1. Specific Aims

Osteochondrodysplasias are a group of skeletal disorders affecting approximately 1:3,000-1:5,000 births and caused by a defect in the development of bone and cartilage (Rasmussen et al., 1996; Stevenson et al., 2012). These diseases are often linked to the abnormal development of osteoblasts and/or chondrocytes, which must differentiate from a common pool of mesenchymal progenitors prior to skeletal formation (Baldridge et al., 2010). Because the differentiation of osteoblasts and chondrocytes must be coordinated in order to maintain the proper balance of these two cell types, this process is tightly regulated and influenced by multiple signaling pathways, including BMP, FGF, IHH, and WNT. Although significant advances have been made in recent years to identify the core regulatory networks operating during skeletal development, our current understanding of osteoblast maturation and the cross-talk that occurs between signaling pathways during bone development and maintenance is incomplete. Because of this, current treatment strategies for osteochondrodysplasias such as achondroplasia and osteogenesis imperfecta are limited by undesirable side effects or the lack of an effective molecular treatment (Castillo et al., 2009; Jin et al., 2012). The goal of this proposed research is to further our understanding of the mechanisms that control osteoblastic differentiation in order to facilitate the development of more effective therapies for patients with these diseases. Our findings will also provide insights into the coordination of bone maintenance throughout life, leading to improved treatments for common diseases such as osteopenia and osteoporosis, which are predicted to adversely affect nearly 50 million Americans by the year 2020 and incur an estimated $25.3 billion healthcare burden per year (Dempster, 2011).

PR domain containing protein 5 (PRDM5) belongs to a conserved family of epigenetic regulators with diverse functions that were identified based on the presence of a characteristic PR domain sharing 20-30% identity with the SET domain (Fumasoni et al., 2007; Huang et al., 1998). Prdm5−/− mice exhibit delayed ossification of the long bones, reduced bone mineral density, and impaired osteoblastic differentiation, suggesting that PRDM5 may play a role in regulating bone formation (Galli et al., 2012). In osteoblasts, PRDM5 promotes the expression of a large number of collagen genes and extracellular matrix components, both by directly recruiting RNA polymerase II to promote transcriptional elongation and by binding to distal enhancers to activate transcription. This function is likely to explain the low bone mineral density of Prdm5−/− mice; however, the reason for their impaired osteoblastic differentiation is not well understood. Recent data suggests PRDM5 may bind to Special AT-rich sequence-binding protein 2 (Satb2), which encodes a transcription factor that is critical during terminal osteoblast
maturation, to regulate its expression (Galli et al., 2012). In addition, PRDM5 has also been shown to regulate WNT signaling by promoting or repressing the expression of many components of both canonical and non-canonical WNT signaling pathways (Meani et al., 2009; Shu et al., 2011). Based on these findings, we hypothesize that PRDM5 promotes terminal osteoblastic differentiation by two mechanisms: 1) regulating the expression of WNT signaling pathway components and 2) enhancing Satb2 expression through an interaction with the lineage-specifying transcription factor Osterix (Figure 1).

**Aim 1: To determine the effect of PRDM5 on Satb2 expression and osteoblastic differentiation.** To investigate whether PRDM5 regulates Satb2 expression, we will examine the effects of Prdm5 knockdown and overexpression on the expression of Satb2 in MC3T3 cells. To confirm that Prdm5 is an upstream regulator of Satb2, we will determine the effects of simultaneous ectopic Prdm5 expression and siRNA-mediated Satb2 knockdown on the expression of downstream markers of osteoblastic differentiation. To determine which stages of osteoblastic differentiation require PRDM5 activity, as well as to study the effects of its aberrant expression, we will assay for defects in skeletal development in Prdm5 conditional null and overexpression mouse models. Transcription activator-like effector nuclease (TALEN) technology will be used to generate floxed Prdm5 and Rosa26-LSL-Prdm5 alleles, which will then be crossed to various existing Cre lines.

**Aim 2: To determine the role of PRDM5 in regulating WNT signaling during terminal osteoblast differentiation.** To demonstrate that PRDM5 regulates WNT signaling in osteoblasts *in vitro*, we will measure and compare the expression of several known targets of WNT signaling using qRT-PCR in wild-type and Prdm5 mutant primary osteoblasts. We will also compare the levels of canonical and non-canonical WNT signaling in these cells using reporter constructs specific to each pathway. To test this relationship *in vivo*, Prdm5 mutant mouse lines...
will be crossed to an existing Dkk2-null mouse model that lacks expression of the canonical WNT antagonist DKK2 (Li et al., 2005). We expect that Prdm5 depletion will enhance the osteopenic phenotype of Dkk2-null mice and Prdm5 overexpression will suppress it. We also expect that ectopic Prdm5 expression in Dkk2⁻/⁻ primary osteoblasts will rescue the mineralization and WNT signaling defects in these cells.

**Aim 3: To determine the upstream signaling pathways that activate the expression of endogenous Prdm5 during bone development.** Characterization of the upstream pathways that induce endogenous Prdm5 expression will lead to a better understanding of the extrinsic signaling cues that regulate bone development and maintenance. Several lines of evidence point to the transcription factor Osterix as a potential upstream regulator of Prdm5. The dependency of Prdm5 expression on OSX will be tested using a Tet-off inducible expression system. To identify additional candidate pathways that potentially regulate the expression of Prdm5, we will perform DNA pulldown experiments in MC3T3 cells using a conserved fragment of the Prdm5 promoter as bait to identify transcription factors that bind to the Prdm5 promoter during osteoblastic differentiation.

