2 cells treated with rWNT3A for 24hours. (D) Relative system activity calculated from experiments in (C). 3 independent trials were performed in each experiment. Error bars depict SD. * p≤ 0.05, unpaired 2-tailed Student’s t-test.
2.3.2 Slc1a5 and Slc7a7 regulate basal and WNT stimulated glutamine uptake

To determine how WNT signaling regulated glutamine uptake, we first evaluated the mRNA expression of genes encoding glutamine transporters in ST2 cells. To do this we analyzed our previously generated RNAseq dataset of ST2 cells treated with rWNT3a for up to 72 hours (Karner et al., 2016). In unstimulated ST2 cells, the system ASC transporter Alanine Serine Cysteine Transporter 2 (ASCT2, encoded by Slc1a5) was the highest expressed glutamine transporter in either System ASC or γ(+)-L (Table 1). Conversely, the System γ(+)-L transporter γ(+)-L transporter 1 (γ(+)-LAT1, encoded by Slc7a7) was expressed at low levels in unstimulated ST2 cells (Table 1). It is important to note that ASCT2 and γ(+)-LAT1 are the only members of their respective transport systems that accept glutamine as a substrate. Despite being expressed at lower levels in unstimulated ST2 cells, Slc7a7 was significantly increased by WNT at 6, 24 and 72 hours (Table 1). On the other hand, Slc1a5 was not significantly increased until after 72 hours WNT treatment (Table 1). We confirmed the temporal regulation of both Slc1a5 and Slc7a7 using qPCR. Both Slc7a7 and Slc1a5 were significantly induced by WNT signaling albeit with differing kinetics (Fig. 10). Slc7a7 was induced rapidly, with peak induction occurring within 6-24 hours of WNT stimulation. By 96 hours, Slc7a7 had returned to baseline expression (Fig. 10). Conversely, Slc1a5 was significantly induced beginning 72 hours after WNT stimulation (Fig. 10). These data demonstrate WNT regulates two amino acid transporters with differing kinetics in ST2 cells.
Table 1: Amino Acid Transporter Expression in ST2 cells

<table>
<thead>
<tr>
<th>GENE</th>
<th>System</th>
<th>Alias</th>
<th>Control</th>
<th>6hr WNT</th>
<th>24hr WNT</th>
<th>72hr WNT</th>
<th>Fold Change</th>
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<tr>
<td>Slc1a5</td>
<td>ASC</td>
<td>ASCT2</td>
<td>35.3</td>
<td>1.1</td>
<td>0.9</td>
<td>3.0*</td>
<td></td>
</tr>
<tr>
<td>Slc7a7</td>
<td>(+)LAT</td>
<td>(+)LAT1</td>
<td>1.1</td>
<td>5.7*</td>
<td>4.6*</td>
<td>3.8*</td>
<td></td>
</tr>
</tbody>
</table>

* p≤0.05

Figure 10. WNT regulates the expression of glutamine transporters Slc7a7 and Slc1a5 with distinct dynamics.

WNT regulates the expression of glutamine transporters Slc7a7 and Slc1a5 with distinct dynamics. qRT-PCR analyses of Slc7a7 and Slc1a5 expression in ST2 cells treated with WNT3A for up to 96 hours. 3 independent trials were performed in each experiment. Error bars depict SD. * p≤ 0.05 by an unpaired 2-tailed Student’s t-test.
We next sought to determine the necessity of these amino acid transporters for glutamine uptake. We first evaluated the role of *Slc1a5* in glutamine uptake as it was highly expressed in ST2 cells (Table 1). To do this, we used γ-glutamyl-p-nitroanilide (GPNA), a specific inhibitor of *Slc1a5/ASCT2* (Esslinger et al., 2005). GPNA treatment reduced basal glutamine uptake by 77% indicating the majority of glutamine uptake is mediated by *Slc1a5/ASCT2* (Fig. 11A). We next sought to determine the effects on WNT induced glutamine uptake. Interestingly, despite significantly reducing basal glutamine uptake, glutamine consumption was stimulated equally by WNT in the presence or absence of GPNA (Fig. 11A,B). To confirm the specificity of GPNA, we targeted *Slc1a5* using short hairpin RNAs (shRNA). This approach significantly reduced both ASCT2 protein and basal glutamine uptake similar to GPNA treatment (Fig. 11C,D). Similar to the GPNA experiment, glutamine uptake was stimulated equally by WNT in both control and ASCT2 knockdown cells (Fig. 11D,E). These data indicate *Slc1a5* mediates the majority of glutamine uptake in ST2 cells but is not responsible for the acute increase in glutamine consumption in response to WNT. We next sought to determine if *Slc7a7* mediates glutamine uptake in ST2 cells. To do this, we targeted *Slc7a7* using shRNA. This approach effectively reduced γ(+)-LAT1 protein levels (Fig. 11F). *Slc7a7* knockdown minimally affected basal glutamine uptake but completely abrogated increased glutamine consumption in response to WNT stimulation (Fig. 11G,H). Collectively these data indicate WNT signaling rapidly increases *Slc7a7* expression to increase glutamine uptake.
Figure 11. WNT signaling induces *Slc*7a7 and *Slc*1a5 with disparate kinetics.

(A) Measurements of radiolabeled glutamine uptake in response to WNT3a in the presence of GPNA.  (B) Graphical depiction of WNT induced glutamine uptake calculated from data shown in (A).  (C) Western Blot analyses of ASCT2 protein expression in level in ST2 cells infected with shRNA targeting *Slc1a5* or RFP as a control.  ASCT2 normalized to β-actin.  Fold change ± SD for 3 independent experiments.  (D-E) Effect of *Slc1a5* knockdown on glutamine uptake in response to WNT3A.  (F) Western Blot analyses of γ+LAT1 protein expression in ST2 cells infected with shRNA targeting *Slc7a7* or RFP as a control.  γ+LAT1 normalized to β-actin.  Fold change ± SD for 3 independent experiments.  (G-H) Effect of *Slc7a7* knockdown on glutamine uptake in response to WNT3A.  3 independent trials were performed in each experiment. Error bars depict SD. * p≤ 0.05, ** p≤ 0.005, *** p≤ 0.0005, **** p≤ 0.00005, by one-way ANOVA (A,D,G) or by an unpaired 2-tailed Student’s *t*-test (B,C,E,F,H).
whereas Slc1a5 is responsible for the majority of glutamine uptake in ST2 cells.

2.3.3 Glutamine uptake and Slc7a7 are regulated by canonical WNT signaling.

We next sought to understand how WNT signaling regulated Slc7a7 expression. The rapid induction kinetics of Slc7a7 suggested it may be regulated directly by β-catenin. Inhibition of LRP5/6 using recombinant DKK1 reduced WNT induced β-catenin stabilization and prevented the induction of alkaline phosphatase (Fig. 12A). Moreover, DKK1 treatment prevented WNT induction of known β-catenin target genes (e.g. Tcf7) as well as Slc7a7 (Fig. 12B,C). Expression of a dominant-negative form of TCF4 that is unable to interact with β-catenin also prevented induction of Slc7a7 suggesting Slc7a7 is a β-catenin target gene (data not shown). To test the role of β-catenin directly, we targeted the gene encoding β-catenin (Catnb1) using shRNA (Fig. 12D). β-catenin knockdown prevented osteoblast differentiation as shown by inhibition of alkaline phosphatase induction (Fig. 12D). Importantly, β-catenin knockdown significantly reduced the basal expression of Slc7a7 and prevented the induction of Slc7a7 in response to WNT treatment (Fig. 12E). Moreover, β-catenin knockdown reduced WNT induced glutamine uptake without affecting Slc1a5 expression (Fig. 12F and data not shown). These data indicate β-catenin is necessary for the induction of Slc7a7 and acute glutamine consumption in response to WNT. We next sought to determine the sufficiency of β-catenin by inhibiting GSK3β using LiCl (Klein and Melton, 1996). LiCl treatment stabilized β-catenin and
Figure 12. Slc7a7 is regulated by canonical WNT signaling.

(A–C) Effect of rDKK1 treatment on WNT3a-induced β-catenin expression, alkaline phosphatase staining or gene expression in ST2 cells. (D–F) Effect of β-catenin knockdown (shCatnb1) on WNT3a-induced β-catenin expression and alkaline phosphatase staining (D), gene expression (E), or glutamine uptake (F). (G–I) Effect of LiCl treatment on protein expression, alkaline phosphatase staining (G), gene expression (H), or glutamine uptake (I). Error bars depict SD. β-catenin or γ(+)LAT1 normalized to α-tubulin and depicted as fold change ± SD for WNT3A over vehicle calculated from 3 independent experiments. 3 independent trials were performed in each experiment. * p≤ 0.05, ** p≤ 0.005, *** p≤ 0.005, **** p≤ 0.00005, one-way ANOVA (A,B,C,D,E,F) or by an unpaired 2-tailed Student’s t-test (G,H I).
induced both alkaline phosphatase staining and Tcf7 mRNA expression similar to WNT treatment (Fig. 12G,H). Moreover, LiCl stimulated both Slc7a7 mRNA and γ(+) Lat1 protein expression and increased glutamine uptake similar to WNT treatment (Fig. 12G-I). Importantly, LiCl did not increase Slc1a5 mRNA expression (Figure 12H). These data indicate β-catenin dependent WNT signaling rapidly stimulates glutamine uptake via transcriptional upregulation of Slc7a7/γ(+) Lat1.

2.3.4 Basal glutamine uptake and Slc1a5 expression is regulated by mTORC1 signaling

We next evaluated the regulation of Slc1a5 expression by WNT. Unlike Slc7a7 and other canonical WNT target genes which are rapidly induced by WNT, Slc1a5 mRNA levels were not significantly increased until 72 hours after WNT treatment (Fig. 10A and Table 1). Indeed, we observed no change in Slc1a5 expression in β-catenin knockdown cells or in response to LiCl treatment, suggesting Slc1a5 is not regulated by canonical WNT/β-catenin signaling (Fig. 12H and data not shown). Rather, the induction of Slc1a5 followed a time course reminiscent of protein anabolism genes induced by WNT through secondary activation of the transcription factor ATF4 (Karner et al., 2015). We previously determined WNT signals through mTORC1 to activate ATF4 and protein anabolism genes during osteoblast differentiation (Karner et al., 2015). Indeed, we observed a significant increase in ATF4 protein expression after 72 hours of WNT treatment (Fig. 13A). Inhibition of mTORC1 signaling using rapamycin prevented ATF4 protein induction by WNT (Fig. 13A). Importantly, rapamycin treatment reduced ASCT2 expression in both
unstimulated and WNT treated ST2 cells (Fig. 13A). Consistent with decreased ASCT2 expression, rapamycin significantly reduced glutamine uptake under both basal conditions and in response to WNT stimulation (Fig. 13B). Despite a significant reduction in overall glutamine uptake, WNT was able to stimulate glutamine consumption in the presence of rapamycin (Fig. 13B,C). This is likely due to normal induction of Slc7a7 as rapamycin treatment did not affect WNT induction of Slc7a7 despite completely abrogating Slc1a5 induction by WNT (Fig. 13D,E). These data indicate mTORC1 activity is required for both glutamine uptake and the normal expression of both ATF4 and ASCT2. We next sought to determine if ATF4 activity is required for Slc1a5 induction by WNT. Atf4 knockdown using shRNA reduced both ATF4 protein expression and Slc1a5 mRNA expression in ST2 cells (Fig. 13F,G). Moreover, Atf4 expression was necessary for Slc1a5 induction by WNT as Atf4 knockdown completely prevented the induction of Slc1a5 by WNT (Fig. 13F,G). Likewise, Atf4 knockdown significantly reduced basal glutamine uptake (Fig. 13G). Similar to the rapamycin treatment, Atf4 knockdown did not affect Slc7a7 induction by WNT or WNT induced glutamine uptake (Fig. 13G,H). Collectively, these data indicate ATF4 is critical for the majority of glutamine uptake by regulating Slc1a5 mRNA expression downstream of mTORC1.
Figure 13. Slc1a5 is regulated by non-canonical WNT signaling.

(A-D) Effect of rapamycin treatment on WNT3a-induced protein expression (A), glutamine uptake (B-C), or mRNA expression (D-E). pSer240/244 S6 normalized to total S6, ATF4 and ASCT2 normalized to β-Actin and depicted as fold change ± SD for WNT3A over vehicle calculated from 3 independent experiments. (F) Western blot analysis of ATF4 expression. (G-J) Effect of Atf4 knockdown on WNT induced Slc1a5 expression (G), glutamine uptake (H-I), or Slc7a7 expression (I). 3 independent trials were performed in each experiment. Error bars depict SD. * p≤ 0.05, ** p≤ 0.005, *** p≤ 0.0005, **** p≤ 0.00005, one-way ANOVA (A,B,G,H) or an unpaired 2-tailed Student’s t-test (C,D,E,F,I,J).
2.3.4 Glutamine uptake is required for osteoblast differentiation

We next sought to determine the role of these glutamine transporters during WNT induced osteoblast differentiation. Knockdown of Slc7a7 using shRNA reduced the induction of both early osteoblast marker genes (exemplified by Akp2) and terminal osteoblast marker genes (exemplified by Bglap) and prevented matrix mineralization in response to WNT (Fig. 14A-D). Interestingly, Slc1a5 knockdown did not affect early osteoblast marker gene induction (e.g. Akp2) but specifically reduced the induction of terminal osteoblast marker genes (e.g. Bglap) and prevented matrix mineralization in response to WNT (Fig. 14E-H). These data indicate both Slc7a7 and Slc1a5 are required for WNT induced osteoblast differentiation in ST2 cells.

2.4 Discussion

Increased matrix synthesis associated with bone formation increases the demand for amino acids. It stands to reason that osteoblasts must increase amino acid production or acquisition to meet this biosynthetic demand. Here, we describe the intricate regulation of glutamine acquisition by WNT signaling in an osteoblast progenitor cell line. We identified two amino acid transporters, Slc1a5 and Slc7a7, responsible for the majority of glutamine uptake in ST2 cells undergoing osteoblast differentiation in response to WNT (Fig. 15). Slc1a5 functions as the primary glutamine transporter in ST2 cells whereas Slc7a7 mediates WNT stimulated glutamine uptake associated with osteoblast differentiation. These transporters appear to work in concert to provide sufficient glutamine, and likely
Figure 14. *Slc7a7* and *Slc1a5* are both required for WNT induced osteoblast differentiation.

Effects of *Slc7a7* (A-D) or *Slc1a5* knockdown (E-H) on WNT induced gene expression (A-C, E-G), or alkaline phosphatase (ALP) and von Kossa staining (D, H). Fold change ± SD for 3 independent experiments. Error bars depict SD. * p≤ 0.05 (one-way ANOVA).
Figure 15. Schematics of biphasic regulation of glutamine consumptions during osteoblast differentiation.

WNT regulates Slc7a7 (encodes γ+LAT1) through canonical β-catenin-dependent pathway, responsible for rapid induction of glutamine uptake. 4F2hc encoded by Slc3a2 is required for the stability of γ+LAT1. WNT regulates Slc1a5 (encodes ASCTS) via mTORC1-ATF4 cascade, responsible for sustained glutamine uptake.
other amino acids, to initiate and sustain osteoblast differentiation and matrix production

\textit{in vitro}.

Osteoblast differentiation is characterized by rapid proliferation of osteoblast progenitors followed by differentiation into mature matrix producing osteoblasts. This process is associated with increased glutamine consumption and metabolism (Fig. 9) (Karner et al., 2015; Yu et al., 2019). Blocking glutamine consumption (Fig. 14) or metabolism prevents osteoblast differentiation and matrix formation (Brown et al., 2011; Karner et al., 2015; Yu et al., 2019). It is not clear why osteoblasts have such an acute requirement for glutamine to facilitate these processes. Osteoblast differentiation is associated with altered energetic and biosynthetic demands (Guntur et al., 2014; Karner and Long, 2017; Riddle and Clemens, 2017). For example, proliferating cells require nucleotides and must duplicate their cell mass in order to divide (Hosios et al., 2016). Similarly, differentiation and matrix production is associated with increased protein synthesis and secretion which may increase reactive oxygen species and oxidative stress detrimental to osteoblast differentiation (Almeida et al., 2007; Mody et al., 2001). Glutamine is a multifunctional amino acid uniquely suited to fulfill these anabolic demands. For example, glutamine nitrogen is important for the \textit{de novo} synthesis of both nucleotides and amino acids. Similarly, glutamine carbon is used for the synthesis of both amino acids and the antioxidant glutathione. Finally, glutamine derived $\alpha$KG can provide energy to fulfill energetic demands through entry in the TCA cycle (Newsholme et al.,
Thus, osteoblasts likely utilize glutamine disparately during differentiation to fulfill distinct metabolic purposes.

