WNT/β-CATENIN PATHWAY IN CRANIAL BONE PROGENITOR SPECIFICATION

By

LAWRENCE HENRY GOODNOUGH

Submitted in partial fulfillment of requirements

For the degree of Doctor of Philosophy

Dissertation Adviser: Radhika Atit, PhD

Department of Pathology

CASE WESTERN RESERVE UNIVERSITY

August, 2013
We hereby approve the thesis/dissertation of

Lawrence Henry Goodnough

Doctor of Philosophy

candidate for the ________________________________degree *

(signed)___________James Anderson, MD, Phd____________(committee chair)

____________Nicholas Ziats, PhD_________________

____________George Dubyak, PhD_________________________

____________Veronique Lefebvre, PhD_____________________

____________Clive Hamlin, PhD___________________________

____________Radhika Atit, PhD___________________________

(date) ____March 25, 2013___________________

*We also certify that written approval has been obtained for any proprietary material contained therein.
# TABLE OF CONTENTS

## LIST OF TABLES AND FIGURES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>iii</td>
</tr>
</tbody>
</table>

## CHAPTER 1. INTRODUCTION AND SIGNIFICANCE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>I.A. CLINICAL SIGNIFICANCE</td>
<td>1</td>
</tr>
<tr>
<td>I.B. EVOLUTION AND DEVELOPMENT OF THE CRANIAL BONES</td>
<td>3</td>
</tr>
<tr>
<td>I.B.1. CRANIAL BONES</td>
<td>3</td>
</tr>
<tr>
<td>I.B.2. EVOLUTIONARY ORIGINS OF CRANIAL BONES</td>
<td>4</td>
</tr>
<tr>
<td>I.B.3. CELL ORIGINS OF THE CRANIAL BONES</td>
<td>8</td>
</tr>
<tr>
<td>I.B.4. MORPHOGENESIS OF THE MAMMALIAN CRANIAL BONES</td>
<td>10</td>
</tr>
<tr>
<td>I.B.5. INTRAMEMBRANOUS OSSIFICATION</td>
<td>13</td>
</tr>
<tr>
<td>I.C. MOLECULAR BASIS OF CRANIAL BONE DEVELOPMENT AND DISEASE</td>
<td>14</td>
</tr>
<tr>
<td>I.C.1. GENETIC MUTATIONS AND CRANIAL BONE DEFECTS IN HUMANS AND MICE</td>
<td>15</td>
</tr>
<tr>
<td>I.C.2. TRANSCRIPTIONAL REGULATION AND CELL SIGNALING IN MOUSE CRANIAL BONE DEVELOPMENT.</td>
<td>17</td>
</tr>
<tr>
<td>I.C.3. WNT SIGNALING PATHWAYS</td>
<td>29</td>
</tr>
<tr>
<td>I.C.4. MUTATIONS OF WNT PATHWAY IN BONE BIOLOGY</td>
<td>34</td>
</tr>
<tr>
<td>I.C.5. WNT SIGNALING AND CONGENITAL CRANIOFACIAL ANOMALIES</td>
<td>35</td>
</tr>
<tr>
<td>I.C.6. ROLES OF WNT PATHWAY IN MURINE BONE DEVELOPMENT</td>
<td>36</td>
</tr>
<tr>
<td>I.C.5. WNT SIGNALING AND CONGENITAL CRANIOFACIAL ANOMALIES</td>
<td>35</td>
</tr>
<tr>
<td>I.C.7. ROLES OF WNT PATHWAY IN MOUSE CRANIOFACIAL DEVELOPMENT</td>
<td>38</td>
</tr>
<tr>
<td>I.C.8. TISSUE-TISSUE INTERACTIONS</td>
<td>39</td>
</tr>
</tbody>
</table>

## CHAPTER 2. Twist1 Mediates Repression of Chondrogenesis by Beta-Catenin to Promote Cranial Bone Progenitor Specification

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.A. ABSTRACT:</td>
<td>41</td>
</tr>
<tr>
<td>II.B. INTRODUCTION</td>
<td>42</td>
</tr>
<tr>
<td>II.C. RESULTS:</td>
<td>46</td>
</tr>
<tr>
<td>II.C.1. beta-catenin is required for cranial osteoblast lineage commitment</td>
<td>46</td>
</tr>
<tr>
<td>II.C.2. beta-catenin activity is necessary for expression of Twist1 in cranial mesenchyme.</td>
<td>52</td>
</tr>
<tr>
<td>II.C.3. beta-catenin activity promotes expression of Twist1 in cranial mesenchyme</td>
<td>53</td>
</tr>
<tr>
<td>II.C.4. beta-catenin forms a molecular complex that acts on the Twist1 promoter</td>
<td>55</td>
</tr>
<tr>
<td>II.C.5. deletion of Twist1 is sufficient to induce chondrogenesis in cranial mesenchyme</td>
<td>58</td>
</tr>
<tr>
<td>II.C.6. beta-catenin and the Twist family interact in cranial mesenchyme IN VIVO.</td>
<td>62</td>
</tr>
<tr>
<td>II.C.7. Twist1 binds to Sox9 3'UTR IN VIVO in cranial mesenchyme</td>
<td>67</td>
</tr>
<tr>
<td>II.D. DISCUSSION:</td>
<td>71</td>
</tr>
<tr>
<td>II.E. MATERIALS AND METHODS</td>
<td>76</td>
</tr>
<tr>
<td>II.E.1. MICE AND GENOTYPING:</td>
<td>76</td>
</tr>
<tr>
<td>II.E.2. IN SITU HYBRIDIZATION, IMMUNOHISTOCHEMISTRY, AND HISTOLOGY</td>
<td>77</td>
</tr>
<tr>
<td>II.E.3. CELL LINES, PLASMIDS, TRANSFECTION, AND LUCIFERASE ASSAYS</td>
<td>78</td>
</tr>
<tr>
<td>II.E.4. IMMUNOPRECIPITATION (CHIP) AND REAL-TIME PCR</td>
<td>79</td>
</tr>
<tr>
<td>II.F. ACKNOWLEDGEMENTS:</td>
<td>80</td>
</tr>
<tr>
<td>II.H. CONFLICT OF INTEREST:</td>
<td>81</td>
</tr>
</tbody>
</table>
CHAPTER 3. SEQUENTIAL REQUIREMENTS FOR CRANIAL ECTODERM AND MESENCHYME-DERIVED WNTs IN SPECIFICATION AND DIFFERENTIATION OF OSTEOBLAST AND DERMAL PROGENITORS ................................................................. 82

III.A. ABSTRACT: ........................................................................................................ 82
III.B. INTRODUCTION: .................................................................................................. 83
III.C. RESULTS: ............................................................................................................ 85
III.D. DISCUSSION: ..................................................................................................... 101
III.E. MATERIALS AND METHODS: ........................................................................... 106
   III.E.1. MICE AND GENOTYPING: ........................................................................ 106
   III.E.2. IN SITU HYBRIDIZATION, IMMUNOHISTOCHEMISTRY, AND HISTOLOGY: ... 106
   III.E.3. ALCIAN BLUE AND ALIZARIN RED STAINING: ...................................... 107
   III.E.4. RT-PCR ....................................................................................................... 107
III.F. ACKNOWLEDGEMENTS: .................................................................................. 109

CHAPTER 4. FUTURE DIRECTIONS ............................................................................. 110

IV.A. INTRODUCTION: ............................................................................................... 110
IV.B. FUTURE DIRECTION #1 ROLE OF TWIST 1 IN CRANIAL BONE PROGENITOR
   SPECIFICATION ...................................................................................................... 111
   IV.B1. RATIONALE ................................................................................................... 111
   IV.B2. EXPERIMENTAL STRATEGY ....................................................................... 112
   IV.B3. INTERPRETATION ......................................................................................... 113
IV.C. FUTURE DIRECTION #2 GENOME-WIDE ROLE OF TWIST1 IN REGULATING CRANIAL
   BONE PROGENITOR SPECIFICATION .................................................................. 113
   IV.C1. RATIONALE ................................................................................................... 113
   IV.C2. EXPERIMENTAL STRATEGY ....................................................................... 114
      a. RNA-SEQ ........................................................................................................ 114
      b. CHIP-SEQ ....................................................................................................... 115
      c. IDENTIFICATION OF CANDIDATES FOR FUNCTIONAL ANALYSIS ................ 116
         i. IDENTIFICATION OF TWIST1 TARGET GENES ........................................... 116
         ii. DIRECT TWIST1 TARGET GENES ............................................................ 116
         iii. IDENTIFY TRANSCRIPTION FACTORS THAT ARE DIRECT TARGETS OF
              TWIST1 .................................................................................................. 117
         iv. IDENTIFY DIRECT TRANSCRIPTION FACTOR TARGETS THAT MAY
             BIND COOPERATIVELY WITH TWIST1 ................................................ 118
             v. DIRECT TWIST1 TARGET GENES ......................................................... 118
   IV.C3. INTERPRETATION ......................................................................................... 119
IV.D. FUTURE DIRECTION #3. IDENTIFY A SET OF TRANSCRIPTION FACTORS SUFFICIENT
   FOR DIRECT INDUCTION OF BONE-FORMING CELL FATE FROM DIFFERENTIATED CELLS 119
   IV.C1. RATIONALE ................................................................................................... 119
   IV.C2. EXPERIMENTAL STRATEGY ....................................................................... 120
   IV.C3. INTERPRETATION ......................................................................................... 121