2. **Research Strategy**

2.1 **Significance**

The role of PRDM5 in regulating osteoblastic differentiation has implications during skeletal development and in the postnatal maintenance of proper bone density. Understanding the specific functions of PRDM5 at different stages of skeletal development would provide valuable insight into the pathogenesis of osteochondrodysplasias, for which very few effective therapies currently exist; however, perhaps more significant is the role that PRDM5 may play in governing the balance between bone deposition and resorption throughout life by regulating the balance between osteoblast and osteoclast numbers. Tight regulation of these two processes is critical; diseases such as osteoporosis occur when bone resorption is uncoupled from and allowed to outpace bone deposition. This can occur either because of increased bone resorption (high-turnover osteoporosis) or because of decreased bone deposition (low-turnover osteoporosis), a common occurrence during aging and during the use of glucocorticoids. Currently available treatments for osteoporosis, such as bisphosphonates, are primarily antiresorptive and act by inhibiting osteoclast differentiation and function. However, in the case of low-turnover osteoporosis, in which reduced bone deposition is the primary defect, an anabolic
therapy would be a more ideal treatment. In addition, the side effects associated with commonly used anti-resorptive treatments highlight the need for an anabolic solution (Khosla et al., 2008; Zaidi, 2007). Based on the relatively subtle role PRDM5 plays in promoting osteoblastic differentiation, gene therapy strategies based on Prdm5 may represent safe and effective anabolic therapies for osteoporosis and other osteopenia-related conditions. The healthcare burden and increasing prevalence of osteoporosis, especially in postmenopausal women, due to the increasingly elderly population makes research into the underlying pathology of and potential treatments for osteoporosis of significant importance and impact.

A clue to the potential function of PRDM5 during skeletal development is its role as an inhibitor of canonical WNT signaling. In this capacity, PRDM5 suppresses tumor formation in multiple tissues and is critical to proper nervous system development in zebrafish (Deng and Huang, 2004; Duan et al., 2007; Meani et al., 2009; Shu et al., 2011; Watanabe et al., 2007). Understanding of the mechanisms by which PRDM5 regulates canonical and non-canonical WNT signaling in osteoblasts could be applied to PRDM5’s functions outside of the context of bone development. This could be especially relevant to the study of cancers that are dependent on WNT signaling and often associated with dysregulation of Prdm5, such as colorectal cancer, or cancers which invade bone tissue, including breast cancer and multiple myeloma. In addition, because other PRDM family members have also been linked to regulation of WNT signaling (Wang et al., 2013b), the mechanisms underlying PRDM5-mediated inhibition may be shared among multiple PRDM family members. Uncovering these mechanisms will provide insight not only to the role of PRDM5 during skeletal development, but also potentially to the general role of PRDM family members in a diverse array of developmental contexts.

2.2 Innovation

PRDM5, as a protein bridging the gap between chromatin modifications and direct transcriptional regulation, provides a unique opportunity to study the relationship between epigenetic and genetic mechanisms orchestrating the differentiation of a pool of multipotent stem cells into competing lineages. Classically, lineage commitment is thought to be governed by the expression of so called “master regulators.” These are transcription factors that are expressed in only a single cell type within a given lineage and are both necessary and sufficient for the differentiation of a cell toward a particular fate. Several members of the PRDM family have been implicated as master regulators controlling these types of developmental switches. PRDM1 plays a critical role in the differentiation of antibody-producing plasma cells, PRDM14
controls the production of primordial germ cells from epiblast-derived stem cells, and PRDM16 regulates the switch between myoblasts and brown adipocytes (Kajimura et al., 2009; Shapiro-Shelef et al., 2003; Yamaji et al., 2008, 2013).

Although this paradigm has proven successful in describing certain developmental scenarios, a focus on the identification of “master regulators” has prevented us from appreciating the significance of other factors that play important roles during cell fate decisions (Ciofani et al., 2012). Recent evidence gathered by studying the differentiation of naïve CD4+ T-lymphocytes into various types of T-helper cells suggests that lineage determination involves combinatorial regulation by multiple factors and cannot be explained solely through the expression of a single master regulator (Oestreich and Weinmann, 2012). Coexpression of multiple lineage-specifying transcription factors occurs during T-lymphocyte differentiation and allows even mature, differentiated cells to exhibit a significant degree of lineage plasticity during a specialized immune response. Foundational to this new model of lineage commitment is the idea that cell fate decisions do not represent “all-or-nothing” transitions from one master regulator to another, but that in certain circumstances, low-level expression of competing lineage-specifying factors allows for lineage reprogramming in response to the appropriate external stimuli. In addition, this model recognizes the contributions of epigenetic regulators and other factors during lineage commitment. For instance, evidence points to the importance of establishing a competent epigenetic landscape early in differentiation, permitting lineage-specifying transcription factors to access specific subsets of competent loci at later stages and drive progression toward a particular cell fate (Samstein et al., 2012; Vahedi et al., 2012). Cofactors and other complex components may lie pre-assembled at competent binding sites prior to the expression of a master regulator, setting the stage for commitment to a particular lineage in response to the appropriate stimuli.