WNT is a potent regulator of osteoblast differentiation and bone formation (Cui et al., 2011; Hill et al., 2005). One of the earliest events associated with WNT induced osteoblast differentiation is increased glutamine consumption (Fig. 9 and (Karner et al., 2015)). It is interesting to note that glutamine uptake is not only rapidly increased, but also sustained throughout the differentiation process. Here we identified two glutamine transporters, Slc1a5 and Slc7a7, that mediate glutamine uptake in ST2 cells. Slc1a5 is critical for the majority of glutamine uptake (Fig. 11). Conversely, Slc7a7 is responsible for mediating the acute increase in glutamine uptake in response to WNT. Importantly, the transcription of these transporters is regulated disparately by WNT. First, Slc7a7 is upregulated within 6 hours whereas Slc1a5 is not increased until 72 hours after WNT stimulation. Mechanistically, β-catenin is both necessary and sufficient for the rapid induction of Slc7a7 and the acute increase in glutamine uptake in response to WNT. Conversely, β-catenin is dispensable for Slc1a5 expression and basal glutamine consumption in ST2 cells (Fig. 12 and not shown). Rather, Slc1a5 expression is regulated downstream of mTORC1 and ATF4 in ST2 cells (Fig. 13). We previously determined that WNT induces GCN2-dependent integrated stress response (ISR) through mTORC1 (Karner et al., 2015). We show here that the ISR transcriptional effector ATF4 is critical for both basal and WNT induced Slc1a5 expression and glutamine uptake. This is consistent
with recent data demonstrating that Slc1a5 is directly regulated by ATF4 (Han et al., 2013; Hu et al., 2020a).

Collectively these data support a biphasic model in which WNT signaling regulates glutamine consumption via canonical and noncanonical pathways to facilitate osteoblast differentiation. Interestingly, inhibiting Slc7a7, but not Slc1a5, inhibited Akp2 induction whereas inhibition of either transporter inhibited terminal osteoblast differentiation and matrix mineralization. The precise mechanism underlying this discrepancy is not clear, however it is important to note that in addition to glutamine, ASCT2 can transport alanine, serine and asparagine. Conversely, γ(+)-LAT1 mediates the influx of ornithine, arginine and lysine in exchange for the efflux of cationic amino acids (Chillaron et al., 1996; Pfeiffer et al., 1999; Torrents et al., 1999) which may be contributing to the disparate early osteoblast differentiation phenotypes observed. It will be important to elucidate the substrates of these amino acid transporters in osteoblast progenitors and determine the precise role they play during WNT induced osteoblast differentiation.
Chapter 3. *Slc38a2/SNAT2* provides proline critical for protein synthesis, bioenergetics and redox homeostasis in osteoblast

Chapter 3 is adapted from a manuscript in progress, which will be submitted in near future. The authors of this work include Leyao Shen (myself), Yilin Yu, Yunji Zhou, Dr. Elizabeth Rendina-Ruedy, Dr. Guofang Zhang and Dr. Courtney Karner. I performed most of the experiments with the assistance from Yilin Yu and wrote the manuscript. Yunji Zhou provided his biostatistics expertise. Dr. Rendina-Ruedy performed Seahorse analysis. Dr. Zhang worked on mass spectrometry. This study is designed, finished and written under the supervision of Dr. Karner.

**3.1 Introduction**

Osteoblasts are the chief bone forming cells. Osteoblast differentiation is involved in skeletal development, playing a critical and indispensable role in both endochondral and intramembranous ossification (Berendsen and Olsen, 2015). Particularly, intramembranous ossification depends solely on osteoblast differentiation, unlike endochondral ossification with an intermediate chondrocyte-involving step. During intramembranous ossification, mesenchymal progenitors condense and are committed to osteoblast progenitor cells, expressing master transcription factors like *Runx2* and *Sp7* (Ducy et al., 1997; Nakashima et al., 2002; Otto et al., 1997; Takarada et al., 2016). These osteoblast progenitors further differentiate into active osteoblasts that produce and secrete the Collagen Type I (COL1) enriched extracellular matrix (termed osteoid)
Osteoblasts then undergo terminal differentiation and are characterized by the expression of *Ibsp* and *Bglap* (Bianco et al., 1991; Ducy et al., 1996). At this stage, the osteoblast cluster forms the ossification center which secretes alkaline phosphatase and other proteins necessary for osteoid mineralization.

Protein synthesis and matrix secretion increase and are required for osteoblast differentiation (Elefteriou et al., 2006; Franceschi et al., 1994; Rached et al., 2010). This increased anabolism results in enhanced biosynthetic and energetic demands (Guntur et al., 2014; Karner and Long, 2018; Riddle and Clemens, 2017). One of the most important nutrients is glucose. Predominant amount of glucose is metabolized to lactate even in the presence of oxygen, which is known as aerobic glycolysis (Esen et al., 2013). This process provides around 80% ATP in mature osteoblasts (Lee et al., 2020). Additionally, oxidation of fatty acids also provides critical amount of ATP required for osteoblast differentiation (Kim et al., 2017). Besides glucose and fatty acid, osteoblasts must acquire amino acids via either de novo synthesis or uptake from extracellular environment to fulfill the elevated energetic and metabolic needs (Karner and Long, 2017). Amino acids serve many functions during osteoblast differentiation. First, amino acids are the building blocks of protein. Furthermore, amino acids can also be metabolized and contribute to energy production, redox regulation, biosynthesis of amino acids and nucleotides (Mody et al., 2001; Riddle and Clemens, 2017; Yu et al., 2019). The role of amino acids in osteoblast differentiation has recently been paid increasing attention. For example, studies have
shown ATF4-mediated amino acid transport and protein synthesis are required for osteoblast differentiation during bone formation (Elefteriou et al., 2006; Karner et al., 2015; Rached et al., 2010). Recently, glutamine metabolism has been demonstrated as a critical regulator of skeletal stem cell proliferation and specification, as well as osteoblast differentiation and bone formation (Karner et al., 2015; Yu et al., 2019). Directly targeting glutaminase, the rate-limiting enzyme of glutamine catabolism, in osteoprogenitors also affects postnatal bone mass (Stegen et al.). Deteriorated osteogenic potential of skeletal stem cells in aged mice partially results from gradual loss of glutaminase expression (Huang et al., 2017). However, limited studies have been devoted to understand the role of other amino acids in osteoblast differentiation and bone formation.

Multiple amino acids play critical roles in cancer cell survival and tumorigenesis. For example, proline, a multifunctional amino acid, has been widely studied in cancer and embryonic stem cell research. In addition to being incorporated into protein, proline can be oxidized into pyrroline-5-carboxylate (P5C) by the flavin dinucleotide (FAD) dependent enzyme proline dehydrogenase (PRODH). P5C can then be converted into glutamate by P5C dehydrogenase. Alternatively, P5C can also be reduced to proline by one of three enzymes of the P5C reductase family (PYCR1, PYCR2 and PYCRL) through NAD(P)H oxidation. Proline oxidation and P5C reduction forms the proline cycle, which has been implicated in energy and redox regulation in cancer cells. For example, PRODH-mediated proline oxidation donates electrons to electron transport chain (ETC) for ATP
generation (Elia et al., 2017; Liu et al., 2012a; Olivares et al., 2017; Phang et al., 2012).

Moreover, proline oxidation is a source of reactive oxygen species (ROS) that can have positive or negative effects depending on the cellular context. PRODH-mediated ROS generation promotes senescence or induces apoptosis in cancer cells (Liu et al., 2012a; Nagano et al., 2017; Phang et al., 2012). However, ROS generated from proline oxidation contributes to pathogen defense in plants and C. elegans (Cecchini et al., 2011; Tang and Pang, 2016). Conversely, PYCRs mediated P5C reduction contributes to proline biosynthesis and replenishes the NAD(P)+ pool for TCA cycle and pentose phosphate pathway (Hollinshead et al., 2018; Liu et al., 2012b; Phang, 2019b). Despite its well-known role in cancer cells, the role of proline and its subsequent metabolism in osteoblast differentiation and bone development remains unclear.

Here we identify proline as a critical nutrient in osteoblasts. Using a multifaceted approach, we demonstrate Slc38a2/SNAT2 acts cell autonomously to provide proline necessary for osteoblast differentiation and bone development. Mechanistically, we describe a bifunctional requirement for proline in osteoblasts. First proline is significantly enriched in osteoblast proteins including those that regulate osteoblast differentiation (e.g. RUNX2 and OSX) and bone matrix production (e.g. COL1A1). Second, proline oxidation in the proline cycle provides ATP and regulates osteoblast redox homeostasis. Collectively, these data highlight a previously unknown requirement for proline to fulfill
biosynthetic and energetic requirements associated with osteoblast differentiation and bone formation.

3.2 Results

3.2.1 Proline is enriched in osteoblast-associated proteins and consumed more by osteoblasts during differentiation.

Osteoblasts obtain amino acids actively during differentiation. However, it is unknown whether osteoblasts differentially demand certain amino acids than the others during differentiation. To investigate whether there is such an amino acid standing out, we first merged the transcriptomic data set of differentiated calvarial osteoblasts (cOBs) with the amino acid composition of corresponding proteins. In the transcriptomic data set, the expression of osteoblast differentiation markers including Runx2, Sp7, Col1a1 and Bglap are upregulated as expected, which is validated by qPCR (Fig. 16A,B). Interestingly, these critical osteoblast regulators unanimously have higher proline proportion compared to the average of all proteins (Fig. 16C). Conversely, glycine and glutamine are enriched in one or two proteins, whereas all of four proteins have lower proportion of glutamate and isoleucine compared to the average. To investigate this question more unbiasedly, we found multiple osteoblast differentiation associated proteins are among the ones that are proline-rich and significantly upregulated during differentiation (Fig. 16D). Conversely, most of these proteins are not enriched with other amino acids, for example, glycine, glutamine, glutamate and isoleucine (Fig. 16E-H). Furthermore, proteins associated with osteoblasts (GO: 0001649) have significantly higher proline proportion (7.2%) when
Figure 16. Proline is enriched in osteoblast differentiation associated proteins.

(A) Alkaline phosphatase, alizarin red and von Kossa staining in cOBs treated with osteogenic medium (OM) for 10 days. (B) qRT-PCR analysis of osteogenic marker genes Runx2, Osx, Col1a1 and Bglap in cOBs treated with osteogenic medium for 7 days. (C) Graphical representation of the relative amino acid proportion for select amino acids in the indicated osteogenic marker genes. (D-H) Scatterplots of amino acid proportions and fold induction of all genes in response to osteogenic medium. Genes marked as red are associated with osteoblast differentiation.
compared to all other proteins (7.2% vs 5.9% proline for osteoblasts vs all proteins). Moreover, this increase in proline enrichment was specific to osteoblasts as we did not observe similar enrichment in other differentiated cell types including cardiomyocyte (GO:0001649), muscle (GO:0055007) or neuron (GO:0030182) (Fig. 17A). Consistent with this, specific osteoblast proteins were found to be enriched for proline. Additionally, proline is more enriched in proteins encoded by induced genes during osteoblast differentiation compared with the average of all genes and with those that are suppressed (Fig. 17B,C). To test whether osteoblasts have increased demand for proline, we next established a model in which the predicted osteoblast demand for certain amino acids is proportional to the transcriptional changes. Our model predicts that osteoblasts have increased demand for proline for protein synthesis during differentiation (Fig. 17D). This suggests that osteoblasts may have a particular need of obtaining proline during differentiation. Consistent with this inference, we also observed that about proline consumption is increased by 20% during osteoblast differentiation (Fig. 1E). Collectively, our data predict that proline is an important nutrient in osteoblasts and may play a critical role in osteoblast differentiation.
Figure 17. Proline is predicted to be more demanded during osteoblast differentiation.

(A) Proline proportions of osteoblast (GO: 0001649), cardiomyocyte (GO: 0055007), muscle cell (GO: 0042692) and neuron (GO: 0030182) GO term associated proteins. (B,C) Amino acid proportions of top 500 induced and suppressed, and all genes in response to osteogenic medium. (D) Predicted relative amino acid demand change in cOBs treated with osteogenic medium. (E) Proline uptake in cOBs after 7 days of differentiation. Error bar depicts SD. *p≤0.05.
3.2.2 *Slc38a2/SNAT2* is the highest expressed proline transporter expressed in osteoblast lineage cells.

Since both proline demand and consumption increase during osteoblast differentiation, next we investigated how osteoblasts acquire proline from the extracellular milieu. Proline transport is facilitated by a number of membrane-tethered proline transporters, characterized by its sodium dependency, pH and MEAIB sensitivity. We profiled our transcriptomic data and identified *Slc38a2/SNAT2* as the highest expressed proline transporter (Fig. 18A). To determine whether *Slc38a2* is expressed in bone tissue, we acquired a mouse model with LacZ knock-in (*Slc38a2*LacZ) (Fig. 18B). β-galactosidase staining demonstrates that *Slc38a2* was expressed in osteoblasts lining around trabecular bones, chondrocytes in cartilage and osteocytes embedded in bone matrix in both calvaria and limbs of newborn mice (Fig. 18C-D, C’-D’). The specific expression of *Slc38a2* in osteochondral lineage cells suggest that *Slc38a2* may play an essential role in osteoblast differentiation during bone development.

We next sought to determine whether SNAT2 transports proline. To test this, we first utilized the CRISPR/Cas9 system and designed 5 short guide RNAs (sgRNA), which were delivered via lentivirus into *Rosa*<sup>Cas9/Cas9</sup> cOBs (Fig. 19A). Deletion of *Slc38a2* is confirmed by Western blot (Fig. 19B). We performed radiolabeled proline uptake assay and found proline uptake was reduced by more than 40% in cOBs lacking *Slc38a2* (Fig. 19C). These results suggest that SNAT2 is responsible for majority of proline uptake in osteoblasts.
Figure 18. Slc38a2 is expressed in osteoblast lineage cells.

(A) Relative expression of proline transporters in cOBs. (B) Schematics of mouse model with Slc38a2<sup>LacZ</sup> allele. β-galactosidase staining of limb sections (C,C’) and skull sections (D,D’) of Slc38a2<sup>LacZ</sup> (C,D) and Slc38a2<sup>+/+</sup> (C’,D’) newborn mice (magnification: 10X). The insets are the 40X magnified images of C and D.
Figure 19. Slc38a2 transports proline and is required for osteoblast differentiation in vitro.

(A) Schematics of short guide RNAs that target exon 8-10 of Slc38a2 (B) Western blot of SNAT2 protein expression in Rosa26Cas9/Cas9 cOBs infected with sgRNA targeting Slc38a2 or Luciferase as a control. Effect of Slc38a2 knockout on (C) proline uptake (D) alkaline phosphatase and von Kossa staining treated with OM for 10 days and (E) mRNA expression of osteogenic genes treated for 7 days. Error bar depicts SD. *p≤0.05, ***p≤0.0005.
Next, we sought to determine whether $Slc38a2$ is required for osteoblast differentiation. Osteoblasts lacking $Slc38a2$ had normal alkaline phosphatase activity while reduced mineralization (Fig. 19D). Additionally, these cells also had reduced expression of terminal osteogenic markers including $Ibsp$ and $Bglap$ (Fig. 19E) This indicates that $Slc38a2$ is dispensable for early differentiation whereas required for late-stage or terminal osteoblast differentiation. To investigate its functional role in vivo, we generated $Slc38a2^{LacZ/LacZ}$ knockout mice, which died at birth with skeletal defects. These mice had smaller body size, suggesting there was an overall growth defects. These mice also had wavy rib phenotype, which reminiscent of patients with osteogenesis imperfecta. $Slc38a2^{LacZ/LacZ}$ newborn mice also had shorter limbs and wider unmineralized regions in the skull (Fig. 20E,E',F,F'). This phenotype was evident as early as E14.5, the onset of mineralization (Fig. 20A,A',B,B'). All of the mutants had no mineralization in their limbs (Fig. 20A',B'). This phenotype persisted until E15.5 (Fig. 20C,C',D,D'). At this timepoint, $Slc38a2^{LacZ/LacZ}$ had reduced mineralization in their limbs as well as their skull (Fig. 20E',F',G,G'). The undermineralized phenotype in the limb was confirmed by von Kossa staining (Fig. 20J,J') whereas alkaline phosphatase was normal (Fig. 20 K,K'), recapitulating in vitro phenotype. This suggests that $Slc38a2$ does not regulate early osteoblast differentiation. In addition, the limbs of $Slc38a2^{LacZ/LacZ}$ were characterized by lower expression of $COL1A1$ and OSX proteins though $Col1a1$ mRNA expression is normal (Fig. 20L-N;L'-N'). Collectively, the undermineralized phenotype of $Slc38a2^{LacZ/LacZ}$
Figure 20. \textit{Slc38a2}^{LacZ/LacZ} mice have bone developmental defects.

(A-F, A’-F’) Skeletal preparations of \textit{Slc38a2}^{LacZ/LacZ} or \textit{Slc38a2}^{+/+} littermate controls at E14.5, E15.5 and P1 (n=5). The values in (A’-B’) are the number of animals with apparent mineralization at E14.5. The values in (E’-F’) represent the quantification of the unmineralized calvarial area. n=5. (G,G’) Humeri dissected from skeletal preparations at E15.5. Quantification of the limb length (H) and mineralized region (I). von Kossa staining[J,J’], alkaline phosphatase staining (K,K’), ISH for \textit{Col1}, \textit{Col2}, \textit{Col10} and \textit{Mmp13}(L’,L’,O’-Q,O’-Q’), and IF for \textit{COL1} and OSX (M,M’,N,N’) of E15.5 femur sections of \textit{Slc38a2}^{LacZ/LacZ} and the littermate controls. Error bar depicts SD. *p≤0.05, ***p≤0.0005.
suggests that Slc38a2 is required for osteoblast differentiation. The shortened limb length also led us to hypothesize that Slc38a2\textsuperscript{LacZ/LacZ} had defects in chondrocyte differentiation (Fig. 20H,I). To test this hypothesis, we performed ISH to examine the expression of chondrocyte markers. The expression of Col2a1 was less separated in the knockout animals (Fig. 20O,O'). We also found chondrocyte hypertrophy marked by Col10a1 mRNA expression is delayed in at E15.5 (Fig. 20P,P'). In addition, Slc38a2\textsuperscript{LacZ/LacZ} animals had no expression of terminal chondrocyte marker Mmp13 (Fig. 20Q,Q'). Collectively, these phenotypes suggest that Slc38a2 is required for chondrocyte hypertrophy during endochondral ossification.