BIBLIOGRAPHY ............................................................................................................. 123
LIST OF FIGURES AND TABLES

FIGURE 1.1 Diagram of fetal/newborn human skull .................................................. 4
FIGURE 1.2 Embryonic origins of mouse cranial bones ........................................... 8
FIGURE 1.3 Separation of cranial neural crest- and paraxial mesoderm-derived
meenchyme ........................................................................................................... 10
FIGURE 1.4 Primary ossification centers in humans ............................................... 11
FIGURE 1.5 Primary ossification centers in mice .................................................. 12
TABLE 1.1 Genes with mutations associated with congenital cranial bone defects
in humans ............................................................................................................... 16
FIGURE 1.6 Cell signaling and transcriptional control of skull bone fate ........... 17
FIGURE 1.7 Proposed roles of signals and transcription factors in CNC
differentiation into osteoblasts and chondrocytes ........................................... 22
FIGURE 1.8 Wnt ligand secretion machinery ......................................................... 30
FIGURE 1.9 Canonical Wnt signaling through beta-catenin to the nucleus ....... 32
FIGURE 1.10 Comparison of canonical and beta-catenin-independent (non-
canonical) signaling pathways ........................................................................ 34
TABLE 1.2 Wnt pathway mutations in bone diseases and craniofacial defects ... 36
FIGURE 1.11 Examples of sequential inductive diseases ectodermal-mesenchymal
interaction and intrinsic autonomous mesenchymal signaling during
avian jaw development ....................................................................................... 39
FIGURE 2.1 Beta-catenin is required for cranial osteoblast lineage commitment
........................................................................................................................... 47
FIGURE 2.S1 Beta-catenin is required for cranial bone fate in paraxial mesoderm
osteoprogenitor cells ......................................................................................... 48
FIGURE 2.S2 Lack of contribution of En1Cre-lineage to skull base and
description of temporal differences in CRE activity ...................................... 49
FIGURE 2.2 Requirement of beta-catenin in cranial mesenchyme for Twist1
expression ........................................................................................................... 54
FIGURE 2.S3 Beta-catenin is sufficient for ectopic Twist1 expression ............... 56
FIGURE 2.3 Beta-catenin activates Twist1 expression ........................................... 57
FIGURE 2.4 Loss of Twist1 is sufficient for chondrogenesis in cranial
mesenchyme ....................................................................................................... 60
FIGURE 2.5 Beta-catenin and the Twist family interact in cranial mesenchyme in
vivo ....................................................................................................................... 63
FIGURE 2.S4 Activation of beta-catenin results in ectopic Lef1 expression;
deletion of Twist1 restores Ap in Lef1 expressing cells ..................................... 65
FIGURE 2.6 Twist1 binds to Sox9 3' UTR in vivo in cranial mesenchyme .......... 69
FIGURE 2.S5 Primer sequences used for Chip-qPCR ........................................... 70
FIGURE 3.1 Expression of Wnt-ligands and Wls in cranial mesenchyme ............ 86
FIGURE 3.2 Removal of Wls from cranial ectoderm or mesenchyme ............... 88
FIGURE 3.S1 Ossification defects in mesenchyme Wls mutants .......................... 90
FIGURE 3.S2 En1Cre labels meningeal progenitors and deletion of Wls results in
mineralization defects ....................................................................................... 90
FIGURE 3.3 Comparison of mineralization defects and ectopic chondrogenesis in
ectoderm- and mesenchyme Wls-deficient mutants ......................................... 91
FIGURE 3.4 Ectoderm-Wls is required for cranial bone and dermal progenitor
specification ....................................................................................................... 93
FIGURE 3.S3 Decreased osteoblast progenitor differentiation, not
SPECIFICATION, USING THE MESENCHYMAL En1cre TO DELETE WLS...........95

FIGURE 3.5 MESENCHYMAL WLS IS REQUIRED FOR OSTEOBLAST AND DERMAL PROGENITOR DIFFERENTIATION, NOT SPECIFICATION ..............................................96

FIGURE 3.6 ECTODERM WLS IS REQUIRED FOR GRADED EXPRESSION OF TARGET GENES DURING CRANIAL MESENCHYME SPECIFICATION ..................................................98

FIGURE 3.7 ECTODERM WLS IS REQUIRED FOR EXPRESSION FOR A SUBSET OF MESENCHYMAL Wnt ligands .................................................................99

TABLE 3.S4 RT-PCR PRIMERS FOR WNT LIGANDS ........................................108

FIGURE 4.1 DELETION OF TWIST1 WITH En1cre RESULTS IN REGIONAL LOSS OF OSSIFICATION NEAR THE CORONAL SUTURE ......................................................111

FIGURE 4.2 REMOVAL OF TWIST1 FROM EARLY CRANIAL BONE PROGENITORS RESULTS IN LACK OF SKULL OSSIFICATION ......................................................112

FIGURE 4.3 PROPOSED WORKFLOW TO ANALYZE TWIST1 TARGET GENES AND DEFINE A ROLE OF TWIST1 IN CRANIAL BONE PROGENITOR SPECIFICATION .................115
Wnt/β-Catenin Pathway In Cranial Bone Progenitor Specification

Abstract

By

LAWRENCE HENRY GOODNOUGH

The skull is comprised of flat bones that protect the brain and are separated by fibrous suture joints, which accommodate brain growth. The mammalian skull has complex cellular origins and arises through direct ossification, an atypical skeletogenic pathway. Congenital skull defects are relatively common, yet only a handful of causative mutations have been identified. Skull morphology exhibits tremendous variation across species, and is nearly synonymous with vertebrate evolution. In spite of the importance of skull bones, our understanding of the initial genetic events that pattern the skull is limited. Here I demonstrate in the mouse embryo that β-catenin signal transduction in the supraorbital mesenchyme comprises a definitive signal fate selection of cranial bone progenitors. The Wnt/β-catenin pathway is active in cranial bone progenitors undergoing fate specification. Removal of β-catenin from the cranial mesenchyme results in transformation of skull ossification to ectopic chondrogenesis. β-catenin directly induces Twist1 expression, which is required to prevent chondrogenesis in the skull, where it binds to the Sox9 gene. These data implicate a β-catenin-Twist1 pathway in the repression of alternate cell fates during cranial bone progenitor cell fate specification. A suite of Wnt ligands, along with the specific Wnt ligand trafficking regulator, Wntless (Wls), is
expressed throughout cranial surface ectoderm and mesenchyme during skull patterning. Removal of Wls from surface ectoderm results in loss of ossification and skin patterning due to defects in cranial bone and dermal progenitor specification. Ectoderm Wnt ligand secretion also initiates expression of a subset of mesenchyme Wnt ligands via β-catenin. Mesenchymal Wnt ligands, as shown by removal of Wls, are required for downstream differentiation of bone and dermis effects. Collectively, our data implicate the cranial surface ectoderm as the source of a patterning Wnt signal to the supraorbital mesenchyme, initiating cranial bone specification and Wnt ligand expression. Characterization of the signaling sequences that initiate skull morphogenesis has implications for understanding evolutionary variation of skull morphology as well as the pathogenesis of congenital skull defects in humans, and for improving our approach to engineering skeletal cells.

Chapter I. Introduction and Significance

I.A. Clinical Significance

Craniofacial birth defects, which affect the face and the skull, are among the most common developmental abnormalities in humans. Congenital defects of the skull vault occur in as many as 1 in 2500 births (Cohen Jr and MacLean, 2000). At one end of the spectrum of congenital cranial malformations is insufficient bone, as in enlarged parietal foramina (PFM), in which delay or lack of ossification results in widely patent skull. Untreated, PFM can result in headache, defects in the scalp, and vascular malformations of the brain (Wilkie et al., 2000).
At the opposite end, precocious or excessive bone formation underlies the abnormal fusion of skull bones at the cranial sutures in craniosynostosis. Beyond congenital cranial anomalies, skull bone fractures can occur in settings ranging from motor vehicle accidents to the military battlefield, where up to 42% of injuries present with craniofacial trauma (Chan et al., 2013). Congenital or acquired/traumatic craniofacial defects frequently require reconstruction with bone, and existing sources and grafting techniques are sub-optimal (Kwan et al., 2008). The requirement for a functional, durable replacement for lost tissue has intensified the research focus on skeletal development and regeneration. Ongoing studies of the developmental origins of craniofacial tissues coupled with advances in stem cell research offer the promise of regeneration of bone tissue, rather than replacement. Understanding the signaling pathways required for development of the cranial bones could in turn delineate for regenerative pathways in adults. This study proposes to offer basic insights into the signaling requirements of cranial bone development, findings that would be applicable to future cell-based therapies for calvarial repair.