The lineage flexibility exhibited by osteoblast and chondrocyte precursors indicates that the differentiation of these cell types is unlikely to be controlled solely by a small number of master regulators. Instead, it is likely that multiple epigenetic and genetic factors must cooperate and be maintained in a fine balance to establish stable cell fates. PRDM5 is likely to contribute to the lineage commitment of osteoblasts and chondrocytes by promoting an epigenetic landscape conducive to osteoblastic differentiation through the recruitment of chromatin remodeling enzymes and other cofactors to specific target sites throughout the genome. Elucidating the specific mechanisms by which this occurs and the interactions between PRDM5 and other lineage-specifying factors during osteoblastic differentiation will advance our understanding of how the balance between these cell types is maintained during skeletal
development and throughout life. In addition, general principles regarding the integration of genetic and epigenetic mechanisms to regulate initial cell fate commitment, lineage reprogramming, and terminal differentiation in this system can be applied to similar processes occurring in other developmental contexts.

2.3 Approach

2.3.1 Preliminary Studies

Transcriptional regulation of osteoblast differentiation during bone development

Bone formation occurs by two distinct mechanisms: the majority of the skeleton is generated by endochondral ossification, while bones of the skull and face are created through intramembranous ossification. Endochondral ossification requires the prior establishment of a cartilage template, whereas intramembranous ossification occurs by direct deposition of bone matrix. Both processes, however, are preceded by the formation of condensations of mesenchymal progenitor cells, from which both osteoblasts and chondrocytes differentiate (de Crombrugghe et al., 2001). Figure 2 summarizes a few of the markers that are expressed at different stages of osteoblast and chondrocyte differentiation, as well as some of the feedback mechanisms and extrinsic signaling cues that guide the highly coordinated development of these two cell types.

One of the earliest markers signifying commitment to the osteoblastic lineage is the transcription factor Runx2. Runx2 was originally cloned by virtue of its binding to a cis-regulatory element within the promoter of Osteocalcin (Bglap), one of the few genes expressed uniquely in osteoblasts and an important marker of osteoblast maturation (Ducy et al., 1997). Runx2 is now known to be a master regulator of osteoblast differentiation, being necessary for skeletal formation and sufficient to induce the expression of many downstream osteoblastic markers (Karsenty, 2008; Komori et al., 1997). Among the downstream targets of RUNX2 are the transcription factors Osterix (Sp7), another major determinant of osteoblast identity that is necessary for skeletal formation, and Satb2 (Nakashima et al., 2002). SATB2 binds AT-rich sequences known as matrix-attachment regions (MARs) and links them to the nuclear matrix to establish higher-order chromatin structures and promote long-range promoter-enhancer interactions. SATB2 is also known to promote osteoblast maturation and the expression of Bglap by physically interacting with ATF4 and RUNX2, the transcription factors primarily
responsible for controlling *Bglap* expression, to enhance their DNA-binding and transactivation potential (Dobreva et al., 2006; Yang et al., 2004).

**WNT signaling in bone development and maintenance**

WNT proteins are a family of secreted glycoproteins that play important roles in a number of developmental processes, including cellular proliferation, migration, and establishment of cell polarity. WNT signaling pathways can be generally divided into two categories: those that activate canonical WNT/β-catenin signaling and those that utilize non-canonical (β-catenin-independent) mechanisms. Canonical WNT signaling involves the simultaneous binding of a canonical WNT ligand (WNT1, WNT3A, etc.) to a member of the Frizzled family of surface receptors and either LRP5 or LRP6. This interaction leads, through various mechanisms, to the stabilization of cytoplasmic β-catenin. Once β-catenin is stabilized, it can be imported into the nucleus where it is free to interact with members of the TCF/LEF family of transcription factors and activate transcription of target genes such as *c-Myc* and

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**Figure 2: Control of osteoblast and chondrocyte differentiation.** Osteoblasts and chondrocytes differentiate from a common pool of mesenchymal progenitor cells. During endochondral ossification, chondrocyte precursors develop first into Sox9 expressing chondrocytes, which deposit the cartilage template that will be later converted to bone. Pre-hypertrophic chondrocytes secrete IHH, which triggers the differentiation of Runx2-positive osteoblast precursors from osteochondroprogenitors. Runx2 enhances the transcription of many downstream osteoblast-specific markers, including Osg, Satb2, and Bglap, which signal progressive stages of osteoblast maturation. WNT signaling generally promotes osteoblast proliferation while simultaneously suppressing chondrocyte differentiation, however, inhibition of WNT signaling is important during the final stages of osteoblast maturation and may be mediated in part by Osg. Red boxes indicate Cre lines to be used in this study (Aim 1).
Two non-canonical WNT signaling pathways have been studied thoroughly, the planar cell polarity (PCP) pathway and WNT/Ca\(^{2+}\) signaling (Clark et al., 2012). Although poorly described in most circumstances, significant overlap exists between canonical and non-canonical WNT signaling pathways, creating the potential for complex feedback mechanisms that regulate the balance between these two types of signaling in a given cell type. For example, interaction between WNT5A and the ROR2 tyrosine kinase receptor has been specifically implicated in PCP signaling through activation of JNK and the AP-1 transcriptional complex; however, this interaction competes with canonical WNT3A for binding to the FZD2 and LRP5 coreceptors, thereby inhibiting canonical WNT signaling (Figure 3) (Clark et al., 2012; Liu et al., 2008; Mikels and Nusse, 2006; Oishi et al., 2003; Sato et al., 2010).