3.2.3 Slc38a2/SNAT2 is required for endochondral ossification.

To test whether Slc38a2 acts cell autonomously, we crossed the Slc38a2 floxed allele with Prx1Cre which targets mesenchymal progenitors of the limb bud (Prx1Cre;Slc38a2\textsuperscript{fl/fl}). Prx1Cre;Slc38a2\textsuperscript{fl/fl} animals phenocopied the limb phenotype of Slc38a2\textsuperscript{LacZ/LacZ} in endochondral ossification. At E15.5, Prx1Cre;Slc38a2\textsuperscript{fl/fl} animals had shorter limbs and reduced mineralized region, which was confirmed by von Kossa staining on the sections (Fig. 21A-E,A',B',E'). The expression of hypertrophy marker Col10a1 mRNA expression was delayed in at E15.5 (Fig. 21G,G'). In addition, the terminal chondrocyte marker Mmp13 was also not present in Prx1Cre;Slc38a2\textsuperscript{fl/fl} animals (Fig. 21H,H'). These phenotypes suggest that Slc38a2 acts in osteochondral lineage cells and plays an indispensable role in chondrocyte hypertrophy during endochondral ossification. In addition, protein
Figure 21. Slc38a2 is required for chondrocyte hypertrophy and osteoblast differentiation during endochondral ossification.

(A,A’,B,B’) Skeletal preparations of Prx1Cre;Slc38a2fl/fl or Slc38a2fl/fl littermate controls at E15.5 (n=5). (C,D) Quantifications of limb length and mineralized region (n=5). Von Kossa staining (E,E’), ISH for Col1, Col2, Col10 and Mmp13(F-H,I,K,F-H’,I’K’), and IF for COL1 and OSX (J,J’,L,L’) of E15.5 humerus sections Prx1Cre;Slc38a2fl/fl or Slc38a2fl/fl littermate controls at E15.5. (M,N) Quantification of OSX+ cell number and COL1A1 intensity (n=4). Error bar depicts SD. *p≤0.05, **p≤0.005
expression of OSX and COL1A1 were reduced in Prx1Cre;Slc38a2^{fl/fl} animals (J,J’,L-N,L’). Interestingly, similar to Slc38a2^lacZ/lacZ, Prx1Cre;Slc38a2^{fl/fl} animals had normal mRNA expression of Sp7 and Col1a1. This suggests that Slc38a2 may regulate COL1A1 and OSX at translational but not transcriptional level. Collectively, Slc38a2 is required for both chondrocyte and osteoblast differentiation during endochondral ossification.

3.2.4 Slc38a2/SNAT2 acts cell autonomously in osteoblast lineage cells.

Next we sought to determine whether Slc38a2 acts in osteoblasts in a cell autonomous manner. We conditionally ablated Slc38a2 in specified preosteoblasts using Sp7tTA;tetO-EGFP/Cre (Sp7Cre; Slc38a2^{fl/fl}). Western blotting analysis confirmed Slc38a2 was knocked out in bones (Fig. 22A). To confirm whether proline is a substrate for Slc38a2/SNAT2, we isolated non-cartilaginous parts of the limbs from Sp7Cre;Slc38a2^{fl/fl} animals at birth and performed ex vivo amino acid uptake assay (Fig. 22B). Interestingly, uptake of proline but not the other known SNAT2 substrates including glutamine and alanine was reduced in the limbs of Sp7Cre;Slc38a2^{fl/fl} (Fig. 22B). Therefore, our data suggest that SNAT2 is a predominant proline transporter in osteoblasts.

To investigate the functional role of Slc38a2 specifically in osteoblast differentiation in vivo, we decided to use the skull as the major model as Sp7Cre also targets a subset of hypertrophic chondrocytes in the limbs. Sp7Cre is specifically
Figure 22. *Slc38a2* acts in osteoblast lineage cells required for their differentiation.

(A) Western blot of SNAT2 protein expression and (B) amino acid uptake assays in the isolated bones of *Sp7Cre;Slc38a2*^fl/fl^ or *Sp7Cre;Slc38a2*^fl/+^ littermate controls. (C-F, C’-F’) Skeletal preparations of *Sp7Cre;Slc38a2*^fl/fl^ or *Sp7Cre;Slc38a2*^fl/+^ littermate controls at E15.5 and P1 (n=5). von Kossa staining (G,G’), alkaline phosphatase staining (H,H’) and ISH for *Spp1*, *Ibsp* and *Bglap* (I-I’,I’-K’) of skull section of *Sp7Cre;Slc38a2*^fl/fl^ or *Sp7Cre;Slc38a2*^fl/+^ littermate controls at P1. (M,N). (n=5). Error bar depicts SD. ***p≤0.0005
Figure 23. Slc38a2 acts cell autonomously critical for osteoblast differentiation in endochondral ossification.

(A) Skeletal preparations of the humeri isolated from of Sp7Cre;Slc38a2fl/fl or Sp7Cre;Slc38a2fl/+ littermate controls at E15.5 (n=4). Blue dashed line for the overall length and the red line for the alizarin red region in the control. (B) Quantification of the mineralized (region red length/blue length). ISH for Spp1, Ibsp, Sp7 and Col1a1 (C-E,C'-E',G,G'), and IF for OSX and COL1A1 (F,F',H,H') on humerus section of Sp7Cre;Slc38a2fl/fl or Sp7Cre;Slc38a2fl/+ littermate controls at E15.5. Error bar depicts SD. (n=4). *p≤0.05
expressed in the osteoblasts lining around calvarial bones and suture. *Sp7Cre; Slc38a2^fl/fl* have less mineralization in the skull at E15.5 and P1, phenocopying *Slc38a2^LacZ/LacZ* mutants (Fig. 22C-F,C'-F',I). Similar to in vitro and *Slc38a2^LacZ/LacZ* phenotype, *Sp7Cre; Slc38a2^fl/fl* mice had less mineralization shown by von Kossa staining while had normal alkaline phosphatase activity (Fig. 22G,H,G',H'). To determine whether osteoblast differentiation was affected by the loss of *Slc38a2*, we performed ISH of osteogenic markers on skull sections of newborn *Sp7Cre; Slc38a2^fl/fl* and the littermate controls. We found the expression of late osteoblast differentiation markers including *Spp1, Ibsp* and *Bglap* was reduced in *Sp7Cre; Slc38a2^fl/fl* animals (Fig. 22I-K,I'-K'). Of note, the phenotype was also observed in the limb. The limbs of *Sp7Cre; Slc38a2^fl/fl* animals were less mineralized and had less expression of osteogenic marker genes like *Spp1* and *Ibsp* and osteoblast proteins like COL1A1 and OSX. (Fig. 23). The limb phenotype of *Sp7Cre; Slc38a2^fl/fl* animals also phenocopied *Prx1Cre; Slc38a2^fl/fl* and *Slc38a2^LacZ/LacZ* animals. Collectively, these data suggest that *Slc38a2*, acting cell autonomously, is required for terminal osteoblast differentiation during bone development.

3.2.5 *Slc38a2* is required for skeletal stem cell proliferation and osteoblast differentiation.

To test whether *Slc38a2* plays a role in the maintenance of skeletal stem cell, we used *Prx1Cre; Slc38a2^fl/fl* as Prx1Cre also targets skeletal stem cells postnatally. *Prx1Cre; Slc38a2^fl/fl* animals had reduced bone volume in 3-month-old females and 4-month-old males (Fig. 24A,A’, Table 2,3). This might result from less expression of
terminal osteogenic marker OCN, suggesting Prx1Cre;Slc38a2Δ/Δ animals had fewer mature osteoblasts (Fig. 24B,B'). Dynamic histomorphometry also demonstrated that Prx1Cre;Slc38a2Δ/Δ animals had reduced mineral apposition rate and bone formation rate despite normal mineral surface area. This suggests that knocking out Slc38a2 leads to reduced osteoblast activity (Fig. 24C-E,C'). To investigate whether Slc38a2 is required for SSC proliferation, we isolated and cultured the SSCs from bone marrow at clonal density. SSCs with deletion of Slc38a2 had less fibroblast colony-forming units (CFU-F) (Fig. 25A,B). Three factors may contribute to this phenotype: fewer progenitor cells, less proliferation, and decreased cell viability. To test whether Slc38a2 regulates progenitor population, we performed flow cytometry and found PDGFα+Sca1+ population was not affected in the cells harvested from Prx1Cre;Slc38a2Δ/Δ (Fig. 25C). This suggests that Slc38a2 is dispensable for maintaining skeletal stem cell progenitor population. Using EdU incorporation assay, we found that SSCs lacking Slc38a2 were less proliferative compared to the control (Fig. 25D,E). In addition, SSC apoptosis (Annexin V+) increased in female Prx1Cre;Slc38a2Δ/Δ mice whereas it was not affected in males (Fig. 25F,G). Additionally, SSCs isolated from Prx1Cre;Slc38a2Δ/Δ mice had less osteoblast specification and differentiation, represented by CFU-AP and CFU-OB respectively (Fig. 26A-C). High-density culture of SSCs also showed less expression of osteogenic markers and mineralization in SSCs from Prx1Cre;Slc38a2Δ/Δ animals (Fig. 26D,E). This suggests that Slc38a2 is required for osteoblast differentiation in SSCs, which is consistent with our data.
pertinent with calvarial osteoblasts. In summary, Slc38a2 is required for skeletal stem cell proliferation, differentiation, critical for bone formation.

Figure 24. Slc38a2 in mesenchymal progenitors is required for postnatal bone acquisition.

(A, A') MicroCT images of distal femur trabecular bone of 4-month-old Prx1Cre;Slc38a2<sup>−/−</sup> or Slc38a2<sup>+/−</sup> controls (n=9). IF for OCN (B,B') and calcein/alizarin red double labeling (C-C') of femur sections of 4-month-old Prx1Cre;Slc38a2<sup>−/−</sup> or Slc38a2<sup>+/−</sup> littermate controls (n=4). Error bar depicts SD. *p≤0.05, ****p≤0.0005
Table 2. Bone parameters for 4-month-old male \textit{Prx1Cre;Slc38a2}^{0/0} and \textit{Slc38a2}^{0/0} controls.

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<td>13.93±3.7</td>
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Table 3. Bone parameters for 3-month-old female \textit{Prx1Cre;Slc38a2}^{0/0} and \textit{Slc38a2}^{0/0} controls.

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<td>Conn.D</td>
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<td>55.75 ± 17.30 **</td>
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<td>4.277 ± 0.1099</td>
<td>3.441 ± 0.1414 **</td>
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<td>Tb. Sp</td>
<td>0.2360 ± 0.007260</td>
<td>0.2944 ± 0.01282 *</td>
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Figure 25. *Slc38a2* is required for colony forming potential due to less proliferation in skeletal stem cells.

Images (A) and quantification (B) of colony-forming unit (CFU) assays stained with crystal violet, isolated and cultured from 4-month-old male *Prx1Cre;Slc38a2fl/fl* or *Slc38a2fl/fl* controls (n=9). A colony has ≥ 20 cells. (C) Percentage of PDGFα+, Sca-1+ skeletal stem cells from bone marrow. Quantification of EdU incorporation (D,E) and Annexin V (F,G) using flow cytometry in SSCs cultured at clonal density for 7 days, isolated from of 2-month-old *Prx1Cre;Slc38a2fl/fl* or *Slc38a2fl/fl* littermate controls (n=5). Error bar depicts SD. *p≤0.05, **p≤0.005, ***p≤0.0005
Figure 26. *Slc38a2* is required for osteogenic specification and differentiation of SSCs.

Images (A) and quantification (B,C) of colony-forming unit (CFU) assays stained with alkaline phosphatase or von Kossa, isolated and cultured from 4-month-old male *Prx1Cre;Slc38a2*+/− or *Slc38a2*+/− littermate controls (n=9). A colony has ≥ 20 cells. (D) Alizarin red staining of SSCs treated with osteogenic medium for 10 days. (E) mRNA expression of osteogenic genes treated for 7 days. *p≤0.05,**p≤0.005,****p≤0.00005
3.2.6 *Slc38a2/SNAT2* is required for postnatal bone acquisition.

To determine if *Slc38a2* is similarly required for postnatal bone acquisition, we conditionally deleted *Slc38a2* using BglapCre, which targets mature osteoblasts and starts being active from E18.5. *BglapCre;Slc38a2*\textsuperscript{fl/fl} male mice had reduced bone volume at 4-month-old, while females did not have such phenotype (Fig. 27A-B,A', Table 4). Dynamic histomorphometry demonstrates that mineral surface was slightly reduced while mineral apposition rate and bone formation rate were affected to a greater extent in male *BglapCre; Slc38a2*\textsuperscript{fl/fl} mice (Fig. 27C-F,C'). This indicates that osteoblast number might be slightly affected whereas osteoblast activity was more impaired in *BglapCre; Slc38a2*\textsuperscript{fl/fl} mice. Consistently, *BglapCre; Slc38a2*\textsuperscript{fl/fl} also had less OCN+ osteoblasts, suggesting that osteoblast maturation was affected in *BglapCre; Slc38a2*\textsuperscript{fl/fl} (Fig. 27G-H,G'). In addition, tartrate-resistant acid phosphatase positive (TRAP+) osteoclast number was increased, indicating that bone resorption might be elevated (Fig. 27I-J,J'). Collectively, *Slc38a2* plays an indispensable role in mature osteoblasts, critical for postnatal bone formation by regulating osteoblast maturation and activity in males but not in females.
Figure 27. *Slc38a2* is required for postnatal bone acquisition.

(A,A',B) MicroCT images and quantification of distal femur trabecular bone of 4-month-old *BglapCre;Slc38a2*^{fl/fl} or *Slc38a2*^{fl/fl} littermate controls (n=6). Calcein/alizarin red double labeling (C-F,C'), IF for OCN (G,G',H) and TRAP staining (I,I',J) of femur sections of 4-month-old *BglapCre;Slc38a2*^{fl/fl} or *Slc38a2*^{fl/fl} littermate controls (n=3). Error bar depicts SD. *p≤0.05, ***p≤0.0005
Table 4. Bone parameters of 4-month-old male $BglapCre;Slc38a2^{fl/fl}$ and control.

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Table 5. Bone parameters of 4-month-old female $BglapCre;Slc38a2^{fl/fl}$ and control.

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3.2.7 The major fate of proline is protein synthesis in osteoblasts

Next we sought to determine whether proline, transported by Slc38a2, can directly regulate protein synthesis in osteoblasts. We performed S\textsuperscript{35}-cysteine-methionine to measure protein synthesis and H\textsuperscript{3}-proline incorporation assays to measure collagen synthesis in Slc38a2 knockout cOBs using CRISPR/Cas9. Our results showed that both proline and collagen synthesis were reduced in osteoblasts lacking Slc38a2 (Fig. 28A,B). Specifically, Western blotting also demonstrated that both COL1A1 and RUNX2 were reduced in Slc38a2 knockout cells (Fig. 28C). This phenotype is independent of mTOR signaling and integrated stress response as phosphorylation of ribosomal protein S6 and EIF2a remained unchanged (Fig. 28D). We then set to investigate the mechanism on how protein synthesis is affected in osteoblasts lacking Slc38a2. Charging of tRNA, or tRNA aminoacylation, is an indication of the abundance of specific amino acids in translation during protein synthesis. Higher uncharged level of tRNA is consistent with the shortage of certain amino acids in translation. Our tRNA charging analysis indicates that tRNA-Proline, AGG, was the only one that has lower charged level in osteoblasts lacking Slc38a2 (Fig. 28E). A similar result was observed in the osteoblasts cultured with proline free medium for 48h, in which only tRNA-Proline AGG was affected (Fig. 28F). This suggests that Slc38a2 knockout osteoblasts undergo shortage of proline during protein synthesis. Interestingly, the other tRNA-Proline CGG was not affected. This suggests the codon bias
may exist during translation. Collectively, limiting proline uptake by knocking out Slc38a2 reduces the synthesis of proline enriched proteins.