I.B. Evolution and Development of the Cranial Bones

I.B.1. Cranial Bones

In vertebrates, the skull bones house the brain. In addition to the dorsally positioned cranial bones (dermatocranium), the brain is protected anteriorly by the facial skeleton (viscerocranium), and is supported ventrally by the skull base (neurocranium). In humans, the cranial bones include the paired frontal and
parietal bones, and the single occipital bone. The frontal bones comprise the anterolateral boundary of the bony skull vault, and the parietal bones are positioned posterolaterally. The occipital bone forms the most posterior wall of the skull vault (Fig. 1.1).

As they enclose the brain during development, the skull bones are each connected by thin layers of fibrous tissue called sutures that provide flexibility and allow the skull to accommodate further brain growth. The coronal suture is located between the frontal and parietal bones, the sagittal suture is between the paired parietal bones, and the lambdoid suture connects the parietal and occipital bones. Thus, skull bones must serve opposing purposes: accommodating brain growth during development while providing protection from the environment.

**I.B.2. Evolutionary Origins of Cranial Bones**
The evolution of the skull is a defining feature of craniates, a clade of the phylum chordata that includes vertebrates and hagfishes. The skull bones, as part of the vertebrate face and head, were neomorphic additions to the primitive chordate filter feeding structure, according to the New Head hypothesis (Gans and Northcutt, 1983). One central innovation during vertebrate skull evolution was the development of a novel cell type, an ectoderm-derived mesenchymal (ectomesenchyme) progenitor cell called the cranial neural crest (CNC), which gave rise to most of the “new” tissue in the vertebrate face and head. The New Head hypothesis proposed that evolution of the cranial neural crest-derivatives (facial skeleton, teeth, tongue), together with ectodermal placodes giving rise to sensory structures, allowed ancient vertebrates to ascend from filter feeders to predators (Gans and Northcutt, 1983).

The first mineralized tissue enclosing the brain arose in craniates some 500 million years ago (Hanken and Hall, 1993a). Early craniates and jawless vertebrates possess a craniofacial skeleton, an early version in which cartilage-like braincases and nasal baskets allow feeding but do not enclose the brain (Hanken and Hall, 1993a). However, the same point in the Silurian-Devonian contains fossil records of now-extinct jawless fish that possessed well developed dermal exo-skeletons, in which the skin possessed either small scales or large bony plates over the face and top of the head (Hanken and Hall, 1993a). Some jawless fish with dermal skeletons, such as osteostracans, possessed dorsal head shields bearing exo-skeletal scales, as well as early evidence of bone mineralization (Hanken and Hall, 1993a). These data suggest that both bone and
dermal skeletal elements are quite ancient innovations that arose from now-extinct jawless fish which are likely distinct from ancestors of existing jawless fish.

Jawed osteichthyans (bony fish) first developed the modern skull vault body plan that has subsequently undergone tremendous modification from fish to mammals. The evolution of the modern skull plan probably begins with an array of dermal scales atop the heads of fossilized jawless fish, and then passes to ray-finned (teleost) bony, jawed fishes. The pattern of early scales and skull vaults of fossilized jawed fishes is so varied that no conclusive homology exists (Hanken and Hall, 1993a). However, the most recent fossils of bony fish show a progressive reduction in the number of cranial bones throughout evolutionary history. Modern teleost fish possess a skull vault body plan that contains paired frontal, parietal, and post-parietal bones upon which the tetrapod skull vault generally resembles. Subsequently, amphibians and reptiles faithfully reproduce the paired bones of the skull vault. In reptiles, the skull vault also gives rise to spines, horns, and frills (e.g. chameleon). In reptiles, the frontal and parietal bones are small, probably because the jaws have expanded for feeding at the expense of the skull vault (Hanken and Hall, 1993a). Avian skull morphology differ from reptiles in that the brain is slightly larger in birds, and thus the accompanying skull vault bones are more dome-shaped and enlarged. In mammals, a common modification was the fusion of the post-parietal bones into an interparietal bone, and in higher mammals the interparietal bone fuses with the supraoccipital or occipital bones (Hanken and Hall, 1993a). In spite of the
tremendous diversity of skull morphology, the general trend is that early vertebrates had smaller cranial bones with larger jaws for feeding. As human ancestors developed larger brains, the face shortened but maintained the ability to withstand powerful mastication forces, while the skull vault bones grew to accommodate the much larger forebrain. Thus, within vertebrates, skull shape variation is incredibly diverse, yet evolution and development of skull bones are almost synonymous with the rise of vertebrates.

The basis for evolution and development of skull morphology relies on the communication among the many tissues that comprise the vertebrate head, as well as within the cells that actually mineralize to form bone. Tissue patterning in development can be defined as “the events by which cells are organized into predictable spatial arrangements of tissues in their proper locations” (Hanken and Thorogood, 1993). Such signals can be divided into extrinsic patterning events, in which spatiotemporal cranial bone patterning is induced by a non-cell autonomous source (e.g. the ectoderm), and intrinsic patterning, in which species-specific identity and timing are encoded for within the cell type itself (Hanken and Thorogood, 1993). Reciprocal transplantation experiments have shown that cranial neural crest-derived viscerocranium (facial skeleton) contains intrinsic patterning cues (Schneider and Helms, 2003). By contrast, similar studies have demonstrated that the initiation of neural crest formation relies on extrinsic patterning cues. Whether the cranial bones receive extrinsic patterning cues, contain intrinsic patterning information, or respond to a mix of both signals, awaits investigation.
I.B.3. Cell origins of the cranial bones

The mammalian cranial bones have complex cellular origins, and the differing properties of regionally distinct skull bones may offer insight into some congenital skull defects. The cranial bones, unlike the rest of the vertebrate skeleton, arise from mesoderm progenitors as well as from a broadly multipotent progenitor population called the cranial neural crest (CNC). The bones of the axial skeleton (ribs, vertebral column, and pelvis) and the appendicular skeleton (limbs) are of mesodermal origin, and progenitors originate from paraxial mesoderm (PM), which is located next to the neural tube, or from lateral plate mesoderm, located laterally between ectoderm and endoderm in the body. The mesoderm also contributes to the cranial bones through the anterior paraxial mesoderm. However, the cranial bones receive an additional contribution from the cranial neural crest, a unique population of cells derived from the surface ectoderm. Cranial neural crest cells are specialized cells that undergo epithelial mesenchymal transformation from
the ectoderm located over the neural tube that gives rise to the forebrain, midbrain, and hindbrain. After delaminating from the ectoderm, cranial neural crest cells migrate into the face and branchial arches and give rise to diverse lineages, including the cranial nerves, melanocytes, teeth, salivary gland connective tissue, smooth muscle, dermis, facial cartilage, and craniofacial bone.

The dual origin of the skull initially led to confusion about the progenitor origins of the different cranial bones and remained controversial until the advent of genetic fate-mapping in mice. Early work with avian chimeric transplants concluded that cranial bones were either of mixed mesodermal and neural crest origin ((Le Lievre, 1978; Noden, 1978; Noden, 1983; Noden, 1984; Noden, 1988)) or exclusively of cranial neural crest origin (Le Douarin et al., 1993). Innovations in conditional mouse genetics have allowed definitive lineage tracing to determine the mesodermal and cranial neural crest contributions to the mammalian skull. The cranial neural crest gives rise to the frontal bones and to anterior, dorsal craniofacial skeletal elements, while also contributing to the posteriorly located interparietal bone (Jiang et al., 2002). Paraxial mesoderm gives rise to the parietal bones and the posterior skull base, which is located beneath the brain (McBratney-Owen et al., 2008; Yoshida et al., 2008) Fig. 1.2). The sclerotome compartment of the occipital somites forms the most posterior bone of the skull, the supraoccipital bone. Although the neural crest-derived and mesoderm-derived cranial bones both undergo intramembranous ossification, whether the signaling pathways that guide this process are conserved between the two tissues is still unknown. While one unique property of the cranial bone is
their complex cellular origins, the elucidation of another unique quality of cranial bones, the basis for intramembranous ossification, begins with fate mapping of cranial bone progenitors from their cellular origins.