The importance of canonical WNT/β-catenin signaling in bone formation was originally recognized as a result of the identification of LRP5 mutations in patients with abnormal bone mass (Boyden et al., 2002; Gong et al., 2001; Kato, 2002). Further studies produced three primary lines of evidence demonstrating the critical role of WNT/β-catenin signaling in promoting bone formation during skeletal development and throughout life (Holmen, 2005; Joeng et al., 2011; Krishnan, 2006). First, canonical WNT signaling drives mesenchymal progenitor cells toward the osteoblastic lineage by simultaneously promoting the expression of key osteoblast markers (Osx, Alp, Bglap) and repressing PPARγ and SOX9, the master regulators of adipocyte and chondrocyte differentiation, respectively (Bennett et al., 2005; Hill et al., 2005; Rawadi et al., 2003). Second, WNT/β-catenin signaling promotes the proliferation of early osteoblast precursors and is necessary for proper osteoblast differentiation (Day et al., 2005; Hill et al., 2005; Hu, 2004). Lastly, inhibition of canonical WNT signaling by antagonists such as DKK1 causes severe defects in bone formation (Li et al., 2006; Morvan et al., 2006).
Interestingly, although canonical WNT/β-catenin signaling generally promotes bone formation, inhibition of canonical WNT signaling may be important for terminal osteoblast differentiation and for maintaining the proper balance of osteoblasts and osteoclasts postnatally (Glass et al., 2005; Li et al., 2005; Rodda and McMahon, 2006; Zhang et al., 2008). A common mechanism by which canonical WNT signaling is inhibited is the expression of secreted factors that directly antagonize WNT ligand/receptor interactions. DKK1, DKK2, and SOST are three such secreted factors that are expressed during terminal osteoblast differentiation and mediate inhibition of WNT/β-catenin signaling (Figure 3) (Li et al., 2005; Yang et al., 2010; Zhang et al., 2008, 2012). Another mechanism for inhibiting canonical WNT/β-catenin signaling is the upregulation of alternative, non-canonical WNT pathways, due to the competition between these two pathways for shared coreceptors and intracellular mediators. The cross-talk between canonical and non-canonical WNT signaling has not been studied thoroughly in osteoblasts, but may play an important role in ensuring proper skeletal development and maintenance.

**Prdm5 is a tumor suppressor that regulates WNT signaling**

PRDM5 was first characterized as a tumor suppressor based on its location within a region on human chromosome 4 that is commonly deleted in various types of human tumors. Ectopic Prdm5 expression causes increased rates of apoptosis and cell cycle arrest in breast, ovarian, and hepatic cancer cell lines (Deng and Huang, 2004). Additional studies showed that Prdm5 is also downregulated in a large percentage of gastric, colorectal, and cervical cancers due to promoter hypermethylation, prompting questions about the mechanism by which PRDM5 suppresses tumor formation in such a wide variety of cell types (Cheng et al., 2010; Watanabe et al., 2007). Meani et al. performed expression microarray analysis in a human osteosarcoma cell line that had been transfected with a Prdm5 expression vector and found differential expression of 32 genes related to WNT signaling, including upregulation of the canonical WNT antagonists DKK1, DKK2, and KREMEN1 and the non-canonical WNT ligand WNT5A (Meani et al., 2009). These findings were confirmed by analyzing the effects of Prdm5 knockdown and overexpression in a zebrafish model, which showed that PRDM5 inhibited both canonical and non-canonical WNT signaling in this context. In addition, other studies have found that downstream canonical WNT signaling is reduced upon ectopic Prdm5 expression (Shu et al., 2011). Thus, the tumor suppressive activity of PRDM5 may be derived from its capacity to regulate both canonical and non-canonical WNT signaling.

**Prdm5 is a transcriptional regulator in osteoblasts**
Previously, the effects of reduced Prdm5 expression were modeled in mice using a gene trap system that introduced a β-galactosidase-neomycin cassette into intron 2 of the gene (Prdm5\(^{LacZ}\)) (Galli et al., 2012). Using this system, Prdm5 expression was visible within cartilaginous templates and skeletal elements beginning at E14.5 in the mouse embryo and was restricted to osteoblasts within the periosteum and trabecular bone compartment at E16.5. ChIP-seq performed on the pre-osteoblastic MC3T3 cell line revealed that PRDM5 binds to 42 of the 43 collagen genes in the mouse genome and a number of important extracellular matrix components, including Decorin, Fibromodulin, and Biglycan (Galli et al., 2012). siRNA-mediated knockdown of Prdm5 in MC3T3 cells caused reduced expression of Col1α1, Col1α2, and Decorin, suggesting that PRDM5 may promote transcription of these genes in osteoblasts. In general, genes containing PRDM5 binding peaks tended to be more highly expressed and were associated with increased levels of H3K4me3 and RNA polymerase II occupancy than genes not occupied by PRDM5, although colocalization with H3K9me3 was observed at a subset of loci. This result, along with other evidence, suggests that PRDM5 can act as either a transcriptional activator or repressor depending on the cellular context and its particular binding partners (Duan et al., 2007). In addition to genes encoding collagen and extracellular matrix components, PRDM5 binding peaks were also observed near the promoter of Satb2, a transcription factor that promotes osteoblast maturation by enhancing Bglap and Ibsp expression. Regulation of Satb2 expression by PRDM5 may provide an explanation for the