In multiple cancer studies, proline is an important source for glutamate. To test whether proline is metabolized into glutamate, we performed stable isotopomer analysis using $^{13}$C-proline in cOBs (Fig. 29A). The analysis demonstrated that a negligible

![Graph A]

**Figure 28.** Slc38a2 is required for protein and collagen synthesis.

S$^{35}$ cysteine/methionine (A), H$^3$ proline incorporation assay (B), Western blot analysis (C) and tRNA aminoacylation assay in Rosa26Cas9Cas9 cOBs infected with sgSlc38a2 or sgLuciferase as a control. (D) tRNA aminoacylation assay in cOBs treated with proline free or complete medium for 48h (n=3). Error bar depicts SD. $^*p \leq 0.05$
amount of glutamate or other amino acids were derived from $^{13}$C-u-proline, suggesting proline is not a primary source for amino acid biosynthesis in osteoblasts (Fig. 29B). By comparison, almost 35% of glutamate was derived from $^{13}$C-u-glutamine in 24 hours, suggesting our isotopomer analysis was reliable in our study (Fig. 29D). To quantitatively measure how much proline contributes to biosynthesis of amino acids in proteins specifically, we extracted, purified and hydrolyzed proteins from cOBs cultured with $^{13}$C-u-proline. Samples were then sent to mass spectrometry for measurement. Almost 70% of proline in proteins was $^{13}$C labelled within 72 hours (Fig. 29D). However, a negligible amount of other amino acids including glutamate and aspartate were derived from $^{13}$C-u-proline (Fig. 29C). Conversely, $^{13}$C-u-glutamine had limited contribution to proline in the proteins, which was significantly lower than its contributions to glutamate and aspartate (Fig. 29E). Collectively, our data indicate that proline, which cannot be metabolized into other amino acids, is directly incorporated into proteins.

We next sought to determine whether Slc38a2 is required for the synthesis of proline-rich osteoblast differentiation associated proteins in vivo. Proline rich osteoblast regulators RUNX2, COL1A1 and OSX were all significantly reduced in Sp7Cre; Slc38a2$^{fl/fl}$ mice at birth (Fig. 30A-G,A’-G’). Col1a1 mRNA level was not affected, neither was the Sp7-GFP expression (used as a proxy for Sp7) (Fig. 30B,B’,E,E’). This indicates that Slc38a2 may regulate COL1a1 and OSX at the translational but not at the transcriptional level.
Figure 29. Proline does not contribute to glutamate synthesis in osteoblasts.

(A) Schematics of stable isotopomer analysis using $^{13}$C-Proline. $^{13}$C$_{\omega}$-Proline tracing (B) and $^{13}$C$_{\omega}$-glutamine (C) tracing in osteoblasts. $^{13}$C$_{\omega}$-Proline tracing (D) and $^{13}$C$_{\omega}$-glutamine tracing (E) in proteins extracted from osteoblasts over a time course until 72h. (n=3). Error bar depicts SD.
Of note, GFP is not a proline-rich protein (%proline: 4.2%) (Fig. 30B,B’). In addition, we also looked at another non-proline protein actin (%proline: 5.2%), which was stained by phalloidin. Actin expression (as determined by the phalloidin-stained area per Sp7:GFP area) was not affected in Sp7Cre; Slc38a2fl/fl mice (Fig. 30H,H’). Collectively, these data indicate Slc38a2 provides proline indispensable for osteoblast differentiation and bone formation via direct regulation of the synthesis of proline-rich proteins.

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**Figure 30. Slc38a2 is required for biosynthesis of proline-rich protein in vivo.**

Images and quantifications of IF for RUNX2 (A,A’), OSX (B,B’), COL1A1 (F,F’) merged with endogenous Sp7-GFP (C,C’,D,D’,G,G’), ISH for Col1a1 and Phalloidin staining on skull section of Sp7Cre;Slc38a2fl/fl or Sp7Cre;Slc38a2fl/+ littermate controls at P1. Cell number quantification of RUNX2, OSX and Sp7-GFP ($10^3$ cells/mm²). Intensity measurement of COL1A1 and Phalloidin. (n=5). *p≤0.05
3.2.8 *Slc38a2* and proline metabolism are required for bioenergetics and RUNX2 stability.

In many cancers, proline is metabolized in the proline cycle, which plays a critical role in bioenergetics and redox homeostasis (Fig. 31A). It is known that proline oxidation donates electrons to electron transport chain (ETC) for ATP production (Phang, 2019b). To test whether proline plays a role in ATP production, we first performed Seahorse analysis in osteoblasts with addition of proline to the medium. In the absence of proline, over 80% of ATP was produced via glycolysis in osteoblasts (Fig. 31A). This is consistent with previous findings that osteoblasts are highly glycolytic (Lee et al., 2020). With the addition of proline, oxidative phosphorylation (OXPHOS) derived ATP production as well as total ATP production was increased (Fig. 31A). Conversely, ATP produced through glycolysis was not affected (Fig. 31A). This result was confirmed by direct measurement of intracellular ATP (Fig. 31B). Collectively, our data suggest that proline can be used for energy production through OXPHOS. Consistently, the intracellular ATP level was reduced in osteoblasts lacking *Slc38a2*. Increased phosphorylation of AMP-activated protein kinase (pAMPK) was also observed in cells with proline-free condition or *Slc38a2* knockout cells. (Fig. 31D,E,F). These data suggest that removal of proline or blocking proline uptake leads osteoblasts to energy deficiency. To determine whether proline regulates bioenergetics through the proline cycle, we used a small molecule inhibitor, tetrahydro-2-furoic acid (THFA), to specifically target PRODH, which is the enzyme that converts proline to P5C (Fig. 31A). Similarly, increased pAMPK and reduced
Figure 31. Proline metabolism regulates bioenergetics in osteoblasts.

(A) Schematics of Proline cycle. (B) Seahorse analysis of BMSC induced by osteogenic medium for 2 days with or without addition of 0.3mM proline (n=3). (C) Measurement of ATP concentration in cOBs with or without addition of 0.3mM proline (n=3). (D) Western blot of cOBs treated with complete medium, proline free medium or 5mM THFA treatment (n=6). Western blot (E), ATP concentration (F) or CM-H2DCFDA flow cytometry (K,L) of Rosa26Cas9/Cas9 cOBs infected with sgSlc38a2 or sgLuciferase as a control (n=6). Measurement of ATP concentration (G), glucose uptake (H) or CM-H2DCFDA flow cytometry (I,J) in cOBs treated with 5mM HCl or THFA (n=6). (M) Schematics of Sp7-GFP gating strategy. (N) Western blot of Sp7-GFP+ cells isolated from P1 Sp7Cre;Slc38a2fl/fl or Sp7Cre;Slc38a2fl/+ controls. Error bar depicts SD. *p≤0.05, **p≤0.005
ATP level were found in osteoblasts with acute treatment of THFA (Fig. 31D,G). We also observed THFA treatment increased glucose uptake, a sign of cellular energy deficit (Fig. 31H). Reactive oxygen species level was also reduced in both THFA-treated cells and cells lacking Slc38a2, indicating the electron transport chain is less active or impaired in these cells (Fig. 31I-L). These data suggest that disruption of proline cycle reduces ATP production and triggers energy deficit in osteoblasts. To confirm this mechanism in vivo, we performed cell sorting of Sp7-GFP+ osteoblasts isolated from the skeleton of newborn Sp7Cre;Slc38a2fl/fl or Sp7Cre;Slc38a2fl/+ littermate controls (Fig. 31M). Phosphorylation of AMPK was increased in the Sp7-GFP+ osteoblasts from knockout mice (Fig. 31N). This suggests that osteoblasts lacking Slc38a2 in vivo were also under energy shortage.

Furthermore, PRODH inhibition by THFA significantly reduced the expression of osteogenic markers including Akp2, Ibsp and Bglap, as well as matrix mineralization indicated by Alizarin Red and von Kossa staining (Fig. 32A,B). Interestingly, THFA treatment does not affect alkaline phosphatase activity, similar with the Slc38a2 knockout cell and animal phenotype. This suggests that proline metabolism may not regulate early osteoblast differentiation. Additionally, removal of proline or inhibition of proline oxidation by THFA reduced colony forming capacity of skeletal stem cells (Fig. 32). This phenotype corresponds with the reduced colony forming units in SSCs isolated from Prx1Cre;Slc38a2fl/+ animals (Fig. 25). These results also indicate that proline transported by Slc38a2 may also regulate SSC viability and proliferation.
Figure 32. Proline oxidation is required for osteoblast differentiation and SSC proliferation.

(A) Alkaline phosphatase or alizarin red staining in cOBs treated with 5mM HCl or THFA cultured OM for 10 days. (B) mRNA expression of osteogenic genes in cOBs treated with 5mM HCl or THFA cultured OM for 7 days. (C,D) Images and quantification of colony-forming unit (CFU) assays stained with crystal violet in SSCs with or without 0.3mM proline. A colony has ≥ 20 cells. (E,F) Images and quantification of colony-forming unit (CFU) assays stained with crystal violet in treated with 5mM HCl or THFA. with Error bar depicts SD. *p≤0.05, **p≤0.005.
A recent study shows that activation of AMPK destabilizes RUNX2, the master transcription factor of osteoblast differentiation, through ubiquitination by ubiquitin ligase SMURF1, in osteoblasts (Wei et al., 2015). Therefore, we hypothesized that RUNX2 may be under dual regulation by both the availability of proline for protein synthesis and bioenergetics to regulate AMPK activity. RUNX2 protein level was reduced in Slc38a2 knockout cells (Fig. 28C), which was also confirmed in vivo in the sorted cells from Sp7Cre;Slc38a2fl/fl (Fig. 31N). However, it is difficult to distinguish whether reduced RUNX2 protein is due to destabilization via AMPK phosphorylation or protein synthesis in Slc38a2 knockout cells since RUNX2 is proline rich. To tackle this obstacle, we performed protein stability assay using cycloheximide to inhibit protein synthesis. Half-life of RUNX2 protein was significantly reduced in Slc38a2 knockout osteoblasts (Fig. 33A-C). This indicates that Slc38a2 is required for RUNX2 stabilization. Furthermore, RUNX2 protein degradation rate was significantly higher in the cells treated with THFA for 48h (Fig. 33D-E). PRODH inhibition by THFA also significantly reduced half-life of RUNX2 (Fig. 33F). These data suggest that both proline availability and proline oxidation are important to stability of RUNX2. Collectively, proline, transported by Slc38a2, regulates RUNX2 stability likely through the proline cycle-AMPK axis.
Figure 33. Proline metabolism regulates AMPK phosphorylation which modulates RUNX2 stability

Representative image (A) and quantification (B) of Western blot, and half-life (C) of RUNX2 protein in Rosa26Cas9/Cas9 cOBs infected with sgSlc38a2 or sgLuc as control (n=3). Representative image (D) and quantification (E) of Western blot, and half-life (F) of RUNX2 protein treated with 5mM HCl or THFA for 48h. Error bar depicts SD. *p≤0.05, ***p≤0.0005.
3.2.9 *Slc38a2* and proline metabolism regulate redox homeostasis independent of pentose phosphate pathway.

Proline cycle can also regulate redox metabolism exemplified by balancing NAD+/NADH and NADP+/NADPH ratios. P5C reduction to proline requires oxidation of NADH or NADPH (Fig. 31A). Therefore, we then sought to determine whether proline is required for redox homeostasis in osteoblasts. We measured NAD+/NADH, and NADP+/NADPH ratios in osteoblasts treated with THFA or deprived of proline for 3 hour. Both THFA treatment and proline deprivation increased NAD+/NADH, and NADP+/NADPH ratios (Fig. 34A-D). NADP+/NADPH ratio but not NAD+/NADH was increased in *Slc38a2* knockout osteoblasts (Fig. 34E,F). However, the NAD+ level is increased in *Slc38a2* knockout cells (Fig. 34G). Since P5C reduction to proline requires oxidation of NADH or NADPH, we hypothesized that more glutamate flux into proline when proline oxidation or uptake is inhibited. To test the hypothesis, we first performed $^{13}$Cu-glutamine tracing in osteoblasts treated with THFA and cells deleted with *Slc38a2*. We found that both THFA treatment and *Slc38a2* deletion increased $^{13}$Cu-glutamine derived proline (Fig. 34H,I). This suggests that an increased flux from glutamine from proline upon inhibition of proline oxidation or uptake. Collectively, *Slc38a2* provides proline regulates redox homeostasis by balancing NAD+/NADH, and NADP+/NADPH through the proline cycle.
Figure 34. Proline metabolism regulates redox homeostasis.

Measurement of NAD+/NADH ratio (A,C,E) or NADP+/NADPH ratio (B,D,F) in cOBs treated with 5mM HCl or THFA for 3 hours (A,B), or in cOBs cultured with complete or proline-free medium for 3 hours (C,D), or Rosa26Cas9/Cas9 cOBs infected with sgSlc38a2 or sgLuciferase as a control (E-G) (n=3). 13C-glutamine tracing to proline in cOBs 5mM HCl or THFA (H), or Rosa26Cas9/Cas9 cOBs infected with sgSlc38a2 or sgLuc (I). n=3. Error bar depicts SD. *p≤0.05, **p≤0.005, ***p≤0.0005.

One of the major sources for NADPH production is via the pentose phosphate pathway. Since NADPH is more oxidized upon proline oxidation or uptake inhibition, we were interested in whether proline metabolism regulates NADPH level through the pentose phosphate pathway (PPP). To test this hypothesis, we performed 1,2-13C-glucose tracing in osteoblasts treated with THFA or lacking Slc38a2. 1,2-13C-glucose is converted to M+2 lactate through glycolysis while to M+1 lactate through PPP. Therefore, the ratio of M+1 and M+2 lactate is an indicative of the flux of glucose to PPP relative to glycolysis (Fig. 35A). Our tracing results showed that the ratio of M+1 and M+2 lactate was unaffected in osteoblasts treated with THFA and or deleted with Slc38a2 (Fig. 35B,C). M+1
and M+2 lactate labeling was also unchanged (Fig. 35D,E) suggesting that aerobic glycolysis is not affected upon proline oxidation inhibition. This result is consistent with our Seahorse analysis that proline supplement increased ATP production through OXPHOS while did not affect glycolysis. Collectively, Slc38a2 provides proline required for redox homeostasis but independent of the pentose phosphate pathway or glycolysis.

Figure 35. Proline metabolism does not regulate glycolysis and the pentose phosphate pathway.

(A) Schematics of [1,2-¹³C]-glucose tracing. M+1/M+2 ratio (B,C), and M+1 and M+2 levels (D,E) in cOBs treated with 5mM HCl or THFA (B,D), or Rosa26Cas9/Cas9 cOBs infected with sgSlc38a2 or sgLuc as control (C,E) (n=3). Error bar depicts SD.
3.3 Discussion

We show here that proline is enriched in osteoblast-associated proteins (Fig. 16). Our prediction model shows that osteoblasts have increased demand for proline during differentiation (Fig. 17). Consistently, osteoblasts also obtain more proline during differentiation. We identified Slc38a2 as the highest expressed proline transporter in osteoblasts and responsible for 50% of proline uptake in bone tissues (Fig. 18). Genetically ablating Slc38a2 in the osteoblast lineage cells prevents osteoblast differentiation, resulting in defective bone development and reduced postnatal acquisition (Fig. 20-27).

Mechanistically, proline metabolism can regulate osteoblast differentiation in multiple ways. First, proline cannot be metabolized to glutamate but can be directly incorporated into proteins (Fig. 29). Osteoblasts lacking proline transporter, Slc38a2, have reduced protein and collagen synthesis (Fig. 28). Slc38a2 conditional knockout mice have less proline-rich proteins despite the normal level of non-proline-rich proteins (Fig. 30). Second, Slc38a2-deficient osteoblasts have increasing energy deficit and redox imbalance (Fig. 28-33). Consistently, inhibiting proline cycle or removing proline from the medium also leads to deficiency in energy and redox homeostasis (Fig. 28-33). This suggests that proline is a critical regulator of bioenergetics and redox homeostasis. Collectively, our study pioneered the research of proline metabolism in the bone field and found proline
metabolism, regulating bioenergetics and biosynthesis of protein, is required for osteoblast differentiation during bone development (Fig. 36).

Figure 36. A model for metabolism of proline transported by Slc38a2/SNAT2.