**l.B.4. Morphogenesis of the Mammalian Cranial Bones**

The localization of mammalian cranial bones precursors and subsequent fate mapping of their progeny, have been recently described using several elegant mouse models. At around five somites, the midbrain/hindbrain region is divided into three prorhombomeres. The first and second prorhombomeres are separated by the pre-otic sulcus. Rostral to the sulcus a population of cranial neural crest called the trigeminal cranial neural crest cells delaminate and migrate from the neural folds (Morriss-Kay and Wilkie, 2005). The cells destined to become frontal bone migrate over the rostral forebrain beneath the surface ectoderm, forming the frontonasal mesenchyme. Cells from the paraxial mesoderm migrate from positions close to the neural tube at the same time as the neural crest, with the neural crest migrating between the surface ectoderm
and the mesoderm. At E11.5 (mouse embryonic day), the cells that give rise to the frontal and parietal bones of the skull reside in supraorbital mesenchyme underneath the surface ectoderm, and a clear boundary forms between cranial neural crest and paraxial mesoderm populations (Fig. 1.3 (Morriss-Kay and Wilkie, 2005)). The cranial neural crest-derived progenitors of the frontal bone segregate anterior to the paraxial mesoderm progenitors of the parietal bone. By this time, cranial bone progenitors likely segregate from dermal progenitors since the cranial neural crest-mesoderm boundary is located anterior to the cranial bone boundary. When the lineage descendants of cranial bone progenitors were labelled strictly at E11.5, progeny were detected in apical regions of mineralized frontal and parietal bone from E16.5 to post-natal day zero (P0) (Deckelbaum et al., 2012; Tran et al., 2010). Thus, the cranial bones grow dorsally to cover the brain by migration and proliferation of progenitors, resulting in physical baso-apical expansion of the cranial bone primordia. These experiments disagree with earlier speculation that

Figure 1.4. Primary ossification centers in humans. The frontal and parietal bones ossify from ossification centers in the supraorbital mesenchyme, separated by a flexible fibrous joint, the coronal suture
“non-skeletal mesenchyme” such as dura mater could be recruited to the cranial bone progenitor pool (Sperber, 2001).

In each cranial bone, the primary ossification center is where bone mineralization first occurs (Hanken and Hall, 1993c). The primary ossification center of the frontal bone is located above the eye in the supraorbital mesenchyme, immediately anterior to the parietal bone ossification center, the parietal eminence (DeBeer, 1937) (Fig. 1.4). Mice demonstrate similar patterning of ossification centers, which appear in the skull by E14.5 (Figure 1.5).

After the onset of mineralization at the primary ossification centers occurs, the second stage of morphogenesis, appositional growth at the cranial sutures, begins. The fibrous cranial sutures separate the bony plates of the skull to allow expansion of the calvarium during brain growth (DeBeer, 1937). The coronal suture (between frontal and parietal bones), the sagittal suture (between parietal bones) and the lambdoid suture (parietal and interparietal bones) all form at the boundaries between cranial neural crest and paraxial mesoderm-derived tissues (Jiang et al., 2002). The coronal suture is unique in that

![Figure 1.5. Primary ossification centers in the mouse embryonic skull. Image from Trainor Lab, http://www.stowers.org/faculty/trainor-lab](image-url)
the tissue boundary for the suture forms as early as E11.5 with the osteoblast progenitors of each bone closely apposed (Deckelbaum et al., 2012), whereas the mesenchyme of the other sutures forms with the skull bones far apart initially. In appositional bone growth at the sutures, osteoblast progenitors proliferate and differentiate at the osteogenic fronts, adjacent to the suture mesenchyme (Lenton et al., 2005). In humans the sutures remain patent until very late in life, with the exception of the metopic suture between the frontal bones, which fuses at 18 months of age (Morriss-Kay and Wilkie, 2005). The homologous suture in the mouse, the posterofrontal (PF), fuses post-natally (Bradley et al., 1996). Notably, both fusing sutures in mice and humans, the posterofrontal and metopic, form exclusively within a cranial neural crest cell-containing domain (Morriss-Kay and Wilkie, 2005). One explanation for the differing fusion properties between otherwise equivalent suture mesenchyme is that intrinsic, pre-patterned differences in osteogenic potential occur based on tissue origin (Quarto et al., 2010). Such evidence implies that early signaling events during cranial bone patterning influence suture biology during appositional growth. Therefore, elucidation of the molecular basis of initial cranial bone patterning could yield an understanding of how early defects could result in later pathologic suture fusion.

I.B.5. Intramembranous ossification

The cranial bones ossify through an atypical mechanism, intramembranous ossification, in which the identifying feature is the direct differentiation of skeletal progenitors into osteoblasts (Hanken and Hall, 1993c). By contrast, in the predominant osteogenic program in vertebrates, endochondral
ossification, skeletal progenitors first form cartilage condensations that undergo hypertrophy, vascularization, and replacement by bone-forming osteoblasts. In the skull, intramembranous ossification first begins with post-migratory ectomesenchymal cells arising from neural crest or paraxial mesoderm and forming mesenchymal condensations (Dunlop and Hall, 1995; Hall and Miyake, 1992; Hall and Miyake, 2000). The progenitor cells proliferate and migrate apically. Cells in mesenchymal condensations differentiate into osteoblasts. As osteoblast progenitors differentiate they begin to secrete extracellular matrix (ECM) proteins. The vast majority of extracellular matrix proteins secreted by osteoblasts is type I collagen (Rossert et al., 1996), and the initial unmineralized collagenous bone matrix is called osteoid. Osteoblasts additionally produce other ECM components that strengthen the ECM, and proteins that regulate bone mineralization such as osteopontin and alkaline phosphatase. Osteoblasts release mineralizing matrix that accumulate calcium and phosphate for hydroxyapatite (HA), which deposits as crystals on collagenous matrix. Alkaline phosphatase supports HA deposition by inhibiting negative regulators of HA formation such as pyrophosphate, and by promoting formation of inorganic phosphate. At the same time, blood vessels invade the mesenchymal condensations, which take on a trabecular appearance. Blood vessels carry calcium salts, which deposit in bone matrix and produce bone mineral. Calcification spreads outwards from a nucleus of osteoblasts at the ossification center. The outermost layers of mesenchymal cells surrounding the calcified ossification center become the periosteum. Periosteum differentiates into
osteoblasts and adds layers of bone by accretion (Gilbert, 2003). Defining the signaling cues for each of these developmental events will be critical in the development of cell-based therapies to repair bony defects.

I.C. Molecular Basis of Cranial Bone Development and Disease

I.C.1. Genetic mutations and cranial bone defects in humans and mice

The first insights into the molecular basis of cranial bone development came from finding that most gene mutations associated with congenital cranial bone defects were either transcription factors or cell signaling pathway components (Table 1.1). Mutations were identified in genes encoding for transcription factors that were associated with decreased mineralization, such as \textit{RUNX2} in cleidocranial dysplasia (Mundlos et al., 1997), and \textit{MSX2} and \textit{ALX4} in persistent calvarial foramina (Mavrogiannis et al., 2001; Wilkie et al., 2000). Conversely transcription factor mutations are also associated with craniosynostosis, predominantly at the coronal suture, including \textit{TWIST1} mutations in Saethre-Chotzen syndrome (el Ghouzzi et al., 1997), and \textit{MSX2} in craniosynostosis (Jabs et al., 1993). The vast majority of the remainder of mutations associated with congenital cranial bone defects occurs in genes encoding for growth factors, their receptors, and signaling pathway inhibitors. \textit{FGFR} mutations are associated with syndromic and non-syndromic craniosynostoses (Bellus et al., 1996; Wilkie et al., 1995a). In addition to receptors, mutations in ligands, such as \textit{EPHRINA} and \textit{JAGGED1}, are associated with coronal suture synostosis (Kamath et al., 2002; Merrill et al., 2006). Mutations in signaling pathway inhibitors are further indicative of the key
roles of cell signaling as in mutations of $\textit{SOST}$, a secreted regulator of the Wnt pathway, which result in cranial sclerosteosis, with excessive bone deposition and narrowing of cranial foramina. In mice, homozygous null mutations of $\textit{Axin2}$, also encoding for a Wnt pathway inhibitor, result in craniosynostosis, although a similar mutation has not been described in humans (Yu et al., 2005). The task remains to identify more disease-causing gene loci, and one estimate is that only 20% of craniosynostoses can be linked to a genetic mutation (Morriss-Kay and