![Figure 4: Impaired osteoblastic differentiation in Prdm5-null osteoblasts.](image)

Figure 4: Impaired osteoblastic differentiation in Prdm5-null osteoblasts. qRT-PCR was used to measure the relative expression of several significant markers of osteoblastic differentiation in wild-type and Prdm5\(^{LacZ/LacZ}\) calvarial osteoblasts. The expression of Bglap1 and Ibsp, late markers of osteoblastic differentiation, are significantly reduced in cells lacking Prdm5, while the expression of early markers Osx, Opn, and Runx2 remains unchanged.
reduced \textit{Bglap} and \textit{Ibsp} expression observed in \textit{Prdm5}^{\text{LacZ/LacZ}} osteoblasts, which is indicative of impaired osteoblastic differentiation (Figure 4) (Galli et al., 2012).

2.3.2 Aim 1: To determine the effect of PRDM5 on \textit{Satb2} expression and osteoblastic differentiation.

\textit{Regulation of Satb2 expression by PRDM5}

To investigate the role of PRDM5 in regulating \textit{Satb2} expression (Figure 5), MC3T3 cells will be transfected with siRNAs designed to target \textit{Prdm5}. The relative expression of \textit{Satb2} in these cells will be measured using qRT-PCR and compared to its expression in MC3T3 cells transfected with a scrambled siRNA that does not recognize \textit{Prdm5} and unaltered MC3T3 cells. The expression of \textit{Bglap} and \textit{Ibsp} will be measured as a positive control since \textit{Prdm5} depletion has been previously shown to reduce the expression of these two genes (Galli et al., 2012). The effect of \textit{Prdm5} knockdown on \textit{Satb2} expression will be compared to the effect of siRNA-mediated knockdown of \textit{Osx}, which has been previously shown to activate \textit{Satb2} expression in osteoblasts (Tang et al., 2011). We expect that siRNA-mediated knockdown of \textit{Prdm5} will cause a significant reduction in \textit{Satb2} expression (Figure 6). To confirm that \textit{Prdm5} is an upstream regulator of \textit{Satb2}, we will attempt to rescue the effects of \textit{Prdm5} knockdown on \textit{Bglap} and \textit{Ibsp} expression through transient transfection with a \textit{Satb2} expression vector (Figure 6; siRNA-Prdm5/Satb2 OE). Overexpression of \textit{Satb2} should rescue the effects of \textit{Prdm5} depletion if \textit{Prdm5} lies upstream of \textit{Satb2}. Conversely, ectopic expression of \textit{Prdm5} is not expected to rescue the effects of \textit{Satb2} knockdown (Figure 6; siRNA-Satb2/Prdm5 OE).

To confirm the binding of PRDM5 to putative sites within the \textit{Satb2} promoter, ChIP-qPCR will be performed in MC3T3 cells. The relative amounts of PRDM5 binding will be compared between siRNA-transfected MC3T3 cells and control cells transfected with either a
scrambled siRNA or an empty vector. These experiments will be repeated in an ex vivo model system using primary osteoblasts isolated from wild-type and Prdm5-null mice as previously reported (Galli et al., 2012). Because OSX has also been shown to bind to a GC-rich region within the Satb2 promoter, it is likely that PRDM5 and OSX cooperate to regulate Satb2 expression (Tang et al., 2011). To characterize this potential interaction, PRDM5 and OSX co-occupancy at the Satb2 promoter will be measured using sequential-ChIP in MC3T3 cells. The occupancy of OSX at its binding sites will also be measured in MC3T3 cells transfected with Prdm5-targeted siRNAs in order to determine if PRDM5 is responsible for recruiting OSX to the Satb2 promoter.

The effects of aberrant Prdm5 expression on osteoblastic differentiation in vivo

The gene trap system used by Galli et al. was well-suited for an initial investigation of the function of PRDM5 because it allowed the endogenous Prdm5 expression pattern and the effects of Prdm5 knockdown to be simultaneously examined. However, in this system, Prdm5 depletion did not occur with equal efficiency in all tissues examined and stage-specific functions of PRDM5 could not be discriminated because of the organism-wide integration of the gene trap. In order to study the specific role of PRDM5 in regulating osteoblastic differentiation, a system must be developed that is able to achieve efficient knockdown of Prdm5 in a cell type-restricted manner. This will be accomplished by generating conditional null and overexpression Prdm5 alleles and introducing these alleles into mice expressing Cre recombinase under the control of various cell-type specific promoters.
Traditional gene targeting techniques developed in the 1980’s have proven invaluable for the targeted disruption of genes in ES cells (Thomas et al., 1986). However, these classical techniques suffer from the need for time-consuming vector construction and low rates of spontaneous homologous recombination in mouse ES cells. The recent development of revolutionary “designer nuclease” technologies, which utilize a targeted double-strand break at the desired site of modification in order to provide increased rates of homologous recombination, has allowed researchers to make targeted modifications of mammalian genomes.