Slc38a2/SNAT2 provides proline in osteoblasts. The majority of proline is incorporated into protein, particularly into proline-rich osteoblast regulators like COL1A1 and OSX. Proline and P5C forms proline cycle, which regulates bioenergetics and redox homeostasis in osteoblasts. Proline can also regulate RUNX2 stability via modulation of AMPK.
Slc38a2 transports proline in osteoblasts, which supports their differentiation. Proline has been recognized as an important nutrient as proteins like collagens have proline as 20% of the amino acid composition (Albaugh et al., 2017). Besides its direct incorporation into proteins, proline is also a metabolizable and synthesizable amino acid (Phang et al., 2012; Phang et al., 2015). Proline has an alpha-nitrogen of proline located in the pyrrolidine side chain, making it difficult to be metabolized through normal enzymatic reactions like transamination and decarboxylation (Phang et al., 2015). PRODH, specialized in opening up pyrrolidine ring, is the only discovered enzyme capable of metabolizing proline so far. The special location of PRODH at Complex II in electron transport chain (ETC) renders proline another role in bioenergetics. Proline oxidation at Complex II is FADH2 oxidation-dependent, providing electrons to ETC for ATP production. Consistent with this finding, we found osteoblasts lacking proline transporter or with PRODH inhibition had lower ATP level and higher energy deficit (Fig. 31). ROS is a known byproduct of ETC, which has also been linked with proline oxidation (Liu et al., 2012a; Liu and Phang, 2012b). Our data also demonstrate ROS downregulation when PRODH is inhibited by THFA (Fig. 31). This suggests that osteoblasts rely on proline metabolism for proper ETC function in mitochondria. PRODH produced P5C can be further catabolized by P5C dehydrogenase to glutamate (Phang et al., 2012). However, proline does not give rise to glutamate in osteoblasts. The reason underlying the limited proline-to-glutamate metabolic pathway is still unknown. Glutamate is an important
source for proline, which depends on P5C synthase converting glutamate to P5C (Phang and Liu, 2012). Consistently, we found proline was derived from glutamine in osteoblasts (Fig. 29,34). The other possible source is ornithine, converted to P5C by ornithine aminotransferase (Phang et al., 2015). Future studies will determine whether ornithine is a potential source for proline in osteoblasts. In the next step, P5C is reduced to proline by P5C reductases (PYCR). PYCR has three isoforms PYCR1, PYCR2 and PYCRL. PYCR1 and PYCR2 are NADH-dependent located in mitochondria, while PYCRL localizes in the cytosol and uses NADPH as the cofactor (Phang et al., 2015). The oxidation of NADH and NADPH is necessary for the proper enzymatic activity of PYCRs. Therefore, proline metabolism is another regulatory role of mediating NAD+/NADH and NADP+/NADPH ratios, regulating redox homeostasis. Our data also show that inhibiting proline oxidation forces more glutamine metabolized to proline in osteoblasts. Meanwhile, more NADH and NADPH are oxidized in parallel. NAD+ can also regulate osteoblast differentiation as a cofactor with SIRT1, which deacetylates osteoblast regulators β-catenin and RUNX2 (Cantó and Auwerx, 2012; Li et al., 2019; Simic et al., 2013). Despite the fact that the NAD+ level is increased in Slc38a2 knockout osteoblasts, it is unclear what is the precise mechanism through which change of NAD+ regulates osteoblast differentiation. Collectively, proline degradation and synthesis forms “proline cycle”, which is a critical regulator of bioenergetics and redox balance in osteoblast differentiation.
One caveat of our study is that Slc38a2, the proline transporter that we knocked out in our system, has been considered as a transporter for other substrates in the context of other cells and tissues. In many cancer studies, Slc38a2 is identified as glutamine transporter (Bröer et al., 2016; Morotti et al., 2019). Knocking down Slc38a2 in breast cancer cells and osteosarcoma cells leads to decreased glutamine uptake, inhibited cell growth and increased cell death. In one recently published study, Slc38a2 is identified as an alanine transporter in pancreatic ductal adenocarcinoma (PDAC) (Parker et al., 2020). PDAC cells with deletion of Slc38a2 fail in concentrating intracellular alanine level, leading to metabolic crisis and inhibited tumor growth. However, glutamine and proline concentrations are not affected in Slc38a2 knockout PDAC cells. Nonetheless, a few studies suggest Slc38a2 accepts proline as the substrate, but with a lower kinetics compared to glutamine and alanine (Tan et al., 2011). On the contrary, uptake of glutamine and alanine is not affected while proline uptake is reduced in osteoblasts with Slc38a2 knockout in bones, suggesting that Slc38a2 is a proline transporter, which does not preferentially accept glutamine and alanine as substrates. It is unknown why the same transporter Slc38a2 has different kinetics for the same substrate in the context of different cells and tissues. One possibility is that Slc38a2 has distinct conformational structures that affect particular affinities against substrates in different cellular systems. Future investigation is needed to test this possibility.
Slc38a2 is responsible for around 50% of the proline uptake in osteoblasts. Thus, there must be other proline transporters that account for the rest of proline uptake in osteoblasts. In our transcriptomic analysis, Slc1a4, encoding ASCT1, and Slc38a4, encoding SNAT4 are the second and third highest expressed proline transporters in osteoblasts. Canonical proline transporter Slc6a7, which encodes PROT, is also expressed in osteoblasts, however, at a very low level. There is a possibility that these proline transporters account for the remaining 50% of proline uptake; unfortunately, none of these proline transporters has been studied in the context of osteoblasts and bones. Studies from a couple of decades ago have demonstrated that proline uptake is sodium-dependent and MEAIB sensitive (Adamson and Ingbar, 1967a; Finerman and Rosenberg, 1966; Hahn et al., 1969; Yee, 1988). Nearly all the known proline transporters have these two characteristics. This leads to obstacles to identify individual proline transporters until genetic silencing or ablation is widely applied in the field. Our study is the first to identify a major proline transporter, Slc38a2, in osteoblasts.

In summary, our study demonstrates that proline, transported by Slc38a2, regulates bioenergetics and protein biosynthesis necessary for osteoblast differentiation during bone development. However, there are potentially other mechanisms through which proline metabolism regulates osteoblast differentiation. A study showed that proline treatment promotes the transformation of embryonic stem cells to a mesenchymal-like state likely through epigenetic regulation via histone methylation of H3K9 and H3K36.
(Comes et al., 2013). Furthermore, the source of proline remains unidentified in the system of osteoblasts and bone. A study using PDAC as the model finds these cancer cells depend on proline released from collagens in the extracellular matrix environment (Olivares et al., 2017). This is reminiscent of the type I collagen-rich bone matrix, which may serve as a natural reservoir for proline. The increased TRAP+ osteoclasts in BglapCre; Slc38a2fl/fl mice may be a way for osteoblasts that demand proline for bioenergetics and redox homeostasis to release proline from the matrix. The dynamic equivalence between proline-involved collagen synthesis and degradation will be another interesting topic to investigate in the future. In addition, it remains unknown how the proline transporter Slc38a2 is regulated. Serving as a glutamine transporter, Slc38a2 has been shown to be transcriptionally regulated by HIF and estrogen signaling pathways (Morotti et al., 2019; Velázquez-Villegas et al., 2014). On the contrary, our preliminary data showed that Slc38a2 expression is not altered in HIF1α knockout cells suggesting HIF signaling may not directly regulate Slc38a2 in the bone system (data not shown). The phenotype we observed in BglapCre; Slc38a2fl/fl mice was only in males but not in females. The sex dimorphism in conditional knockout mice indicates that estrogen may play a role in Slc38a2. Future investigations are warranted to determine whether HIF and estrogen signaling pathways are regulators of Slc38a2 in osteoblasts.
Chapter 4. Methods

Radiolabeled amino acid uptake assays in primary bone cells and bone explants.

Radiolabeled amino acid uptake assays are a highly sensitive method used to characterize the uptake of amino acids by cells or tissues in culture. This method is an excellent tool to quantify changes in amino acid consumption that are associated with states of cellular differentiation and/or disease. The methods presented here can be adapted to measure the transport of all amino acids and can be applied to cultured cells and bone explants. Here, we describe two modified protocols for analyzing radiolabeled amino acid uptake using L-[3,4-3H]-proline in both cultured primary cells and in bones ex vivo. Importantly, these protocols are easily adaptable allowing for the evaluation of uptake of other radiolabeled amino acids in bone cells both in vitro and ex vivo. Finally, we include important troubleshooting information about working with radioactivity.

Amino acid uptake in primary osteoblasts

1. Isolate calvarial osteoblasts (see Note 3)

2. Seed 1x10^5 primary calvarial cells on 12-well tissue culture plates in α-MEM containing 15% FBS. Seed cells in extra wells for normalization in the final step (3.1.13). Place the plate in a humidified cell culture incubator at 37°C with 5% CO₂.

3. Culture the cells for 2-3 days until confluent.

4. Prewarm 1x PBS and KRH to 37°C.

5. Wash cells two times with 1x PBS, pH 7.4.
6. Wash cells once with KRH.

7. Perform all the experiments associated with radiation behind the protection shield.

8. Make 4µCi/mL L-[2,3-3H]-Proline working media by diluting 4µL of [1 µCi µL⁻¹] L-[2,3-3H]-Proline stock in 1mL KRH (see Note 4).

9. Incubate cells with 4µCi/mL L-[2,3-3H]-Proline for 5 minutes.

10. Remove the radioactive medium. Wash the cells three times briefly with ice-cold KRH. Discard all the washes in the radioactive liquid waste container (see Note 1).

11. Lyse cells with 1mL 1% SDS. Pipette up and down for 10 times. Transfer cell lysates to 1.5 mL Eppendorf tubes. Discard cell culture plates and pipette tips in radioactive solid waste container (see Note 1).

12. Centrifuge at >10000 rpm for 10 min. Transfer supernatants to scintillation vials containing 8mL scintillation solution. Discard tubes and pipette tips in radioactive solid waste container (see Note 1).

13. Read radioactivity in counts per minute (cpm) using Scintillation counter. Discard scintillation vials in radioactive glass waste container (see Note 1).

14. Quantify cell numbers in the extra wells from 3.1.2. Normalize the cpm to cell number. (see Note 5)

15. Spray the cell culture hood, instruments and bench with radioactivity decontaminant. Perform wipe tests to eliminate the possibility of radioactive contamination.
Figure 37. L-[2,3-³H]-Proline uptake assays in calvarial osteoblasts (cOBs).

Example of proline uptake assay in cOBs treated with the amino acid analog 2-(methylamino)-isobutyric acid (MeAIB). MeAIB is a competitive inhibitor of System A amino acid transporters known to transport proline. cOBs were cultured with labeled proline for 5 minutes in the presence of water or 5µM MeAIB.

Amino acid uptake in bone explants (see Fig. 38)
1. Prewarm KRH to 37°C.
2. Dissect both humeri from each mouse. Remove all extemporaneous tissues using a scalpel. Remove the epiphyses from the bone.
3. Flush out marrow from the bone and weigh the bone shafts. (see Note 6).
4. Boil one humerus in 1x PBS on heat block at 100°C for 10 min to decellularize the bone as control. (see Note 7)
5. Equilibrate humeri in KRH for 30min in the cell culture incubator at 37°C.
6. Perform all the experiments associated with radiation behind the protection shield.
7. Make 4µCi/mL L-[2,3-³H]-Proline working media by diluting 4µL of [1 µCi µL⁻¹] L-[2,3-³H]-Proline stock in 1mL KRH.
Figure 38. Evaluating radiolabeled amino acid uptake assay in bones *ex vivo*.

(A) Schematic overview of amino acid uptake assay in humeri cultured *ex vivo*. (B) Sample preparation for amino acid uptake in humeri *ex vivo*. (i) Image of a humerus with extemporaneous tissues removed. (ii) Image of a humerus after epiphyses are removed. (iii) Image of a humerus cut in the middle to ease the removal of marrow using a syringe or centrifugation.
8. Incubate both the experimental and boiled humeri with 4µCi/mL L-[2,3-3H]-Proline for up to 60 minutes in the cell culture incubator at 37°C. The actual incubation time should be determined empirically (see Note 8).

9. Remove radioactive medium. Terminate the reaction by washing humeri three times using ice cold KRH. Discard all the washes in the radioactive liquid waste container (see Note 1).

10. Transfer each bone into 1.5mL Eppendorf tube. Add 500 µL RIPA buffer. Discard culture plates and pipette tips in radioactive solid waste container (see Note 1).

11. Homogenize the humeri by chopping 100 times with scissors in 1.5 mL Eppendorf tubes.

12. Sonicate bone homogenates (Amplitude: 35%, Pulse 1s, Duration: 10s). (see Note 9).

13. Clarify the lysate by centrifugation at >10000 rpm for 10 min. Transfer 200µL of the supernatant to scintillation vials containing 8mL scintillation solution. Discard tubes and pipette tips in radioactive solid waste container (see Note 1).

14. Read radioactivity (cpm) using Scintillation counter. Discard scintillation vials in radioactive glass waste container (see Note 1).

15. Normalize the radioactivity with bone weight from 3.3.3.

16. Spray the cell culture hood, instruments and bench with radioactivity decontaminant. Perform wipe tests to eliminate the possibility of radioactive contamination.
Figure 39. *Ex vivo* L-[2,3-³H]-Proline uptake assays in neonatal humeri.

(A) L-[2,3-³H]-Proline uptake assays performed over 90 minutes in live and boiled humeri isolated from 3-day-old C57BL/6 mice. Proline uptake increases linearly for the first 60 mins and then plateaus. (B) L-[2,3-³H]-Proline uptake assay in humeri performed in the presence or absence of 5µM MeAIB.
Notes

1. For the use of radioactive materials, please refer to Office of Radiation Safety at your home institution before conducting any radiation-associated experiments.

2. Geiger counter is not required in this case since the working isotope in this protocol is $^3$H. It is still important to keep it on the side to alert people that you are working with radiation. If you are working with isotopes with stronger radioactivity (e.g. $^{14}$C and $^{35}$S), Geiger counter is required to be placed along the side.

3. This protocol can be applied to cell lines including ST2, ATDC5, MC3T3, etc. or other primary skeletal cells like chondrocytes, bone marrow stromal cells, and bone marrow macrophages.

4. Other radioactive isotope labels (e.g. $^{14}$C and $^{35}$S) are also appropriate and detectable.

5. Results can also be normalized by DNA content for cells in vitro. This is important for mineralizing cells that are difficult to trypsinize.

6. Flush out the bone marrow by centrifugation for adult mice. In young mice or embryonic bones, it is better to flush out marrow using a syringe and 30G needle.

7. Contralateral bone needs to be boiled as a control because radioactive amino acids can adsorb to bone matrix independent of facilitated transport by bone cells.

8. For adult bone, consider longer incubation in radioactive medium.

9. Sonication of small sample volumes can result in foaming. If this occurs, centrifuge the sample and let rest to remove bubbles.
**Mouse analyses**

Mouse strains used in this study are listed in Table 10 in Appendix B. Whole-mount skeletal preparation was performed on E15.5 or P1 embryos. Embryos are dehydrated in 95% ethanol overnight followed by acetone overnight. Specimens were then stained with 0.03% (w/v) alcian blue and 0.005% (w/v) alizarin red overnight. Stained embryos were then transferred to 1% (w/v) KOH until the clearing of the embryos. Skeletal preparation was stored in 80% (v/v) glycerol. Freshly isolated limbs and skulls were fixed in 4% PFA at 4°C overnight. Limbs were then processed and embedded in paraffin and sectioned at 5μm using Microtome. Fixed skulls were sucrose embedded in OCT and sectioned at 10μm by Cryostats. Immunostaining was performed on paraffin sections of limbs or frozen sections. For immunofluorescence staining, sections were blocked by goat serum (3:200(v/v) in PBST) for 30 minutes and incubated with primary antibodies (1:250(v/v) in blocking solution) at 4°C overnight. Antibodies used for immunofluorescence are listed in Table 7 in Appendix B. Sections were then incubated with Alexa Fluor 568 goat anti-rabbit/-mouse IgG(H+L) antibody at 1:500 dilution at room temperature for 30min. Unfixed frozen sections were used for b-galactosidase staining for LacZ expression at 37°C overnight. Micro computed tomography (VivaCT80, Scanco Medical AG) was used for three-dimensional reconstruction and analysis of bone parameters (threshold=320) from 200 slices underneath the growth plate. Adult bones were fixed in 10% buffered formalin overnight at 4°C and decalcified in 14% EDTA for
two weeks at 4°C before processed for paraffin embedding. Immunostaining was performed on paraffin sections for osteocalcin (OCN). For dynamic histomorphometry, mice were injected with calcein (25mg/kg) intraperitoneally at 7 days and alizarin red (75mg/kg) 2 days prior to sacrifice, respectively. Freshly isolated femurs were fixed in 4% PFA overnight and sucrose embedded in Cryomatrix for non-decalcified sections crosslinked by cryojane. Dynamic histomorphometry parameters including bone surface, mineralizing surface and interlabel width were quantified using ImageJ.

Cell culture

The bone marrow derived cell line, ST2 (RRID:CVCL_2205) was plated at 40,000 cells/ml in α-MEM (GIBCO) supplemented with 10% FBS (Invitrogen). In WNT treatment experiments, 25 ng/ml WNT3A (TIME Bioscience) or vehicle control (0.1% BSA in PBS) was supplemented in the α-MEM. For LiCl treatment, 20mM LiCl or vehicle control (20mM NaCl) was added in the growth medium. When inhibitors were used, cells were pretreated with corresponding inhibitors for 30 min before any other treatment. In indicated experiments, growth medium was supplemented with 100nM Rapamycin (Sigma-Aldrich), 250ng/mL DKK1 (R&D) or 0.03 mM GPNA (MP Biomedicals), or the respective vehicle (DMSO, 0.1% BSA in PBS or 1M HCl for Rapamycin, DKK1 or GPNA respectively) as a control. For WNT3A-induced mineralization, cells were treated with WNT3A for 72 hours followed by osteogenic medium (α-MEM supplemented with 50 mg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich) for
6 days. Primary calvarial osteoblasts were isolated as follows. The calvaria of P4 pups were harvested and extemporaneous tissue was removed. Calvaria was chopped by scissor into small pieces and washed with PBS for two times. Calvaria was then digested by 1.8mg/mL Collagenase D in PBS for 10 minutes at 37 °C for four times. The first digestion was discarded, and the last three digestions were collected and run through 70µm cell strainer. Cells were then centrifuged at 350x g for 5min and cultured at T75 flasks in αMEM containing 15% FBS at 37 °C and 5% CO₂. Cells were plated at 1x10⁵ cells/mL for further experiments when it reached 90% confluency. Primary bone marrow skeletal stem cells (SSC) were isolated as follows. Femur and tibiae were harvested, and all extemporaneous tissue was removed. Bone marrow was collected by centrifugation, followed by red blood cell lysis buffer. For colony forming unit assays, 1x10⁶ SSC were plated in a T25 flask. 3 hours after plating, non-adherent cells were removed by washing vigorously with PBS. Cells were then cultured for 7 days in hypoxia chamber (2%) as indicated for CFU-F and CFU-Ap assays. Osteogenic media (CFU-Ob) was added for an additional 7 days. For high density cultures, SSCs were plated and washed after 5 days to remove non-adherent cells. SSCs were plated for experiments at a seeding density of 50,000 cells/cm². Osteoblast differentiation was induced at 100% confluency using αMEM supplemented with 50 mg/ml ascorbic acid and 10 mM b-glycerophosphate for the indicated time period with a change of media every 48 hours. Osteoblast differentiation was assayed by visualizing alkaline phosphatase activity using 5-bromo-4-chloro-3'-
indolyphosphate/nitro blue tetrazolium (BCIP/NPT) (Katagiri et al., 1994) or von Kossa staining of deposited calcium phosphate (Rungby et al., 1993). Alkaline phosphatase assay was performed 24 hours after WNT stimulation. Von Kossa staining was performed 6 days after addition of osteogenic media.