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Cellular Role</th>
<th>Disease</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\textit{RUNX2}$</td>
<td>Transcrip tion Factor</td>
<td>Cleidocranial dysplasia (OMIM 119600) -delayed intramembranous bone formation</td>
<td>(Mundlos et al., 1997)</td>
</tr>
<tr>
<td>$\textit{TWIST1}$</td>
<td>Transcription Factor</td>
<td>Saethre-Chotzen syndrome (OMIM 101400) -craniosynostosis</td>
<td>(et al., 1997)</td>
</tr>
<tr>
<td>$\textit{MSX2}$</td>
<td>Transcription Factor</td>
<td>Boston-type craniosynostosis (OMIM 604757) -craniosynostosis Parietal Foramina (OMIM 168500) -intramembranous ossification defect</td>
<td>(Jabs et al., 1993; Wilkie et al., 2000)</td>
</tr>
<tr>
<td>$\textit{ALX4}$</td>
<td>Transcription Factor</td>
<td>Parietal Foramina (OMIM 609597) - intramembranous ossification defect</td>
<td>(Mavrogiannis et al., 2001)</td>
</tr>
<tr>
<td>$\textit{FGFR1}$</td>
<td>Receptor</td>
<td>Pfeiffer syndrome (OMIM 101600)</td>
<td>(Schell et al., 1995)</td>
</tr>
<tr>
<td>$\textit{FGFR2}$</td>
<td>Receptor</td>
<td>Apert (OMIM 101200), Crouzon (OMIM 123500), and Pfeiffer (OMIM 101600) syndromes -craniosynostosis</td>
<td>(Jabs et al., 1994; Reardon et al., 1994; Schell et al., 1995; Wilkie et al., 1995b)</td>
</tr>
<tr>
<td>$\textit{FGFR3}$</td>
<td>Receptor</td>
<td>Muenke syndrome (OMIM 602849) -craniosynostosis</td>
<td>(Bellus et al., 1996; Muenke et al., 1997)</td>
</tr>
<tr>
<td>$\textit{EFNB1}$</td>
<td>Receptor</td>
<td>Craniofrontonasal syndrome -craniosynostosis</td>
<td>(Twigg et al., 2004)</td>
</tr>
<tr>
<td>$\textit{EFNA4}$</td>
<td>Receptor</td>
<td>craniosynostosis</td>
<td>(Merrill et al., 2006)</td>
</tr>
<tr>
<td>$\textit{SOST}$</td>
<td>Secreted Inhibitor</td>
<td>Cranial sclerosteosis</td>
<td>(Balemans et al., 2001; Brunkow et al., 2001b)</td>
</tr>
</tbody>
</table>

Table 1.1. Genes with mutations associated with congenital cranial bone defects in humans.
Collectively, however, the known mutations associated with congenital skull defects suggest that intercellular communication via cell signaling and epigenetic regulation by transcription factors are critical events for normal skull development. In order to determine if associated mutations were actually causative of congenital cranial bone defects, previous studies have turned to functional genetics in mice.

I.C.2. Transcriptional regulation and cell signaling in mouse cranial bone development.

The advent of transgenic mouse technology has allowed the initial characterization of cell signaling events and transcriptional regulation underlying cranial bone development. Since only a small subset of causative mutations for congenital skull defects has been identified, large gaps in our knowledge remain about the genetic basis of intramembranous ossification in

Figure 1.6. Cell signaling and transcriptional control of skull bone fate

(A) Cranial mesenchyme undergoing specification
- Hox (-) Notch (negative regulator?)
- Dlx5/6 Twist1
- Msx1/2 Foxc1

(B) Cell Fate Determination of osteoblast progenitors
- BMP2/4 β-catenin
- Runx2 Alx4

(C) Differentiation into osteoblasts
- Osx FGFR2
- TGFb2 TGFbRII
- Ihh/Shh?
development and disease. In addition to mouse models of mutations linked to congenital skull defects, most major signaling pathways have been deleted in mesenchymal progenitors in the mouse embryo. However, our interpretation of cranial bone phenotypes is limited in mouse models that have been generated for functional analysis of pre-migratory cranial neural crest formation or for analysis of endochondral ossification. In spite of the caveats of previous studies, a clearer picture of tightly coordinated transcriptional control of cranial bone formation begins to emerge.

In the embryo, transcription factors confer cell memory during fate selection and differentiation, and several are essential in the cranial bone progenitor lineage. The central transcriptional regulators of the osteoblast lineage are also essential in cranial osteoblast development, although their functions are restricted to osteoblastogenesis in the skull bones. Runx2 encodes a Runt-domain containing transcription factor that is expressed by E12.5 in mouse skull progenitor cells. Runx2 is required for bone formation and osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Osx encodes a zinc finger-containing transcription factor, expressed as early as E13.5 in the mouse skull, and is required for osteoblast formation downstream of Runx2 (Nakashima et al., 2002). Thus osteoblast lineage-determinants initiate intramembranous ossification, although how osteoblast lineage determinants get activated in the skull remains unresolved.

In contrast to the transcriptional determinants of osteogenesis, the master regulator of cartilage fate, Sox9, is dispensable in skull bone formation.
Although Sox9 is expressed in early cranial neural crest cells (Bell et al., 1997; Bi et al., 1999), genetic deletion from cranial neural crest perturbed only cartilaginous elements of the skull (Mori-Akiyama et al., 2003). These studies confirm that intramembranous ossification is independent of chondrogenesis, although the molecular switches that promote direct specification of osteoblasts in the skull instead of chondrocytes remain unresolved.

While the upstream regulators of bone and cartilage cell fate determinants are unknown, there is evidence that the transcription factor, β-catenin may constitute an important input, as well as its upstream regulatory pathway, Wnt signaling. The spatiotemporal localization of Wnt pathway activity in cranial bone progenitors would provide a better context for understanding its role. Our current understanding stems from removal of ctnnb1 (encoding for β-catenin) from skeletal progenitors, which resulted in an absence of endochondral bone formation and intramembranous ossification, as well as an increase in chondrocyte differentiation (Day et al., 2005; Hill et al., 2005). These data suggest that Wnt/β-catenin is therefore an essential starting point for understanding how cranial bone lineage commitment occurs. However, several outstanding questions remain: when and where does cranial bone patterning occur, what are the instructive tissue sources, and what is the role and targets of Wnt signaling?

Cranial bone progenitor cells must “know” their location in the head. Hox genes convey positional identity on cells in the limb and post-cranial axial skeleton. Yet, expression and function studies show that the CNC-derived skull,
along with first branchial arch-derivatives (e.g. jaws), are unique for their Hox-negative status. The most anterior Hox gene, *Hoxa2*, is expressed in the second branchial arch, and homozygous deletion of *Hoxa2* results in homeotic transformation of second arch to first arch, while *Hoxa2* over-expression in the first arch confers second arch-like marker expression (Kanzler et al., 1998; Rijli et al., 1993). Therefore, the absence of Hox expression plays an intriguing role in formation of CNC-derived elements as such as the skull, suggesting other transcription factors may provide location.

The *distalless*-like family of homeobox transcription factors (Dlx) provides positional cues to skeletal progenitor cells in the face, and two members, *Dlx5/6*, are expressed in cranial mesenchyme during early differentiation (Acampora et al., 1999). Other Dlx family members, such as *Dlx1/2*, pattern maxilla versus mandible identity (Depew et al., 2002). In the skull, homozygous deletion of *Dlx5/6* in mice results in loss of cranial bone ossification, suggesting the two factors function redundantly (Robledo et al., 2002). However whether *Dlx5/6* actually function in skull bone patterning remains unknown.

Another set of transcription factors, the forkhead domain-containing Fox family, also plays key roles in regional identity of craniofacial skeletal elements. Multiple Fox family members are expressed throughout the head and face, and *Foxc1* is expressed in early cranial mesenchyme before progressive restriction to meningeal progenitors. The “fox code” hypothesis suggests that combinatorial expression of Fox transcription factors determines identity of skeletal elements in the face (Joeng et al., 2011). A spontaneous mouse mutation, *congenital*
hydrocephalus (ch), was localized to the Foxc1 locus (Kume et al., 1998), and Foxc1<sup>ch</sup> homozygous mutant mice lack cranial bone. Further analysis showed Foxc1 is critical for differentiation of skull bone progenitors (Sun et al., 2013; Vivatbutsiri et al., 2008). If Foxc1 is indeed a “fox code” effector, it is probably not acting alone to pattern the skull bones.

*Msx1* and *Msx2* are muscle segment homeobox-like transcription factors expressed in CNC-derived cranial mesenchyme beginning at E10.5, with expression progressively more restricted to osteoblast progenitors from E12.5 onward (Holland, 1991). Both *Msx2* and *Msx1* homozygous null mutants individually have persistent calvarial foramina (Satokata et al., 2000; Satokata and Maas, 1994). *Msx1/Msx2* compound homozygous null mutants demonstrated an exacerbated phenotype, and failed to even undergo mesenchymal condensation during ossification (Han et al., 2007). Surprisingly, deletion of both *Msx1* and *Msx2* specifically in cranial neural crest cells caused heterotopic bone formation in the skull (Roybal et al., 2010). Opposing results suggest details in Msx1/2 function in the skull remains unresolved, but mice genetics demonstrate that the *Msx* genes are at least essential for early differentiation of cranial bone progenitors. In humans, *MSX2* gain-of-function mutations are associated with Boston-type craniosynostosis (Jabs et al., 1993). Conversely, human loss-of-function *MSX2* mutations result in symmetrical parietal foramina (PFM), a defect phenocopied by *Msx2* homozygous null mice (Wilkie et al., 2000). Tracing the pathogenesis of PFM to early progenitor differentiation in the supraorbital mesenchyme in mice provides further support to
the hypothesis that both normal and pathologic cranial bone formation depend on early patterning cues. Although one layer of Msx gene regulation in cranial bone differentiation certainly comes from BMP signaling (Vainio et al., 1993), phenotypic differences between BMP pathway mutants and Msx pathway mutants could indicate the presence of a separate, yet unidentified cranial bone patterning cue.