![Diagram of the endogenous Prdm5 locus and the donor template, including loxP sites flanking exon 1 indicated by red arrows, to be used in generating the Prdm5 allele. b) Outline of TALEN-mediated genome editing. TALE domains direct TALEN proteins to target sites flanking the desired site of modification. Once localized, FOK1 domains dimerize and create a DSB at the target site. This break can be repaired through either non-homologous end joining (NHEJ) or homologous recombination (HR) mechanisms. NHEJ-mediated repair generates a series of deletion and insertion alleles that are useful for future studies. HR-mediated repair will result in the incorporation of the donor template into the target site.]

**Figure 7: Strategy for generating floxed Prdm5 allele using TALENs.** a) Diagram of the endogenous Prdm5 locus and the donor template, including loxP sites flanking exon 1 indicated by red arrows, to be used in generating the Prdm5 allele. b) Outline of TALEN-mediated genome editing. TALE domains direct TALEN proteins to target sites flanking the desired site of modification. Once localized, FOK1 domains dimerize and create a DSB at the target site. This break can be repaired through either non-homologous end joining (NHEJ) or homologous recombination (HR) mechanisms. NHEJ-mediated repair generates a series of deletion and insertion alleles that are useful for future studies. HR-mediated repair will result in the incorporation of the donor template into the target site.
more quickly and efficiently than could be achieved using traditional methods (Moehle et al., 2007; Mussolino and Cathomen, 2012; Qiu et al., 2013; Smih et al., 1995). TALENs are one such type of designer nuclease, consisting of a TAL effector (TALE) domain, which provides DNA binding specificity, fused to the FOK1 endonuclease domain, which cleaves double-stranded DNA. The specificity of TALE site recognition is determined by a central repeat domain comprised of a variable number of 34 amino acid repeats, each of which recognize a single nucleotide in the target sequence (Moscou and Bogdanove, 2009). The 12th and 13th amino acids in each repeat are the most critical in determining specificity and can be artificially designed to recognize any of the four DNA nucleotides (Miller et al., 2010). Tandem arrays of repeats within the TALE domain allow for the recognition of specific sequences up to 25 base pairs in length.

A major advantage of TALEN technology is that genome modifications can be made directly in one-cell embryos derived from a wild-type donor, bypassing the need to establish cultures of modified ES cells and screen chimeric animals for successful germline transmission. As a result, the speed and ease with which mutant animals can be generated are greatly increased. In order to introduce targeted mutations into one-cell embryos, a pair of TALEN proteins designed to recognize sequences immediately adjacent to the desired point of insertion/mutation are introduced along with a donor construct containing the sequences that are to be inserted and short arms of homology (approximately 500 bp) to allow for homologous recombination (Bogdanove and Voytas, 2011; Wang et al., 2013a; Wefers et al., 2013). Using this strategy, a floxed allele of \textit{Prdm5} (Prdm5\textsuperscript{flo}) will be generated by introducing \textit{loxP} sites into positions flanking exon 1 of the gene (Figure 7). Conditional deletion of exon 1 is predicted to abolish expression of all known \textit{Prdm5} transcripts. In addition to the conditional \textit{Prdm5\textsuperscript{flo}} allele, NHEJ-mediated repair will also generate a series of \textit{Prdm5} deletion mutants that can be compared to other \textit{Prdm5}-knockout models. mESCs containing a Rosa26-LSL-Prdm5 allele (Prdm5\textsuperscript{LSL}) will be generated by knocking \textit{Prdm5} into the ubiquitously expressed \textit{Rosa26} promoter behind a transcriptional/translational termination sequence (STOP) flanked by \textit{loxP} sites using classical mESC manipulations (Lakso et al., 1992). The introduction of the STOP cassette prevents premature expression driven from the \textit{Rosa26} promoter, allowing \textit{Prdm5} expression to be temporally and spatially controlled through the use of cell-type specific Cre recombinase expression (Canalis et al., 2012).

\textit{Sox9-Cre}, \textit{Runx2-Cre}, \textit{Osx-Cre}, and \textit{Bglap-Cre (Oc-Cre)} mouse lines have been previously developed and used to study the role of various regulators of osteoblast differentiation (Akiyama et al., 2005; Rauch et al., 2010; Rodda and McMahon, 2006; Zhang,
The use of these Cre lines in combination with conditional Prdm5 alleles will allow the function of PRDM5 to be studied at different stages of skeletal development (Figure 2). Based on the putative functions of PRDM5, activation of Prdm5 within mesenchymal progenitors through the use of either Runx2-Cre or Sox9-Cre is expected to cause defects in the development of the cartilaginous skeleton. In order to characterize these defects, whole-mount Alcian blue staining will be performed on E13.5-E16.5 wild-type and Prdm5fl/fl mouse embryos (Nagy et al., 2009). Conditional deletion of Prdm5 in more mature osteoblasts using Osx-Cre, and Bglap-Cre is expected to produce a phenotype similar to that observed in Prdm5LacZ/LacZ mice, namely delayed ossification and reduced bone mineral density (Galli et al., 2012). Von Kossa staining will be performed on tibial sections of E16.5 and E18.5 embryos to visualize the degree of ossification in wild-type and Prdm5−/− embryos. Bone mineral density will be analyzed in femoral sections of 5 week old mice using peripheral quantitative computed tomography (pQCT).