**In situ hybridization**

ISH was performed on 10μm cryosectioned skull or 5μm paraffin-sectioned limb. Cryosections were washed with water first for 5min. Paraffin sections were deparaffinized and rehydrated, followed by 20μg/ml proteinase K treatment for 10min. Sections were first fixed using 4%PFA for 10min followed by 10 min acetylation. Sections were then applied with hybridization buffer for 2 hours at room temperature. Digoxigenin-labeled RNA probes (Spp1, Ibsp, Bglap) were applied on the sections incubated at 60°C overnight. Sections were washed with 0.2x SSC 30min twice and then blocked using 2% BR/NTT with 5% HISS for 1 hour. Anti-Digoxigenin antibody (1:4000 in blocking solution) was applied on sections at 4°C overnight. Sections were subsequently washed with NTT 30min for three times followed by NTTML 5min for three times. Sections were developed using BM Purple AP as the substrate at 37 °C until color appeared.

**CRISPR/Cas9 system**

Lentiviral vectors were obtained from Dr. Shondra Miller’s lab at St Jude’s Children Research Hospital. Lentiviral vectors were designed to express short guide RNA (sgRNA), targeting Slc38a2 or Luciferase as a negative control. The sgRNA carrying
lentiviral vector was cotransfected in 293T cells with the plasmids pMD2.g (RRID:Addgene_12259) and psPax2 (RRID:Addgene_12260). Virus containing media was collected and run through 0.45μm filter. Calvarial osteoblasts harvested from Rosa26Cas9/Cas9 pups were infected for 24 hours and recovered for 24h in regular media before further experiments. Puromycin (5μg/ml) was used to select infected cells.

**Mass spectrometry**

Calvarial osteoblasts were cultured in 6cm plates until confluency before sample preparation for mass spectrometry. Cells were treated with 1mM (U-^{13}C)proline or 2mM (U-^{13}C)glutamine for 24 hours. Reaction was terminated by cold PBS and cells were scrapped with -20°C methanol on ice. 20nmol norvaline as internal control was added into each methanol extract, followed by centrifuge at 10000x g force for 15 minutes. Supernatants were taken for further preparation. For tracing experiments into protein, cells were treated with 1mM (U-^{13}C)proline or 2mM (U-^{13}C)glutamine for 0, 12, 24 or 72 hours. Cell were scrapped in RIPA lysis buffer. Protein was precipitated by 1M perchloric acid. The protein pellet was washed with 70% ethanol for three times. 20nmol norvaline was added as internal control. The pellet was then incubated with 1mL of 6M HCl at 110°C for 18hours to hydrolyze the proteins. 1mL of chloroform was then added to each sample followed by centrifuge at 400x g for 10 minutes. Supernatants were taken for further preparation. The supernatant was dried by N2 gas at 37°C. GC-MS method for small polar metabolites assay used in this study was adapted from Wang et al. (2018).
dried residues were resuspended in 25μL methoxylamine hydrochloride (2%(w/v) in pyridine) and incubated at 40℃ for 90 minutes. 35 μL of MTBSTFA + 1% TBDMS was then added, followed by 30-minute incubation at 60℃. The derivatized sampled were centrifuged for 5 minutes at 10000 x g force. Supernatant from each sample was transferred to GC vials for analysis. 1μL of each sample was injected in split or splitless mode depending on analyte of interest. GC oven temperature was set at 80 ℃ for 2 minutes, increased to 280 ℃ at a rate of 7 ℃/min, and then kept at 280 ℃ for a total run time of 40 minutes.

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 mm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (Meister, 1975) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230 ℃, the MS quad temperature at 150 ℃, the interface temperature at 280 ℃, and the inlet temperature at 250 ℃. Mass spectra were recorded in selected ion monitoring (SIM) mode with 4 ms dwell time.

**Amino acid uptake assay**

Amino acid uptake assay was adapted from a recent published protocol (Shen and Karner, 2021). ST2 Cells were washed with PBS and two additional washes with Krebs Ringer Hepes (KRH, 120mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 25mM NaHCO₃, 5mM HEPES, 1mM D-Glucose, pH 8). Cells were then treated with KRH containing 4
μCi/mL (2,3,4-³H)-Glutamine for 5 min. Glutamine uptake and cellular metabolism was terminated by ice cold KRH. Cells were scraped with 1mL ice-cold dH₂O. The lysate was centrifuged and counts per minute (CPM) were measured using a Beckman LS6500 scintillation counter. All results are normalized to cell number. In experiments to measure glutamine transport system activity the recipe for KRH was altered as follows:

- Complete KRH (120mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 0.4mM KH₂PO₄, 5mM HEPES, 1mM D-Glucose, pH 8), complete KRH plus MeAIB (120mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 0.4mM KH₂PO₄, 5mM HEPES, 5mM MeAIB, 1mM D-Glucose, pH 8), complete KRH plus GPNA (120mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 0.4mM KH₂PO₄, 5mM HEPES, 0.3mM GPNA, 1mM D-Glucose, pH 8), Na⁺ free KRH (120mM choline chloride, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 0.4mM KH₂PO₄, 5mM HEPES, 1mM D-Glucose, pH 8), Na⁺ free plus Li⁺ KRH (120mM LiCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 0.4mM KH₂PO₄, 5mM HEPES, 1mM D-Glucose, pH 8).

We calculated the relative glutamine transport system activity as follows:

\[
% \text{System A Activity} = \frac{CPM_{complete \ KRH} - CPM_{MeAIB}}{CPM_{complete \ KRH}} \times 100%
\]

\[
% \text{System N Activity} = \frac{(CPM_{Li^+}) - (CPM_{Na^+ free})}{CPM_{complete \ KRH}} \times 100%
\]

\[
% \text{System L Activity} = \frac{CPM_{Na^+ free}}{CPM_{complete \ KRH}} \times 100%
\]

\[
% \text{System ASC} & \gamma(+)L Activity = 100% - %\text{System N} - %\text{System L}
\]
Calvarial osteoblasts were first washed three times with PBS and incubated with Krebs Ringer Hepes (KRH) with 4µCi/mL L-[2,3-3H]-Proline, L-[2,3,4-3H]-Glutamine, L-[2,3-3H]-Alanine, L-[3,4-3H]-Glutamate or α-[1-14C]-methylaminoisobutyric acid for 5 minutes at 37°C. Uptake assay was then terminated with ice cold KRH and scraped with 1%SDS. Cell lysates combined with 8mL scintillation cocktail and CPM was measured using Beckman LS6500 Scintillation counter. Newborn mouse humeri and femurs were used for ex vivo amino acid uptake acid. Extemporaneous and cartilaginous tissues were removed from the bones and counter lateral parts were harvested and boiled for normalization. Bones were then incubated with KRH containing radiolabeled amino acids for 30min at 37°C. The reaction was terminated by ice cold KRH. Samples were homogenized in RIPA buffer followed by sonication (Amplitude: 35%, Pulse 1s, Duration: 10s) and centrifugation. Supernatant from each sample was combined with 8mL scintillation cocktail and CPM was measured using Beckman LS6500 Scintillation counter. Radioactivity was normalized with the boiled counter lateral bones.

**Metabolic labeling with S35-cysteine/methionine**

Cells were incubated with cysteine/methionine-free DMEM supplemented with 165µCi of EasyTag EXPRESS S35 protein labeling mix for 30 min. Cells were then lysed with RIPA and followed by centrifugation. Lysates were spotted on Whatman paper. Protein is precipitated with cold 5% trichloroacetic acid (TCA) and washed with 10% TCA, ethanol and acetone. The Whatman paper was air dried for 10 minutes and dipped into
8mL scintillation cocktail. Radioactivity was measured using LS6500 Scintillation counter and normalized with cell number.

Proline incorporation assay into collagens

Cells were incubated with KRH supplemented with 4μCi/mL L-[2,3-3H]-Proline for three hours. Cells were lysed with RIPA and followed by centrifugation. Protein is precipitated with TCA and resuspended using 1M NaOH. Each sample was split into two: one was treated with 15mg Collagenase P and 60μM HEPES to digest collagens and the other with only 60μM HEPES as the baseline control. Samples were incubated at 37°C for 3 hours. After incubation, residual proteins and Collagenase P was precipitated using TCA followed by centrifugation. Supernatant from each sample was combined with 8mL scintillation cocktail and CPM was measured using Beckman LS6500 Scintillation counter. Radioactivity was normalized with 60μM HEPES treated the baseline control.

shRNA knockdowns

Lentiviral vectors were obtained from the shRNA consortium at Washington University School of Medicine. All knockdown results were confirmed using 2 or more unrelated shRNA constructs. The shRNA sequence listed first was shown in the results. The lentiviral vector pLKOpuro was modified to express shRNAs targeting Slc1a5 (CCTGTAGAGTTCTCTACCCCT, GCAGTGTTCATCGACAACTA), Slc7a7 (GCTACATGTTTCAGACTTCAT, GCCATCTGTATGGTTCATG), Atf4 (CCAGAGCATCTCATTAGTTA, CCTCTAGTCCAAGAGACTAAT), Catnb1
(GCGTTATCAAACCTAGCCTT, CCATCACAGATGTTGAAACAT) or either RFP (ACAACAGCCACAACGTCTATA) or LacZ (GCGATCGTAATCACCCGAGTG) as a negative control. The shRNA expressing lentiviral vector was cotransfected in 293T cells with the plasmids pMD2.g (RRID:Addgene_12259) and psPax2 (RRID:Addgene_12260). Virus-containing media was collected and filtered. ST2 cells were infected for 24 hours and recovered for 24 hours in regular media prior to further treatment.

**RNA isolation and qPCR**

Total RNA was isolated using the RNAeasy kit with on-column DNase treatment (Qiagen). 500ng of total RNA was reverse transcribed using the Iscript cDNA Synthesis kit (Bio-Rad). Reactions were set up in technical and biological triplicate in a 96-well format on an ABI Quantstudio 3 using SYBR Green chemistry (SsoAdvanced; Bio-Rad). The PCR conditions were 95°C for 3 min followed by 35 cycles of 95°C for 10s and 60°C for 30s. Gene expression was normalized to 18S rRNA, and relative expression was calculated using the $2^{(\Delta\Delta CT)}$ method. Primers were used at 0.1µM, and their sequences are listed in Table 8 in Appendix B. PCR efficiency was optimized and melting curve analyses of products were performed to ensure reaction specificity.

**Western blotting**

ST2 cells or calvarial osteoblasts were scraped in lysis buffer containing 50 mM Tris (pH 7.4), 15 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.1% sodium deoxycholate with a protease and phosphatase inhibitor tablet (Roche). Protein concentration was quantified
using the BCA method (Pierce). Proteins (20µg) were resolved on 12% polyacrylamide gel, transferred onto Immuno-Blot PVDF membrane (Biorad). Proteins were detected using the specific antibodies in Table 7 in Appendix B. The membranes were blocked for 1 hour at room temperature in 5% milk powder in TBS with 0.1% Tween (TBST) and then incubated at 4°C with the primary antibody overnight. Membranes were washed 3 times with TBST and further incubated with Anti-Rabbit/Mouse IgG, HRP-linked Antibody in 5% milk (TBST) for 1 hour at room temperature. All blots were developed using enhanced chemiluminescence (Clarity Substrate Kit, Bio-Rad). Each experiment was repeated with a minimum of 3 independently prepared protein samples.

**ATP, NAD+/NADH, NADP+/NADPH quantification**

Intracellular ATP level was determined using luminescent ATP Detection Assay Kit (abcam). NAD(P)+ and NAD(P)H was measured separately using the NAD/NADH Glo and NADP/NADPH-Glo Assays (Promega).

**Seahorse Analysis**

To measure ATP flux in real time, BMSCs were plated in Seahorse XFe 96-well plates at 2.0 x 10^4 cells/ well and cultured under osteogenic conditions for 2 days. This timepoint was selected as it represents an early, committed osteoblast, like those isolated from the calvaria (Sinnott-Armstrong et al., 2021). ATP assays were performed according to the manufacturers protocol. Briefly, assay medium consisted of basal DMEM supplemented with 10 mM glucose, 1 mM sodium pyruvate, 2mM glutamine, and 100 nM
insulin. Assays were ran using assay medium, or assay medium with proline (40 µg/ L). Basal measurements were collected, followed by an injection of oligomycin (2 µM), and then rotenone/ antimycin A (1 µM/ 1 µM) during the assays. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were monitored over time. Considering the stoichiometry of the glycolytic pathway, the rate of ATP produced via in glycolysis is calculated as such: glycolytic ATP production rate (pmol ATP/min) = glycolytic proton efflux rate (pmol H+/min). Conversely, the rate of oxygen consumption that is coupled to ATP production during oxidative phosphorylation can be calculated as the OCR that is inhibited by addition of the ATP synthase inhibitor, oligomycin: OCR\text{ATP} (pmol O_2/min) = OCR (pmol O_2/min) - OCR\text{Oligo} (pmol O_2/min). Further transformation of OCR\text{ATP} to the rate of mitochondrial ATP production consists of the final equation mitoATP Production Rate (pmol ATP/min) = OCR\text{ATP} (pmol O_2/min) * 2 (pmol O/pmol O_2) * P/O (pmol ATP/pmol O). Finally, the total cellular ATP Production Rate is the sum of the glycolytic and mitochondrial ATP production rates: ATP Production Rate (pmol ATP/min) = glycoATP Production Rate (pmol ATP/min) + mitoATP Production Rate (pmol ATP/min). Hoechst stain was included in the final injection to determine cell number by semi-automated quantification (Cytation 5, BioTek). All final data was analyzed using Wave Controller Software 2.6.1 (Agilent).
Amino acid demand prediction analysis

Amino acid sequences of proteins (Mus_musculus.GRCm38.pep.all.fa) were retrieved from Ensembl (https://uswest.ensembl.org/info/data/ftp/index.html). Amino acid proportion was calculated based on the amino acid sequences. mRNA expression of genes in undifferentiated and differentiated osteoblasts were obtained from transcriptomic analysis done by Karner Lab. Amino acid proportion and mRNA expression were merged using Gene.stable.ID as the bridge. 75 unmatched proteins were excluded from a total of 49665 proteins. To predict the amino acid demand change, we assume that mRNA expression change is proportional to the change of protein translation. Based on this, the change of amino acid demand in each protein is proportional to mRNA expression change:

\[ \Delta AA \propto \Delta R \times Naa \]

\[ AA = \text{amino acid demand} \]

\[ R = \text{mRNA abundance} \]

\[ Naa = \text{number of amino acids} \]

To summarize the overall change of amino acid demand during osteoblast differentiation:

\[ \%\Delta AA = \frac{\sum [(R_{differentiated} - R_{undifferentiated}) \times Naa]}{\sum [R_{undifferentiated} \times Naa]} \times 100\% \]
tRNA aminoacylation assay

The method is adapted from two papers (Loayza-Puch et al., 2016; Saikia et al., 2016). Purified RNA was resuspended in 30mM NaOAc/HOAc (pH 4.5). RNA was divided into two parts (2 μg each): one was oxidized with 50mM NaIO₄ in 100mM NaOAc/HOAc (pH 4.5) and the other was treated with 50mM NaCl in NaOAc/HOAc (pH 4.5) for 15 min at room temperature. Samples were quenched with 100mM glucose for 5 min at room temperature, followed by desalting using G50 columns and precipitation using ethanol. tRNA was then deacylated in 50mM Tris-HCl (pH 9) for 30min at 37°C, followed by another ethanol precipitation. RNA (400ng) was then ligated the 3’adaptor (5’-5rApp/TGGAATTCTCGGTGGCCAAGG/3ddC/-3’) using T4 RNA ligase 2 (NEB) for 4 h at 37°C. 1μg RNA was then reverse transcribed using SuperScript III first strand synthesis system with the primer (GCCTTGGCACCAGGAATTCCA) following the manufacturer’s instruction. Relative charging level was calculated by qRT-PCR using tRNA-specific primers listed in Table 9 in Appendix B.