Twist1 is a basic helix-loop-helix (bHLH) transcription factor that is expressed by E11.5-E12.5 in cranial mesenchyme and bone progenitors (Bialek et al., 2004), and has pleiotropic roles in craniofacial development. Twist1 is required for mesoderm and cranial neural crest formation (Bildsoe et al., 2009; Soo et al., 2002), and for formation of cranial neural crest/mesoderm boundary at the
coronal suture (Merrill et al., 2006; Ting et al., 2009). In addition, Twist1 inhibits osteoblast differentiation (Bialek et al., 2004). Previous studies implicated reciprocal FGF pathway and Twist1 signaling during osteoblast differentiation at the coronal suture (Rice et al., 2000). Collectively, these studies revealed roles for Twist1 in the genesis of cranial neural crest and in later differentiation of its derivatives, yet have not identified functions for Twist1 in mesenchymal cell specification, or determined which signals initiate expression of Twist1 in mesenchyme.

In spite of its central importance, current knowledge about transcriptional control of cell fate in cranial bone progenitors is largely restricted to indirect evidence (Fig 1.6, 1.7). The definitive factor or set of factors remains to be identified, as do the cell signals that induce the transcription factors.

There are likely to be one or more critical signals that induce cranial bone progenitor specification in the right time and the right place for normal development to occur. Fibroblast Growth Factor (FGF), Bone Morphogenetic Proteins (BMPs), Sonic Hedgehog (Shh), and Wnt signaling are important in skeletal patterning of the vertebrate face and limb (Helms and Schneider, 2003; Johnson and Tabin, 1997). A candidate “osteoinductive” signal in the mouse might induce cell fate specification around E11.5 or E12.5, communicating with post-migratory cranial neural crest and paraxial mesoderm (Hanken and Hall, 1993b). An inductive signal would originate in a nearby tissue population, such as the surface ectoderm or meninges. On the other hand previous studies suggest that skeletogenic cranial neural crest cells contain intrinsic patterning
signals (Schneider and Helms, 2003). No signal has been definitively
demonstrated to function as an inductive signal or as an intrinsic factor for cranial
bone specification, yet functional genetics in mice has offered some clues.

Bone morphogenetic proteins (BMPs) are part of the TGFβ super-family of
ligands. Ligand-receptor binding induces Type II Receptors to activate Type I
receptors. Type I receptors phosphorylate and activate intracellular
transcriptional effectors, Smad 1/5/8, while Smad-independent BMP signaling
occurs through the P38/mitogen-activated protein kinase (MAPK) pathway.
BMP2/4 localize to cranial osteoblast progenitors undergoing cell fate
specification at E12.5 (Roybal et al., 2010), while BMP7 expression was
restricted to meningeal progenitors (Vivatbutsiri et al., 2008). Levels of
phosphorylated Smad1/5/8, the activated forms of the downstream effector
complex of BMP signaling, were broadly present in dermal and cranial bone
progenitor mesenchyme at E12.5 (Sun et al., 2013). The autocrine expression of
BMP ligands and effectors suggests an autocrine pathway, which would argue
against an inductive cue for spatiotemporal patterning of skull bones. Perhaps
because of intrinsic redundancy, inroads into the role of BMP signaling in skull
bone osteoblast development have been difficult in vivo, while extensive in vitro
studies show that BMP signaling promotes osteoblast differentiation. The role of
BMP signaling in skull osteoblast formation has remained elusive, in part due to
the highly redundant nature of BMP signaling. There are three BMP type II
receptors in mice, Bmpr2, Acvr2a, and Acvr2b. Removal of Bmpr2 from murine
mesenchymal progenitors did not effect skull bone development, suggesting
BMP signaling may utilize multiple type II receptors in the skull (Gamer et al.). Removal of the type I receptor Alk2 from cranial neural crest cells did not perturb early cranial bone development, but resulted in a delay in ossification (Dudas et al., 2004). Recently limited insights have been gleaned from studies of ligand function. Conditional deletion of BMP4 in cranial neural crest cells resulted in mouse embryos with enlarged frontal fontanelles, and the additional neural crest-specific deletion of BMP2/BMP4 resulted in an exacerbated frontal bone ossification defect. Deletion of BMP7, however, did not affect frontal bone development (Bonilla-Claudio et al., 2012). Collectively, the results of exhaustive in vitro experiments, coupled with mesenchyme-restricted expression pattern of BMPs, provide a crucial role for BMPs in osteoblast differentiation, likely downstream of an inductive patterning event. Similar to the frustrations of BMP signaling in mouse skull bone development, mutations in the BMP pathway have not been implicated in congenital skull bone defects. However, studies of BMP signaling identified several transcriptional effectors, functional studies of which have been informative about cranial bone development.

TGFβ signaling, closely related to BMP signaling, consists of three superfamily ligands, TGFβ1, TGFβ2, and TGFβ3. Binding to receptors activates Type I receptors, which phosphorylate Smads transcriptional effectors, Smad2/3. TGFβ signaling promotes chondrocyte formation in cranial neural crest cells of the face (Ito et al., 2002). Cranial bone progenitors express TGFβ ligands by E14.5, TGFβ2 deletion in mice resulted in diminished ossification of skull bones (Sanford et al., 1997; Visel et al., 2004), and TGFβRII deletion in cranial neural
cells results in loss of calvarial bone (Ito et al., 2003). Differentiation marker analysis and cell survival were unaffected, whereas proliferation of cranial bone progenitors was decreased (Sasaki et al., 2006). Notably, bone development was inhibited in both CNC-derived frontal bone and PM-derived parietal bone. One interpretation is that TGFβ-signaling in meningeal progenitors is essential for ossification, although direct evidence is lacking. Additionally, removal of the TGFβ Type I receptor Alk5 from pre-migratory cranial neural crest cells resulted in complete loss of skull bone mineralization, including mesoderm-derived parietal bone (Dudas et al., 2006). Presently no mutations have been identified in TGFβ signaling components that implicate the pathway in human disease. Therefore while TGFβ signaling is critical for ossification, it appears likely another signaling pathway comprises the inductive patterning cue for initiation of skull bone progenitor cell fate selection.

In the Delta/Notch pathway, three Notch receptors (Notch 1-3) interact with five membrane-bound ligands, Jagged1-2, and Delta-like (Dll1, Dll3, and Dll4). Ligand-receptor interaction results in a cleavage of the Notch intracellular domain (NICD). Cleaved NICD is transcription factor that translocates to the nucleus and converts the repressor, RBP-Jκ, to an activator on Notch target genes (Weinmaster, 1997). Notch pathway components and effectors are present in the mouse coronal suture during specification (E12.5). Removal of Jagged1 specifically from mesoderm allowed interspersion of cranial neural crest cells into coronal suture mesenchyme and caused suture synostosis (Yen et al., 2010), pointing to the critical role for Notch signaling in maintaining tissue
boundaries in the skull. The Notch pathway also inhibits mesenchymal progenitor cell differentiation, although there is no direct evidence for Notch regulation of cranial bone progenitor specification, constitutive pathway activation caused a loss of skull ossification (Dong et al., 2010). Further studies may specifically elucidate a role for Notch in negatively regulating osteoblast progenitor differentiation in the skull.