2.3.3 Aim 2: To determine the role of PRDM5 in regulating WNT signaling during terminal osteoblast differentiation.

Effect of Prdm5 depletion and overexpression on canonical and non-canonical WNT signaling

Several targets of Prdm5 identified by ChIP-seq in MC3T3 cells are components of WNT signaling pathways (Table 1). Among these, Fzd2, Fzd5, Fzd7, Dvl3, Wnt5b, and Ror2 are related to non-canonical WNT5A signaling and Wnt3a and Wnt10b have both been shown to play significant roles in osteoblast differentiation by mediating canonical WNT/β-catenin signaling (Liu et al., 2008; Oishi et al., 2003; Yamagata et al., 2011). The enrichment of WNT signaling components in the set of genes bound by PRDM5, in addition to evidence in other
systems, suggests that it may inhibit canonical WNT signaling during osteoblast differentiation by upregulating components of non-canonical WNT pathways (Figure 8).

To test this hypothesis, we will begin by assessing the effects of PRDM5 depletion and overexpression on canonical and non-canonical WNT signaling by measuring downstream activation of these pathways in primary calvarial osteoblasts isolated from wild-type, Rosa26-Cre; Prdm5^fl/fl, and Rosa26-Cre; Prdm5^LSL mice. The TOPFlash construct (Addgene) comprises a tandem array of seven TCF/LEF response elements fused to Luciferase protein and will be used to measure the relative activation of canonical WNT/β-catenin signaling in transfected cells. Downstream activation of non-canonical WNT5A signaling will be measured by transfecting primary osteoblasts with an AP-1 reporter construct (BPS Bioscience). Because Prdm5 binding sites in the WNT5A receptor genes Fzd2, Fzd5, Fzd7, and Ror2, co-localize with histone modifications associated with active transcription in osteoblasts, it is expected that Rosa26-Cre; Prdm5^fl/fl osteoblasts will exhibit reduced JNK/AP-1 signaling. This reduction in non-canonical WNT signaling is also expected to be accompanied by an elevation of canonical WNT/β-catenin signaling, potentially due in part to competition between WNT3A and WNT5A for the FZD2 receptor (Mikels and Nusse, 2006; Sato et al., 2010). To further characterize the influence of PRDM5 on canonical and non-canonical WNT signaling during skeletal development, we will perform RNA in situ hybridization in E13.5-E16.5 wild-type and Prdm5-mutant embryos to measure the expression of Wnt5a, Wnt5b, Wnt10b, and WNT5A receptor genes bound by PRDM5 during bone development.

**Genetic interaction of Prdm5 and Dkk2 mutations**

Prdm5^LacZ/LacZ and Dkk2^-/- mice display similar phenotypes, including reduced bone mineral density and impaired osteoblastic differentiation (Galli et al., 2012; Li et al., 2005). DKK2, like other members of the dickkopf family of proteins, inhibits canonical WNT signaling, however, it may also promote WNT signaling in an LRP6-dependent manner under certain conditions.
circumstances (Brott and Sokol, 2002). These findings suggest that PRDM5 and DKK2 may function redundantly to inhibit WNT signaling during terminal osteoblast differentiation. To test this relationship, Rosa26-Cre; Prdm5[^6][^7] and Rosa26-Cre; Prdm5[^5][^L][^S][^L] mice will be crossed to Dkk2[^−] mice to generate mice homozygous for both alleles. Loss of Prdm5 in a Dkk2[^−] background is expected to enhance the mineralization defects of osteoblasts isolated from Dkk2[^−] mice (Figure 9), whereas Prdm5 overexpression should have the opposite effect. In addition, osteoblasts isolated from Dkk2[^−] mice exhibit increased levels of canonical WNT signaling (Figure 9). If PRDM5 and DKK2 function redundantly to inhibit WNT signaling, ectopic Prdm5 expression would be expected to suppress the enhanced LEF-1 reporter activity observed in Dkk2[^−] osteoblasts. Mineralization potential will be assessed using Alizarin red staining in cultures of primary calvarial osteoblasts after stimulation of osteogenic differentiation. WNT signaling will be measured using the previously described TOPFlash and AP1 reporter constructs. Bglap and Ibsp expression will also be measured in wild-type and Prdm5, Dkk2 double mutant osteoblasts to assess their differentiation potential upon osteogenic stimulation.

2.3.4 Aim 3: To characterize the upstream signaling pathways that activate the expression of endogenous Prdm5 during bone development.