Flow Cytometry and Sorting

Flow cytometry was used to analyze EdU incorporation, Cell viability and ROS level in calvarial osteoblasts. EdU incorporation was performed using Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit. Cell were incubated with 10 μM EdU (5-ethynyl-2’-deoxyuridine) for 24 hours. Cells were then trypsinized, fixed, permeabilized and incubated with Click-iT reaction cocktail for 30 minutes following manufacturer’s
instructions. Cell viability was analyzed using the Cell Meter™ APC-Annexin V Binding Apoptosis Assay Kit (Cat# 22837). Cells were trypsinized and incubated with APC-Annexin V conjugate and propidium iodide for 30min. ROS was measured using CM-H2DCFDA kit (Invitrogen, C6827). Cells were trypsinized and incubated with CM-H2DCFDA at 37° C for 30 minutes. Cells were all resuspended in 500μL PBS and analyzed using FACSCanto II flow cytometer (BD Biosciences). Data were analyzed and evaluated using FlowJo (v.11). Sp7Cre-GFP+ cells were harvested from newborn Sp7Cre;Slc38a2<sup>fl/+</sup> or Sp7Cre;Slc38a2<sup>fl/fl</sup>. Extemporaneous tissues were first removed. The skeleton was chopped by scissors and treated with 3.6 mg/mL Collagenase P in PBS for 1 hour with agitation. Digestion was filtered using 30μm cell strainer, followed by 2-minute treatment with red cell lysis buffer and centrifuge at 350x g for 7 minutes. Cells were resuspended in PBS, sent to Duke Cancer Institute Flow Cytometry Core and sorted by MoFlo XDP sorter.

**Quantification and Statistical analysis**

Most statistical analyses were performed using Graphpad Prism 6 and R softwares. One-way ANOVA or unpaired 2-tailed Student’s t-test were used to determine statistical significance. All data are shown as mean values±SD. P value < 0.05 is considered as statistically significant. Sample size(n) and other statistical parameters are included in the figure legends. Most experiments were repeated on 3 independent sets of samples unless noted.
Chapter 5. Conclusion

My dissertation has provided novel insights into the role of amino acid transporters in osteoblast differentiation and bone formation. Here I have demonstrated that WNT signaling regulates glutamine consumption through amino acid transporters Slc7a7 and Slc1a5, which are indispensable for osteoblast differentiation. In addition, I have also shown that Slc38a2 provides proline required for osteoblast differentiation and bone formation. Proline serves multiple functions including protein synthesis, bioenergetics and redox homeostasis in osteoblasts. However, many questions associated with these amino acid transporters still remain unclear in the context of bone. In this chapter, I will highlight the potential future directions pertinent with the roles of amino acid transporters in osteoblast differentiation and bone formation.

5.1 Glutamine consumption regulated by WNT signaling in osteoblasts.

Osteoblasts actively consume amino acids including glutamine to meet high energetic and biosynthetic demands. WNT signaling is known to promote glutamine uptake and metabolism critical for osteoblast differentiation. However, it was unknown how WNT regulates glutamine uptake in osteoblasts. In Chapter 2, we identified two amino acid transporters Slc7a7 and Slc1a5 as the primary glutamine transporters in osteoblasts in response to WNT. Slc1a5 maintains the sustained basal uptake in osteoblasts while Slc7a7 is responsible for the rapidly induced glutamine uptake in response to WNT. WNT signaling regulates Slc7a7 expression through β-catenin dependent pathway.
contrary, $Slc1a5$ expression is regulated by transcription factor ATF4 downstream of mTORC1 pathway. Knocking down either $Slc7a7$ or $Slc1a5$ inhibits glutamine uptake and prevents osteoblast differentiation. Collectively, we demonstrated a biphasic role of WNT signaling in regulating glutamine uptake through two amino acid transporters in osteoblasts.

WNT signaling has been targeted for osteoporosis for several years. In 2019, romosozumab was approved by FDA for the medical use against osteoporosis. Romosozumab is a monoclonal antibody that neutralizes WNT antagonist SOST. However, romosozumab is categorized as first-in-class drug that has not been used widely in clinical setting. In rare cases, many side effects are associated with romosozumab including cardiovascular problems, joint pain and osteonecrosis of the jaw after long-term use. This may result from the systemic effect of WNT inhibition by romosozumab. Additionally, neutralization of SOST by romosozumab restores WNT-LRP5 interactions. This could affect multiple WNT downstream pathways governed by WNT-LRP5 signaling like β-catenin and mTORC2. Our study on the role of WNT signaling in regulating glutamine transporters may provide new ideas for the design of therapeutic strategies to treat osteoporosis. In the next step, I aim at investigating whether $Slc7a7$ or $Slc1a5$ is sufficient to induce osteoblast differentiation. If so, it might be worth investing funds and time to find a pharmacological way to activate these two amino acid transporters.
transporters. Targeting Slc7a7 or Slc1a5 might be a better and more specific strategy than interfering the upstream of WNT signaling.

It is also intriguing to understand why osteoblasts need two amino acid transporters for glutamine. Based on our findings, Slc1a5 sustains the basal glutamine uptake while Slc7a7 is responsible for WNT-induced glutamine import. Apparently, glutamine imported by these two different transporters serve for two distinct purposes. At basal level, Slc1a5 maintains a high activity in order to fulfill basic cellular functions that require glutamine. However, it is likely that Slc1a5 is unable to immediately respond to stresses or signals due to its existing peak activity. Therefore, osteoblast needs an amino acid transporter that can meet this demand rapidly. In our scenario, Slc7a7 is the candidate that can be induced rapidly in response to WNT within 6 hours. However, it is unknown whether Slc7a7 is able to respond to other developmental cues or stresses. Meanwhile, it is likely that other glutamine transporters, which act as rapid responders like Slc7a7, may be induced under different signals or stresses. Furthermore, the two distinct roles that Slc7a7 and Slc1a5 play may also account for why Slc1a5 and Slc7a7 are regulated by two distinct WNT downstream cascades. It is likely that WNT-β-catenin signaling acts more rapidly than mTORC1-ATF4. Indeed, WNT treatment increases β-catenin protein level within 6 hours. Conversely, although WNT can activate mTORC1 within 1 hour, ATF4 is not induced by mTORC1 until 72 hours. Different regulating rates of WNT downstream effectors could define the distinct purposes that Slc1a5 and Slc7a7 serve for. Therefore,
Slc7a7 fits better as a rapid responder, which is induced by WNT- β-catenin signaling. Conversely, Slc1a5, regulated by ATF4, acts slower but more robustly in osteoblasts.

Our study used ST2 cell line to understand the role of WNT-mediated glutamine consumption. In the future, we will first confirm the necessity of Slc7a7 and Slc1a5 for osteoblast differentiation in primary cells like calvarial osteoblasts. We are also interested in the in vivo relevance of Slc7a7 and Slc1a5 in osteoblast differentiation and bone formation. Global Slc7a7 knockout mice recapitulates LPI patients with featured phenotypes like delayed skeletal development and kidney development (Stroup et al., 2020). However, it is still unclear whether Slc7a7 acts cell autonomously in osteoblast lineage cells. Due to the role of Slc7a7 in kidney, we cannot exclude the possibility that loss of Slc7a7 in kidney indirectly affects bone forming, since kidney is a critical organ involved in calcium and phosphate homeostasis (Wei et al., 2016). I would like to conditionally knockout Slc7a7 using osteoblast specific Cre lines to directly test its role in osteoblasts. Additionally, our lab has generated a mouse line with Slc1a5 floxed allele. The investigation on the role of Slc1a5 in bone formation is ongoing. To test whether WNT regulates Slc7a7 and Slc1a5 in vivo, we will knockout Slc1a5 or Slc7a7 in the genetic background of WNT hyperactive allele (Lrp5

A214V/+) , which has a high bone mass. If Slc1a5 and Slc7a7 act downstream of WNT signaling, we expect to see deletion of either transporter can rescue the bone phenotype.
5.2 *Slc38a2/SNAT2 provides proline critical for osteoblast differentiation.*

Besides glutamine, we also investigated whether there is a differential demand for certain amino acid during osteoblast differentiation. Signature osteoblast differentiation markers are enriched with proline. Furthermore, using transcriptomic dataset of differentiating osteoblasts and amino acid composition of all proteins, we predict that proline demand is increased during osteoblast differentiation. Consistently, osteoblasts consume more proline during differentiation. Based on the same transcriptomic dataset, we also found *Slc38a2/SNAT2* is the highest expressed putative proline transporter. We further confirmed that *Slc38a2* transports proline and is expressed in osteoblast lineage cells. Knocking out *Slc38a2* in mesenchymal progenitors or preosteoblasts leads to defects in intramembranous and endochondral ossification, and postnatal bone acquisition. We are interested in how *Slc38a2*-transported proline regulates osteoblast differentiation. To test whether proline contributes to protein synthesis, we knocked out *Slc38a2* in osteoblasts using CRISPR/Cas9 system and found protein synthesis, particularly collagen synthesis, is reduced in *Slc38a2* knockout cells. In addition, proline-rich proteins (>9.5%, above 90 percentile) like COL1A1 and RUNX2, are also downregulated in knockout cells while non-proline rich proteins are not affected. We confirmed this phenotype *in vivo* that protein expression of COL1A1 and OSX, which are proline-rich, are reduced in knockout animals without affecting the mRNA expression. Furthermore, isotopomer-labelled proline tracing also revealed that almost 70% of proline in proteins are labeled within 72
hours. Collectively, *Slc38a2*-mediated proline uptake is critical for biosynthesis of proteins, especially proline-rich proteins, in osteoblast differentiation. Proline can also be metabolized for biosynthesis of amino acids like glutamate. Interestingly, proline tracing suggests that proline does not contribute to biosynthesis of any other amino acids in osteoblasts. Additionally, proline can regulate bioenergetics and redox homeostasis through proline cycle mediated by PRODH and PYCRs. Disrupting proline cycle blocks osteoblast differentiation and matrix mineralization. Inhibition of proline oxidation by THFA or removal of proline from media lead to reduced ATP production and increased energy deficit marked by elevated pAMPK. Increased pAMPK reduces the stability of RUNX2, the master transcription factor of osteoblast differentiation, likely through ubiquitin ligase SMURF1. In addition, THFA treatment increases oxidized NAD and NADP levels, which is observed in proline-free treatment and *Slc38a2* knockout cells as well. In summary, *Slc38a2* provides proline critical for protein synthesis, bioenergetics and redox homeostasis in osteoblast differentiation.

### 5.2.1 Proline as an energy source.

Here we identified proline as another important energy source for osteoblasts. Previous studies found glycolysis, fatty acid oxidation and glutamine catabolism provide energy for osteoblasts (Adamek et al., 1987; Karner et al., 2015; Lee et al., 2020). Since the sum of the energy that these three sources provide has exceeded 100%, it is even more confusing that how proline-derived ATP production fits into this “overwhelming” energy
pool. There are several possibilities that may explain why such a discrepancy exists. First, we found proline has the potential to be metabolized for bioenergetics; however, we do not know exactly whether proline is used for energy production in osteoblasts in vivo. Second, the amount of energy derived from proline metabolism may be limited. Proline oxidation inhibition by THFA only reduces about 10% of ATP level. This suggests that around 10% ATP is produced by proline oxidation in osteoblasts. Lastly, previous studies measured ATP level in cells at different stages using different methods. Lee et al. analyzed using Seahorse in mature osteoblasts that had been induced with osteogenic medium for 7 days (2020). Conversely, I measured ATP using a commercial kit in preosteoblasts with no osteogenic induction. In addition, compared to αMEM used for our osteoblast culture, Seahorse analysis uses DMEM-based medium that is supplemented with more glucose but does not contain a number of non-essential amino acids including alanine, asparagine and serine. The inconsistencies between the cell culture media may also cause significant differences in the measurement of ATP production. However, our Seahorse analysis is consistent with the study conducted by Lee et al. (2020) that approximately 40% ATP derives from glycolysis and the rest is from OXPHOS in specified preosteoblasts. Collectively, in order to resolve this confusion, we need to evaluate the percentage of each energy source in osteoblasts at different stages using the same method at the same time in the future.
I have shown that the addition of proline to medium drastically boosted the rate of ATP production from OXPHOS within one hour whereas OXPHOS had limited contribution in the absence of proline. Since PRODH is located at complex II in mitochondria, it is likely that PRODH provides electrons directly to electron transport chain for ATP production. Different from the canonical complex II enzyme SDH which catalyzes succinate, PRODH uses proline as the substrate. In the absence of proline, ATP production through OXPHOS is less than 20%. This is consistent with the study by Lee et al. that OXPHOS provides only 20% of ATP for mature osteoblasts (2020). Thus, SDH does not appear to have high activity in osteoblasts. Instead, osteoblasts might rely on PRODH to fulfill the functional role of complex II for energy production. However, there is a pitfall associated with this hypothesis. It is unknown about the role of proline in fatty acid metabolism, which contributes to energy production through OXPHOS as well. Indeed, proline metabolism is linked with fatty acid metabolism in worms and plants (Kim and Janick, 1991; Pang et al., 2014; Shinde et al., 2016). In plants, proline accumulation is directly associated with fatty acid accumulation (Kim and Janick, 1991; Shinde et al., 2016). In worms, mutations of proline catabolic enzyme alh6 (ortholog of Aldh4a1) leads to accelerated fatty acid mobility and enhanced expression of fatty acid oxidation genes (Pang et al., 2014). Therefore, we need to elucidate whether fatty acid metabolism is altered upon proline addition or proline metabolism inhibition.
5.2.2 Bias in codon usage for proline.

The other interesting observation is the proline tRNA AGG but not CGG is affected in cells treated with proline-free medium or deleted with Slc38a2. Unfortunately, I do not have the results for the other two proline tRNAs (TGG, GGG) potentially due to bad primer design. It will be interesting to investigate whether tRNAs TGG and GGG are affected like AGG in the same condition. In the future, I will redesign better primers for these two tRNAs. It is unclear why cells have such a preference for one tRNA to the other for the same amino acid. One potential possibility is codon usage bias. Codon usage bias is defined as the different frequencies of occurrence of synonymous codons in cDNA. Indeed, proline codon is biased in mouse cells: among CCT (corresponds to tRNA-AGG), CCC (corresponds to tRNA-GGG), and CCA (corresponds to tRNA-TGG), each encodes around 30% proline. In contrast, CCG (corresponds to tRNA-CGG) is only responsible for 10%. If we take a closer look at the osteoblast-associated genes, codon usage bias is even more apparent in individual gene. For example, 58% of proline in COL1A1, one of the highest expressed proteins in osteoblasts, is encoded by CCT while this number is 33% in OSX. Some other proteins like RUNX2 and OCN preferentially use CCC to encode proline. Interestingly, CCG is biased against in all of nine osteoblast proline-rich proteins in our preliminary screening. This is consistent with our finding that proline tRNA CGG is not significantly affected when proline is less accessible. However, what are the advantages of codon usage bias? One possibility is that distinct codons render different translational
efficiency. One study suggests translationally efficient codons may have higher elongation rate and translation accuracy (Plotkin and Kudla, 2011). Thus, it is likely that proline codon CCG might have less translational efficiency compared to the other three synonymous codons. The other intriguing aspect is that codon usage bias differs drastically across species. For example, the frequency of proline codon CCG is much higher in *Drosophila melanogaster*, while the frequencies of four proline codons are evenly distributed in maize. Likely, translational efficiency of the same codon also differs across different species.

5.2.3 The role of *Slc38a2* in chondrocyte differentiation during endochondral ossification.

In chapter 3, I have shown that *Slc38a2* is indispensable for chondrocyte differentiation during endochondral ossification. However, these findings also raise up more questions: What are the substrates for *Slc38a2* in chondrocytes? How does *Slc38a2* regulates chondrocyte differentiation and endochondral ossification? Our preliminary data showed that proline and glutamine uptake were reduced significantly in *Slc38a2* knockout sternal chondrocytes by CRISPR/Cas9 in *vitro* (Fig. 40). This suggests *Slc38a2* is able to transport proline and glutamine in chondrocytes *in vitro*. However, it is unknown whether it happens the same *in vivo*. Therefore, I proposed to test whether glutamine and proline are substrates for *Slc38a2* in cartilage tissues. I developed a protocol in which radio-labeled amino acid uptake assays can be successfully done in bone tissues *ex vivo*. I would like to extend the application of this protocol to *ex vivo* cartilage culture.
Figure 40. Slc38a2 is a proline and glutamine transporter in chondrocytes in vitro.

(A) RT-PCR showing Slc38a2 knockout and (B) amino acid uptake in Rosa26Cas9/Cas9 sternal chondrocytes infected with sgSlc38a2 or sgLuciferase as the control. n=3. Error bar depicts SD. *p≤0.05, **p≤0.005

Figure 41. Loss of Slc38a2 does not affect chondrocyte proliferation and type II collagen synthesis.