There are three Hedgehog homolog ligands, Shh, Ihh, and Dhh (Sonic Hedgehog, Indian hedgehog, and Desert Hedgehog). Hedgehog ligands bind to the Patched1 (Ptc1) Receptor, relieving constitutive inhibition of the Hh pathway transducer Smoothened (Smo) by Ptc1. Subsequently, Smo activates Gli1-2, a pair of transcriptional activators, as well as the transcriptional repressor Gli3. Ihh and Shh transcripts localize to developing skull bone progenitors by E16.5 (Lenton et al., 2011) Endochondral bone formation bears a stringent requirement for Ihh, but there was only a subtle delay in skull bone formation in Ihh homozygous null mutants (Lenton et al., 2011). However, removal of the Gli3 repressor in homozygous null mice caused synostosis of the interfrontal suture (homologous to metopic in human)(Veistinen et al., 2012), phenocopying a rare manifestation of Gli3 mutations in Grieg cephalopolysyndactyly syndrome (McDonald-McGinn et al., 2010; Vortkamp et al., 1991). Perhaps functional analysis of Gli activators or the Ptc1 receptor will yield more insights into Hedgehog signaling in cranial bone biology, since a role for Ihh may be masked by the presence of Shh.
In mice, there are 22 Fibroblast Growth Factor ligands (FGFs) that bind to one of four Receptor Tyrosine Kinases (RTKs) called Fibroblast Growth Factor Receptors (FGFRs) (Ornitz and Itoh, 2001). FGFR1-3 are expressed during cranial bone development as early as E12.5 and exhibit dynamic expression at the osteogenic fronts/suture mesenchyme of the skull (Kim et al., 1998; Rice et al., 2000). Levels of phosphorylated ERK1/2, the activated intracellular effectors of receptor tyrosine kinase signaling, are present in the osteogenic fronts of the frontal and parietal bones at the coronal suture by E14.5 (Ting et al., 2009). Removal of FGFR2 from mesenchymal progenitors resulted in decreased bone mass, and defects in osteoblast progenitor proliferation (Yu et al., 2003). Activating mutations of FGFR2 have been implicated in several human craniosynostosis syndromes (Bellus et al., 1996; Wilkie et al., 1995b). A mouse model of one activating Fgfr2 mutation provided insight into the molecular pathogenesis of FGF signaling-related craniosynostosis: an underlying increase in cranial osteoblast progenitor proliferation and differentiation marker expression (Eswarakumar et al., 2004). FGF2 beads induce Runx2 expression in calvarial explants, as well as BMP2 expression. Runx2 deletion results in loss of BMP2 expression in suture mesenchyme at E15.5 but not loss of FGF2 expression (Choi et al., 2005). Due to the preponderance of FGF pathway mutations in human suture fusion syndromes, a fuller understanding of the upstream and downstream regulation of the FGF pathway in osteoblast biology offer the promise of new therapeutic avenues.
Insights from human and mouse genetics provide the cellular and molecular basis of cranial bone formation and pathogenesis of birth defects (Fig. 1.6, 1.7). Nonetheless, it remains clear that our understanding is incomplete. Clearly, a fundamental gap remains in our knowledge of signals that induce cell fate selection and spatiotemporal patterning of cranial bone progenitors. Since Wnt/β-catenin interconnects skeletal cell fate, and skull bone defects, Wnts are a potential patterning signal in cranial bone progenitor cells.

I.C.3. Wnt signaling pathways

Wnt signaling is a conserved intercellular communication pathway with multiple essential roles in embryonic development (Logan and Nusse, 2004). The Wnt genes are vertebrate homologs of Drosophila wingless, which encodes for a secreted protein required for segment polarity and wing formation (Nusslein-Volhard and Wieschaus, 1980). In mice, there are 19 known Wnt ligands, a staggering number that suggests diverse functions, yet Wnt production and secretion is a tightly controlled, highly conserved process (Fig. 1.8). Accumulating evidence suggests that in mice, as in Drosophila, Wnt protein secretion requires post-translational palmitoylation at a conserved cysteine residue by Porcupine, an acyltransferase in the endoplasmic reticulum (van den Heuvel et al., 1993). Porcupine may additionally acylate a serine residue on Wnt ligands. In spite of its essential role, there are notable phenotypic differences between porcupine mutants and lipid-deficient wingless mutants in Drosophila. Thus, porcupine is either not specific to the Wnt pathway, or the Wnt pathway requires additional acyltransferases (Herr et al., 2009). The secretion of
palmitoylated Wnt ligands is also highly conserved, and requires a G-protein coupled receptor, Drosophila wntless or its mouse homolog, Gpr177(Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009; Goodman et al., 2006). Wls physically interacts with acylated Wnt ligands, (Coombs et al., 2009), and one proposed function of Wls is to shuttle Wnt ligands to the plasma membrane for exocytosis and secretion (Port et al., 2008). Recent studies in mice have shown the stringent requirement for Wls in Wnt-dependent development and homeostasis (Chen et al., 2012; Fu et al., 2009). Following secretion Wnt ligands can either act as morphogens either in a short or long range manner.

One mechanism by which Wnt ligands signal to target cells is the canonical Wnt/β-catenin pathway. Wnt ligands bind to Fzd receptors (Bhanot et al., 1996) on target cells in conjunction with LRP 5/6, essential Wnt co-receptors (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Ligand-receptor binding prevents the assembly of the APC/Axin destruction complex.
The active complex degrades β-catenin through glycogen synthase kinase-mediated (GSK3β) phosphorylation (Aberle et al., 1997; Latres et al., 1999; Liu et al., 1999); Fig. 9). Wnt ligand binding thus inhibits the APC/Axin/GSK3β-mediated destruction of β-catenin in the cytoplasm, β-catenin translocates to the nucleus (Cox et al., 1999; Miller and Moon, 1997; Tolwinski and Wieschaus, 2004) and interacts with the TCF/LEF transcription factor families (Behrens et al., 1996) to alter gene transcription. The TCF/LEF families bind to consensus genomic sequences and activate or inhibit the transcription of specific target
genes (Korinek et al., 1998). At short-range Wnt signals effect gene expression in a ligand concentration-independent manner, whereas at long-range Wnts act as morphogens, producing a graded, dose-dependent effect on gene expression in cells up to 20-30 cell diameters away (Zecca et al., 1996). Thus, Wnt signal transduction results in the production of a specific transcriptional program that alters the growth, division, and differentiation of cells.

β-catenin is not specifically a Wnt pathway nuclear factor; instead, it plays a second, important role intercellular adhesion at the cell membrane ((Nelson and Nusse, 2004b); Fig. 1.9). At the cell membrane, β-catenin participates in adhesion complexes with cadherins. Cadherin-β-catenin interactions are essential in organization of stem cell niches and perturbations can affect cell fate (Nelson and Nusse, 2004a). Therefore, functional studies of β-catenin as a Wnt pathway transducer should be supplemented with other functional studies, to isolate the effects of cadherin-β-catenin interactions in development.

Conversely Wnt ligands also signal through β-catenin independent pathways, which collectively constitute non-canonical Wnt signaling (Fig. 1.10). Three Wnt ligands, Wnt5a, Wnt11, and Wnt4, do not produce classic canonical pathway effects such as malignant transformation of mammalian cells or axis duplication in amphibians (Du et al., 1995). Instead, mutants of the non-canonical Wnt ligands and their signaling pathway components result in defects in cell movements. Furthermore, non-canonical Wnt signaling through Ror2, an RTK, actively inhibits Wnt/β-catenin signaling (Gordon and Nusse, 2006). Thus, many effects on cranial neural crest cells and bone development are attributable
predominantly to β-catenin-dependent, canonical Wnt signaling, but the roles for non-canonical Wnt signaling and Wnt-independent, β-catenin cell adhesion are undoubtedly underappreciated.

I.C.4. Mutations of Wnt pathway in bone biology

While Wnt signaling is nearly ubiquitous in developmental processes, the Wnt pathway is genetically altered in numerous mutations associated with human skeletal diseases, underscoring its central significance in bone biology and skeletal development in particular (Table 2). Mutations in the canonical pathway are associated with both bone mass homeostasis and skeletal patterning. Opposing LRP5 mutations are associated with osteoporosis pseudoglioma syndrome (OPPG) and with hereditary high bone mass (Gong et al., 2001). Additional genes encoding for Wnt pathway regulators, including LRP6, SOST, WTX, and FZD9, can carry mutations associated with bone diseases (Balemans et al., 2001; Brunkow et al., 2001a;
Jenkins et al., 2008; Mani et al., 2007; Wang et al., 1999). Furthermore, single nucleotide polymorphisms in GPR177 are associated with osteoporosis in genome wide association studies (Rivadeneira et al., 2009). The list of Wnt pathway genes mutated in developmental skeletal defects is nearly as long, and implicates both canonical and non-canonical Wnt signaling in early skeletal patterning. For example, mutations in canonical signaling components such as LRP4, WNT7A, WNT3, SOST are associated with congenital limb defects and with digit fusion ((Baron and Kneissel, 2013) and references therein). Thus, the number of Wnt pathway mutations associated with skeletal anomalies in humans offers a glimpse into the crucial role for the Wnt pathway in normal vertebrate skeletal patterning.

I.C.5. Wnt signaling and congenital craniofacial anomalies

While Wnts are clearly linked to vertebrate limb development, a growing body of evidence points to similarly critical roles for Wnts in craniofacial patterning and development (Table 1.2). The most common craniofacial birth defect, orofacial clefting, has been associated with Wnt9b/WNT9B in mouse and man (Chiquet et al., 2008; Juriloff et al., 2006). Mutations in AXIN2 are also infrequently associated with tooth agenesis (Lammi et al., 2004). Mutations in WTX, encoding for a Wnt pathway negative regulator, can cause a cranial skeletal dysplasia with thickened skull bones (Jenkins et al., 2008), suggesting Wnt signaling is an essential regulator of skull development. Likewise, mutations in SOST, encoding for sclereostin, results in cranial sclerosteosis, with thickened skull bones (Balemans et al., 2001). Thus, removal of inhibitors of the
Wnt pathway consistently results in increased bone deposition in humans. These studies suggest that a requirement for the Wnt pathway in skull bone formation should be tested.