*Prdm5 is a potential downstream effector of Osterix*

Several lines of evidence suggest that OSX may control the expression of Prdm5 within developing osteoblasts. As proposed for PRDM5, OSX directly regulates Satb2 expression by binding a GC-rich region within the proximal promoter (Tang et al., 2011). Enhancement of
Prdm5 expression by OSX combined with dual-control of Satb2 expression by OSX and PRDM5 would form a transcriptional network motif known as a coherent feedforward loop (Figure 10). These types of network motifs influence the response time and resiliency of transcriptional networks and appear frequently in E. coli (Mangan and Alon, 2003). Also, OSX negatively regulates WNT/β-catenin signaling during osteoblast differentiation by activating the expression of canonical WNT antagonists Dkk1 and Sost and by disrupting the binding of TCF1 to β-catenin target genes (Yang et al., 2010; Zhang et al., 2008). Enhancing the expression of Prdm5 could be another mechanism by which OSX inhibits canonical WNT signaling in osteoblasts (Figure 11). A final piece of evidence implicating OSX in the regulation of Prdm5 expression is the presence of six consensus SP1 binding sequences, which OSX has been previously shown to recognize, within the proximal promoter of Prdm5.

In order to address the potential regulation of Prdm5 expression by OSX, we will perform qRT-PCR to measure the expression of Prdm5 in primary osteoblasts isolated from wild-type mice. The expression of Prdm5 in these cells will be compared to cells transfected with Osx-targeted siRNAs in which the expression of Osx has been reduced. In addition, primary osteoblasts will be transfected with a vector allowing for the inducible expression of Osx in the absence of doxycycline, a tetracycline derivative (Moriyama et al., 2013). It is expected that siRNA-mediated knockdown of Osx will cause a parallel reduction in Prdm5 expression, whereas overexpression of Osx will cause the opposite expression pattern (Figure 12). To determine whether OSX binds to the SP1 consensus sequences within the Prdm5 promoter, ChIP-qPCR will be performed in MC3T3 cells. The binding of OSX to the Prdm5 promoter under normal conditions will be compared to its binding in MC3T3 cells that have been modified using TALEN-mediated genome editing to lack the SP1 recognition sequences and cells in which the expression of Osx has been reduced through siRNA transfection.
To determine whether PRDM5 is an effector of OSX-mediated canonical WNT signaling inhibition (Figure 11), the effects of Osx expression on WNT signaling will be compared in wild-type and Rosa26-Cre; Prdm5\textsuperscript{fl/fl} osteoblasts transfected with the previously described Tet-off Osx expression vector. Levels of canonical WNT/\(\beta\)-catenin signaling will be assessed in the presence and absence of doxycycline by measuring the expression of Dkk1 and Sost, WNT antagonists that are known to be targeted by OSX, and c-Myc and Ccnd1, which are activated by canonical WNT signaling. Co-transfection with the TOPFlash reporter construct will also allow the level of canonical WNT signaling to be measured. If PRDM5 contributes to the inhibition of canonical WNT signaling mediated by OSX as predicted (Figure 13a), it is expected that the fold change in Dkk1 and Sost expression upon addition of doxycycline will be the same in wild-type and Prdm5\textsuperscript{−/−} osteoblasts, whereas the fold change in c-Myc expression, Ccnd1 expression, and TOPFlash activity will be greater in wild-type osteoblasts than those lacking Prdm5 (Figure 13b).
Unbiased approach to identify transcription factors that control Prdm5 expression

Because bone development is governed by complex interactions between multiple signaling pathways, an unbiased approach may better facilitate identification of the pathways that activate Prdm5 expression. To this end, DNA pulldown experiments will be performed to identify transcription factors that bind to the Prdm5 promoter after stimulation of osteogenic differentiation by exposure to conditioned medium. A conserved 1.5 kb fragment spanning the Prdm5 promoter will be isolated, biotinylated, and immobilized on streptavidin-coupled Dynabeads (Life Technologies) to be used as bait. The immobilized probe will then be incubated with nuclear extracts isolated from MC3T3 cells at various stages of osteogenic differentiation and after being exposed to WNT3A-, BMP2-, or FGF8-conditioned medium, all of which have been shown to stimulate osteoblast proliferation and differentiation (Mukherjee and Rotwein, 2011; Spencer, 2006; Valta, 2006). Proteins with high affinity for the immobilized probe will be separated by high-performance liquid chromatography and characterized by mass spectrometry. Prdm5 expression will be measured during the course of osteogenic differentiation in order to identify the timepoints most relevant for analysis. This experiment should identify transcription factors that bind to the endogenous Prdm5 promoter at various stages of osteoblastic differentiation. Individual proteins implicated in the regulation of Prdm5 by this method can be further validated by confirming their binding to the Prdm5 promoter using ChIP. The identification of transcription factors that are associated with well-characterized signaling pathways will also implicate upstream signaling proteins in the regulation of Prdm5 expression. These associations can be further explored in future work.

Figure 13: Predicted effects Prdm5 depletion on OSX-mediated inhibition of WNT signaling. a) Proposed role of PRDM5 in mediating OSX-mediated canonical WNT inhibition independent of DKK1 and SOST. b) Based on this model, ablation of Osx expression through introduction of doxycycline is expected to cause a greater reduction in canonical WNT signaling in wild-type than Rosa26-Cre; Prdm5<sup>fl/fl</sup> osteoblasts. Change in expression of Dkk1 and Sost should be equal in both cell types if this arm of OSX-mediated inhibition is independent of PRDM5.

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3. Literature Cited


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