Images (A,A’) and quantifications of BRDU incorporation (B,C) and IF for COL2A1 (D,D’) on humerus sections of Prx1Cre;Slc38a2^+/− or Slc38a2^+/+ littermate controls at E15.5. Error bar depicts SD.
If proline and glutamine are the substrates for Slc38a2, how does proline and glutamine metabolism regulate chondrocyte differentiation? Previous studies have shown the role of glutamine in chondrogenesis and cartilage development (Stegen et al., 2019; Stegen et al., 2020b). Although dispensable for bioenergetics, glutamine metabolism is required for chondrocyte proliferation and collagen synthesis. However, our data demonstrate that targeting Slc38a2 in mesenchymal progenitors using Prx1Cre does not affect chondrocyte proliferation and protein expression of type II collagen (Fig. 41). This potentially suggests that Slc38a2 is not a glutamine transporter, or at least not a major glutamine transporter, in chondrocytes in vivo. However, it is still possible that targeting glutamine uptake does not exert the same effect as deletion of glutaminase likely due to the compensation from other glutamine transporters like Slc1a5. Additionally, Prx1Cre targets chondrocyte lineage much earlier compared to Col2Cre, which was used in previous papers (Stegen et al., 2019; Stegen et al., 2020b); by the time of our analysis, chondrocytes may have already adapted to the metabolic abnormality that results from Slc38a2 deletion. To test these possibilities, I will cross Slc38a2fl/fl animals with Col2Cre in the future. If Slc38a2 is a proline transporter, I would like to test whether proline metabolism regulates chondrocyte differentiation in the same way as osteoblast differentiation. I will investigate this question from three aspects: bioenergetics, biosynthesis and redox homeostasis.
5.2.4 Regulation of *Slc38a2*

Based on our studies, *Slc38a2* plays a critical role in osteoblast differentiation. Next, we are interested in what are the signals that regulate *Slc38a2* in osteoblasts? We demonstrated that WNT signaling pathway regulates glutamine consumption via amino acid transporters, *Slc1a5* and *Slc7a7*. To test whether WNT signaling also regulates *Slc38a2*/*SNAT2*, we treated ST2 cells with WNT3A for 24 hours and performed radiolabeled proline uptake assay. Proline uptake was significantly upregulated in response to WNT3A treatment (Fig. 42A). Next, we knocked out *Slc38a2* in ST2 cells and found that not only WNT-induced proline uptake, but also basal proline uptake was significantly reduced (Fig. 42A). This suggests that WNT3A is able to promote *Slc38a2* activity. We were then interested in how WNT signaling regulates *Slc38a2*. We noticed that WNT stimulated proline uptake as early as 1h (Fig. 42B). This led us to hypothesize WNT regulates *Slc38a2* via neither transcription nor translation. To test this hypothesis, we examined mRNA expression by quantitative PCR and protein expression by Western blotting in cells treated with WNT3A for 24h. Neither mRNA nor protein expression was induced by WNT3A, despite increased proline uptake (Fig. 42C,D). Therefore, WNT signaling does not regulate *Slc38a2*/*SNAT2* transcriptionally or translationally.
Figure 42. WNT signaling regulates Slc38a2 activity independent of transcription and translation.

(A) Proline uptake assay in Rosa26<sup>Cas9</sup>/Cas9 ST2 infected with Cas9, and sgSlc38a2 or sgLuciferase as the control in response to 24h WNT3A or vehicle. (B) Proline uptake assay in ST2 cells treated with WNT for 1h. qPCR of Slc38a2 (C) and Western blot analysis of SNAT2 (D) in ST2 cells in response to 24h WNT3A or vehicle. qPCR of Tcf7 (E) and proline uptake (F) in ST2 cells with Catnb1 knock down in response to 24h WNT3A or vehicle. Western blot of pS6 (S240/244) and total S6 (G), and proline uptake (H) in ST2 cells treated with or without rapamycin (H) in response to 1h WNT3A or vehicle (n=3). Error bar depicts SD. *p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005
A previous paper demonstrates that insulin promotes the translocation of SNAT2 from trans-Golgi network to cell membrane within 30 minutes after treatment (Hatanaka et al., 2006). Based on this, I hypothesize that WNT signaling can stimulate SNAT2 translocation to the membrane. To test this hypothesis, I will make a ST2 cell line stably expressing SNAT2 fused with GFP. I will perform live-cell imaging to monitor the movement of SNAT2 in response to WNT in real time. Additionally, since insulin signaling also plays a critical role in osteoblasts, insulin may regulate SNAT2 in osteoblasts through a similar mechanism (Ferron et al., 2010; Fulzele et al., 2010). In the future, I would like to investigate whether insulin regulates SNAT2 activity.

We are also interested in what is downstream effector of WNT signaling that regulates SNAT2 activity. Since WNT can stimulate proline uptake within a relatively short time after WNT treatment, it is less likely to be β-catenin-mediated transcriptional activity. We confirmed that β-catenin knockdown did not affect WNT-induced proline uptake in ST2 cells, suggesting WNT does not regulate SNAT2 through canonical signaling pathway (Fig. 42E,F). Conversely, rapamycin blocked WNT-induced proline uptake in ST2 cells in 1h (Fig. 42G,H). This indicates that WNT regulates SNAT2 via mTORC1 pathway.

To explore whether WNT regulation on SNAT2 has any in vivo relevance, I will measure SNAT2 activity in bones isolated from mice with WNT hyperactive allele (Lrp5<sup>A214V/+</sup>) and the ones with deletion of LRP5. To measure SNAT2 activity, I will perform
proline uptake in these bones *ex vivo*. I predict that proline uptake will increase in bones from WNT hyperactive mice (*Lrp5<sup>A214V/+</sup>*), while decrease in LRP5 knockout mice. If WNT regulates SNAT2 *in vivo*, I will also conditionally knock out *Slc38a2* in the genetic background of WNT hyperactive allele which has high bone mass phenotype. I expect that deletion of *Slc38a2* will reduce bone mass in this WNT hyperactive mouse model.

### 5.2.5 The source of proline for osteoblasts *in vivo.*

Osteoblasts can obtain amino acids through extracellular environment, biosynthesis and protein degradation. We demonstrated that osteoblasts acquire majority of proline from the extracellular milieu through the amino acid transporter *Slc38a2*. However, it is unknown about which extracellular sources provide osteoblasts with proline. One possibility is that osteoblasts obtain proline from the blood stream. To test this possibility, we can infuse mice with 13C-labelled proline and investigate whether proline can be traced from the circulation system into the bone tissue. Additionally, a provocative idea is that proline may be released from extracellular matrix. Pancreatic ductal adenocarcinoma cells upregulate metalloproteases to digest collagen fragments to release proline for the use of bioenergetics (Olivares et al., 2017). Similarly, osteoblasts are also embedded in a collagen-rich environment. Proline-rich collagens may provide osteoblasts with a natural reservoir of proline. Indeed, we found the number of TRAP<sup>+</sup> osteoclasts increased in *BglapCre;Slc38a2<sub>f/f</sub>* animals (Fig. 23). Osteoclasts can break down bone matrix and may release proline for osteoblasts. Interestingly, females who
experienced famine have higher rate of developing osteoporosis at older age (Kin et al., 2007; Kueper et al., 2015; Marcus and Menczel, 2007). It is likely that proper cellular functions might rely on nutrients released from bone matrix under nutrient-limited environment. However, this is at the expense of healthy bone quality. Therefore, we hypothesize that shortage of proline in osteoblast lineage cells signals via unknown mechanism to promote osteoclastogenesis, which frees up collagen fragments from matrix. A detailed investigation is warranted to explore whether osteoblasts obtain proline from collagen-rich matrix. If so, it is also interesting to investigate the mechanism through which osteoblasts signal to osteoclasts to increase the catabolism of collagens in bone matrix.

5.2.6 Sex dimorphism in BglapCre;Slc38a2 conditional knockout mice

We observed male BglapCre;Slc38a2 conditional knockout mice had reduced bone mass while females did not exhibit such a phenotype (Fig. 23, Table 4,5). Several possibilities might account for this sex dimorphism. First, estrogen may regulate Slc38a2 expression or activity in osteoblasts. One study shows that Slc38a2 promoter region possesses an estrogen receptor α (ERα) binding motif. Presence of estrogen promotes Slc38a2 expression in breast cancer cells (Morotti et al., 2019). It is unclear if estrogen regulates Slc38a2 in osteoblasts. In the future, we will directly treat osteoblasts with estrogen to investigate whether estrogen regulates Slc38a2. To directly examine the role of estrogen in Slc38a2 in vivo, we examine the expression of Slc38a2 in bones harvested
from ovariectomized female mice. Second, female adult bones may not even express 
Slc38a2 or have low Slc38a2 activity. In addition, adult female mice may employ a different 
system of proline transporters compared to males or embryos. Older studies 
demonstrated that proline uptake is sodium-dependent in embryonic membranous but 
not in adult diaphyseal bone (Hahn et al., 1969). This suggests that the transport systems 
for proline might be different between embryonic and adult stages. In our study, proline 
uptake is reduced in embryonic bones; however, it is not known whether proline uptake 
is affected in BglapCre;Slc38a2fl/fl mice, especially in females. If proline uptake is not 
affected in female conditional knockout mice, osteoblasts may utilize another set of 
transporters for proline. To directly test this hypothesis, we need to perform proline 
uptake assay in bones isolated from both male and female BglapCre;Slc38a2fl/fl mice.

5.2.7 NADH/NADPH in osteoblast differentiation.

In Chapter 3, we uncovered Slc38a2-mediated proline metabolism is required for 
redox homeostasis. Inhibition of proline uptake or oxidation leads to elevated levels of 
oxidized form of NAD+ and NADP+. Since ROS generation is reduced when proline 
oxidation or uptake is compromised, the role of NADPH in offsetting ROS is very likely 
to be trivial (Fig. 27). Glucose tracing also suggest that pentose phosphate pathway is not 
affected in THFA-treated cells or the ones deleted with proline transporter (Fig. 31). 
However, how NADH and NADPH regulates osteoblast differentiation remains unclear. 
One possibility is that NADH and NADPH may mainly act as cofactors for PYCRs to
facilitate proline cycle. Second, NAD+ is a known cofactor for SIRT1, which is able to deacetylate osteoblast regulators like RUNX2 and β-catenin. Lack of Slc38a2 or inhibition of proline oxidation elevates NAD+ levels, which may affect the expressions of these osteoblast regulators. In the future, we will directly target SIRT1 in order to investigate the potential role of NAD+-mediated proline-SIRT1 axis in osteoblast differentiation.

5.2.8 Slc38a2 as a potential target to increase bone formation and promote bone regeneration

Affecting more than 10 million people in the US, osteoporosis is characterized by low bone mass and vulnerability to fracture. Current treatments mainly focus on bone catabolism and very few regimens directly target bone anabolism. Our study shows that Slc38a2, as a proline transporter, is required for osteoblast differentiation and bone formation. In section 4.1, I discussed the possibilities of Slc7a7 and Slc1a5 as potential therapeutic targets for osteoporosis. I am also curious whether Slc38a2 can be a target to promote bone formation and combat osteoporosis. To test this hypothesis, I overexpressed Slc38a2 in calvarial osteoblasts. Preliminary data demonstrated that overexpression of Slc38a2 upregulated proline uptake (Fig. 43A,B). Additionally, Slc38a2 overexpression also increased the expression of osteogenic markers Ibsp, and matrix mineralization represented by von Kossa or alizarin red staining (Fig. 43C,D). This suggests that Slc38a2 is sufficient for osteoblast differentiation in vitro. To evaluate this question in vivo, I plan to make a mouse model with Slc38a2 overexpression. I expect to see mice overexpressed with Slc38a2 will have higher bone mass and better healing potential to bone fracture. If
so, we can develop therapeutic strategy to stimulate Slc38a2 activity against osteoporosis. To do so, we need to first understand how Slc38a2 is regulated. As mentioned above, Slc38a2 activity is regulated by WNT signaling. Activating WNT signaling pharmacologically may stimulate Slc38a2 activity to increase bone formation. Additionally, we would also like to perform a drug screening to find candidates that can promote the activity or expression of Slc38a2 and enzymes associated with proline metabolism. Meanwhile, it is also interesting to test whether a proline-rich diet is simply able to promote bone formation and bone regeneration.

![Graph A: Fold Change (hsSlc38a2/β-actin)](image)

![Graph B: Proline Uptake (% Control)](image)

![Graph C: Von Kossa and Alizarin Red staining](image)

![Graph D: Fold Change (Ibsp/β-actin)](image)

Figure 43. Overexpression of human Slc38a2 (hsSlc38a2) is sufficient to induce osteoblast differentiation in vitro.

qPCR of Homo Sapiens Slc38a2 (hsSlc38a2) (A) and proline uptake assay (B) in cOBs infected with Homo Sapiens Slc38a2, and luciferase as the control. (C) von Kossa and alizarin red staining of cOBs treated with OM for 10 days. (D) qPCR of lbsp in cOBs treated with OM for 7 days. Error bar depicts SD. **p≤0.005, ***p≤0.0005, ****p≤0.00005
## Appendix A

Table 6. Amino acid proportions of proteins associated with different GO Terms

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## Appendix B – Reagents and materials

Table 7. Antibodies

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Table 8. qPCR primers

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</tr>
<tr>
<td>Bglap</td>
<td>CAGCGGCCCTGCTGCTGA</td>
<td>GCCGGAGCTGTCTGATTTCATTA</td>
</tr>
<tr>
<td>Sp7</td>
<td>CCTTCTCAAGCACAATGG</td>
<td>AAGGGTGGGTAGTCTTGTGCAT</td>
</tr>
<tr>
<td>Runx2</td>
<td>CCAACCGAGTCTTTAAGGCT</td>
<td>GCTCAGTCGCTACATTGG</td>
</tr>
<tr>
<td>Slc7a7</td>
<td>CCTGCCCTCTACTTCTTCT</td>
<td>ATCGGGCGGTACGTTGATA</td>
</tr>
<tr>
<td>Slc1a5</td>
<td>TGGAGATGAAAGACGTTCGC</td>
<td>CAGGCGAGCCTGACACTGAT</td>
</tr>
<tr>
<td>Slc38a2</td>
<td>GGCTATGCACAGCTACCTTC</td>
<td>GTCAAGGGTCGATAACCACA</td>
</tr>
<tr>
<td>Tcf7</td>
<td>AGCGCTGCCATCAACCAGAC</td>
<td>TGGCCCTGCTTTTCGAGATAG</td>
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</tbody>
</table>

Table 9. qPCR primers for tRNA aminoacylation.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>tRNA</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>AGG</td>
<td>GGCTCAGTTGGTCTAGGGGTATG</td>
</tr>
<tr>
<td></td>
<td>CGG</td>
<td>GGTGCGTCTAGGGGTATGCTT</td>
</tr>
<tr>
<td>Leu</td>
<td>CAA</td>
<td>GTCAGGATGGCCAGGTGCTA</td>
</tr>
<tr>
<td></td>
<td>CAG</td>
<td>GTCAGGATGGCCAGGTGCTA</td>
</tr>
<tr>
<td>Glu</td>
<td>CTG</td>
<td>CCATGGTCTAGGGGTATGCTA</td>
</tr>
<tr>
<td></td>
<td>TTG</td>
<td>GTCTCCATGGTCTAGGGGTATG</td>
</tr>
<tr>
<td>Glu</td>
<td>TTC</td>
<td>CCCACATGGTCTAGGGGTATG</td>
</tr>
<tr>
<td>Asn</td>
<td>GTT</td>
<td>GTTTCCATAGGTAGTGGTTAT</td>
</tr>
<tr>
<td>Val</td>
<td>TAC</td>
<td>GCTTCCATAGGTAGTGGTTAT</td>
</tr>
</tbody>
</table>

Reverse | GCCTTGGCAGCCAGAATTC |

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Table 10. Mouse strains

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Source</th>
<th>RRID</th>
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<tbody>
<tr>
<td>Prx1Cre: B6.Cg-Tg(Prrx1-cre)1Cjt/J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:005584</td>
</tr>
<tr>
<td>BglapCre: B6N.FVB-Tg(BGLAP-cre)1Clem/J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:019509</td>
</tr>
<tr>
<td>Sp7Cre: B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:006361</td>
</tr>
<tr>
<td>Wild Type: C57Bl/6J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:000664</td>
</tr>
<tr>
<td>Rosa26^Cas9^: Gt(ROSA)26Sortm1.1(CAG-cas9^+,^-EGFP)Fexh/J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:024858</td>
</tr>
<tr>
<td>Rosa26^Flip^; 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J</td>
<td>The Jackson Laboratory</td>
<td>IMSR_JAX:003946</td>
</tr>
<tr>
<td>Slc38a2^LacZ^: C57BL/6N-A&lt;tm1Brd&gt;Slc38a2&lt;tm1a(KOMP)Wtsi&gt;/Wtsi Ph</td>
<td>Sanger MGP</td>
<td>MGI:5293947</td>
</tr>
</tbody>
</table>
References


Day, T.F., Guo, X., Garrett-Beal, L., and Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8, 739-750.


promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280, 33132-33140.


Lefebvre, V., Li, P., and de Crombrugghe, B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. Embo j 17, 5718-5733.


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Biography

Leyao Shen attended Vassar College for his undergraduate degree and graduated with honors in biology in 2016. At Vassar College, Leyao became interested in developmental biology and performed research with Dr. Jennifer Kennell on how WNT signaling regulates Drosophila wing development. After graduation, he joined Developmental and Stem Cell Biology (DSCB) program at Duke University and later got affiliated with the Department of Cell Biology.

Leyao joined the lab of Dr. Courtney Karner to study the role of amino acid transporters in osteoblast differentiation and bone formation. Leyao was the co-first author of the research article “Biphasic regulation of glutamine consumption by WNT during osteoblast differentiation” published in Journal of Cell Science. Leyao also wrote a book chapter “Radiolabeled amino acid uptake assays in primary bone cells and bone explants” included in Skeletal Development and Repair. Methods in Molecular Biology. Additionally, he contributed to several studies published in Cell Metabolism and Scientific Reports.

Leyao has been selected as a member of Sigma Xi Society since 2016. He is also a recipient of Duke Graduate School Conference Travel Award (2021) and DSCB Travel Award (2018). Leyao presented his work at several regional and national conferences including ORS 2021 annual meeting, ASBMR annual meetings (2019, 2020), SDB national and regional meetings (2018, 2020).