I.C.6. Roles of Wnt pathway in murine bone development

Insights from mouse genetics reveal roles for Wnt signaling in nearly every aspect of skeletal development. For example, homozygous knockouts of Wnt ligands demonstrated the key role of the pathway in limb formation. \textit{Wnt3a}^{-/-} mice have limb pattern defects, and Wnt3a is essential for maintenance of the apical ectodermal ridge (AER) in the limb (Galceran et al., 1999). \textit{Wnt7a}^{-/-} mice have defective dorsoventral polarity of the limb (Yang and Niswander, 1995).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Cellular Role</th>
<th>Skeletal defect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{LRP5}</td>
<td>Co-receptor</td>
<td>Osteoporosis in osteoporosis-pseudoglioma syndrome High Bone mass</td>
<td>(Boyden et al., 2002; Gong et al., 2001)</td>
</tr>
<tr>
<td>\textit{LRP6}</td>
<td>Co-receptor</td>
<td>Osteoporosis</td>
<td>(Mani et al., 2007)</td>
</tr>
<tr>
<td>\textit{SOST}</td>
<td>Secreted inhibitor</td>
<td>Sclerosteosis (high bone density)</td>
<td>(Balemans et al., 2001; Brunkow et al., 2001a)</td>
</tr>
<tr>
<td>\textit{FZD9}</td>
<td>Receptor</td>
<td>Williams-Beueren (low bone density)</td>
<td>(Wang et al., 1999)</td>
</tr>
<tr>
<td>\textit{WTX}</td>
<td></td>
<td>osteopathia striata congenita with cranial sclerosis</td>
<td>(Jenkins et al., 2008)</td>
</tr>
<tr>
<td>\textit{GPR177}</td>
<td>Trafficking regulator</td>
<td>Osteoporosis</td>
<td>(Rivadeneira et al., 2009)</td>
</tr>
<tr>
<td>\textit{PORCN}</td>
<td>Acyltransferase</td>
<td>Focal dermal hypoplasia (osteofathia striata)</td>
<td>(Grzeschik et al., 2007; Wang et al., 2007)</td>
</tr>
<tr>
<td>\textit{WNT7A}</td>
<td>Ligand</td>
<td>Phocomelia and other limb defects</td>
<td>(Woods et al., 2006)</td>
</tr>
<tr>
<td>\textit{WNT3}</td>
<td>Ligand</td>
<td>Tetra-amelia (limb defects)</td>
<td>(Niemann et al., 2004)</td>
</tr>
<tr>
<td>\textit{AXIN2}</td>
<td>Intracellular inhibitor</td>
<td>Tooth agenesis</td>
<td>(Lammi et al., 2004)</td>
</tr>
</tbody>
</table>

Table 1.2. Wnt pathway mutations in bone diseases and craniofacial defects
Collectively, these early inroads into understanding vertebrate limb patterning clearly implicate Wnt ligands in pattern induction.

Skeletal development has proven to be one of the most fruitful areas for defining Wnt/Planar Cell Polarity pathways in vertebrates. Non-canonical Wnt signaling ligands are essential in the proximal-distal outgrowth of the limb and play key roles in cell polarity during cartilage development (Gao et al., 2011; Yamaguchi et al., 1999). While direct evidence for non-canonical Wnt signaling in osteoblast development is lacking, Wnt ligands can stimulate osteoblast formation through β-catenin-independent pathways (Albers et al.; Tu et al., 2007). Future studies will yield further information about possible roles for β-catenin-independent signaling in skeletal cell fate and patterning.

Functional studies of β-catenin, the central transducer of canonical Wnt signaling offer the strongest rationale studying the Wnt pathway in skeletal progenitor cell fate. Removal of Ctnnb1, encoding for β-catenin, from limb bud mesenchyme recapitulated defects of the ectoderm ligand knockouts. These data revealed that an ectoderm Wnt signal to the underlying mesenchyme is an essential patterning event in the limb. In limb mesenchymal progenitor cells, Ctnnb1 removal from mesenchyme pushed the osteochondroprogenitor cell that gives rise to bone and cartilage predominantly towards a cartilage cell fate pathway (Day et al., 2005; Hill et al., 2005). Paradoxically, a ctnnb1 gain-of-function mutation in mesenchymal limb progenitors caused a premature arrest in skeletogenesis, including reduced cell fate marker expression (Hill et al., 2005). Collectively, these experiments suggest β-catenin has complex functions during
skeletal fate determination, yet how β-catenin promotes osteoblast cell fate remains elusive. Insights from mouse limb development thus highlight the key roles of Wnt/β-catenin in patterning and skeletogenesis.

I.C.7. Roles of Wnt pathway in mouse craniofacial development

Mouse genetics has served an invaluable role characterizing the signals essential for the emergence of the fascinating cranial neural crest lineage and its derivatives, and the Wnt pathway has emerged as a key player in CNC biology. Compound deletion of \textit{Wnt1} and \textit{Wnt3a} results in loss of cranial neural crest derivatives, probably through defective formation of cranial neural crest cells (Ikeya et al., 1997). Severe arrest in craniofacial development occurred upon removal of Wnt pathway components from the cranial neural crest such as the trafficking regulator, \textit{Wls/Gpr177}, the central transducer, \textit{ctnnb1}, or the negative regulator, \textit{APC} (Brault et al., 2001; Fu et al., 2011; Hasegawa et al., 2002). Wnt signals regulate the morphogenesis of structures populated by cranial neural crest cells such as the first branchial arch-derived facial prominences, where the Wnt pathway underlies both species-specific pattern and pathologic phenotypes such as a mouse-model of DiGeorge syndrome (Brugmann et al., 2007; Huh and Ornitz, 2010). While a role for Wnt signaling in craniosynostosis in humans is lacking, evidence is accumulating for a role for Wnt signaling in skull bone biology. \textit{Axin2} homozygous null mice, deficient for the negative regulator of Wnt signaling, exhibit a craniosynostosis phenotype (Liu et al., 2007; Yu et al., 2005). Continued efforts may yield links between the Wnt pathway and factors underlying human craniosynostosis, cementing the role of Wnt pathway in the
latter stages of skull morphogenesis. Does the Wnt pathway govern early skull morphogenesis? Removal of \textit{ctnnb1} from skull progenitors resulted in an absence of intramembranous ossification (Day et al., 2005; Hill et al., 2005). In this thesis, my work makes initial inroads linking the Wnt pathway to initiation of skull bone cell fate and morphogenesis, and, through subsequent regulation of downstream targets, to human disease.

\textit{I.C.8. Tissue-tissue interactions}

As a class of secreted proteins, Wnt ligands signal between two cells in a paracrine fashion, or the proteins feedback on the secreting cell in an autocrine loop.

The mouse limb has served as a model of tissue-tissue interactions during skeletal patterning and lineage determination. In the limb, reciprocal signals between ectoderm and underlying mesenchyme shape pattern of the skeletal elements. Endochondral ossification
occurs subsequently deep within the limb, but involves both autocrine and paracrine signals among chondrocytes, epithelium-like perichondrium, and osteoblast progenitors. (Cohen, 2006). A similarly rigorous analysis of sequential tissue-tissue interactions during skull morphogenesis awaits investigation.

Both the early formation of cranial neural crest, as well as subsequent differentiation of those cells into distinct structures, involves precise communication between tissues. Formation of many important CNC-derived structures require first ectoderm-mesenchyme interactions, and subsequently mesenchyme-mesenchyme interactions (Fig. 1.11(Jheon and Schneider, 2009). The identification of tissue-tissue interactions has clearly been informative in craniofacial development, and continues to be of great interest for understanding the pathogenesis of craniofacial birth defects.

In the skull bones, craniosynostosis in mice and humans results, in part, from dys-regulated signaling between tissues. The coronal and sagittal sutures lie at the boundaries between cranial neural crest and paraxial mesoderm (Jiang et al., 2002), and proper segregation of populations requires repulsive signaling events to prevent cell mixing and craniosynostosis (Merrill et al., 2006; Yen et al., 2010). Paracrine mesenchymal signals between cells of the suture and osteogenic fronts also set the timing of appositional bone growth, and pathologic cell signaling leads to precocious differentiation of osteoblasts and craniosynostosis (Eswarakumar et al., 2004; Kim et al., 1998). Thus tissue-tissue interactions play multiple key roles throughout cranial bone formation, from the
early induction of cranial neural crest to the final stages of skull morphogenesis at the cranial sutures.

The signaling mediators of tissue-tissue interactions during initiation of skull bone fate selection remain unidentified, and their initial description represents a major goal of this thesis. Precise elucidation of the initial events in skull bone morphogenesis has implications for evolution of skull shape, engineering of skeletal tissue from stem cells, and the upstream regulation of events leading to cranial bone pathology such as craniosynostosis.
II. A. Abstract: The bones of the mammalian skull vault form through intramembranous ossification. Skull bones ossify directly, in a process regulated by β-catenin, instead of passing through a cartilage intermediate. We tested whether β-catenin is necessary for fate selection of intramembranous bone progenitors in the skull. Here, we show in mice that removal of β-catenin from skull bone progenitors resulted in the near complete transformation of the skull bones to cartilage, whereas constitutive β-catenin activation inhibited skull bone fate selection. β-catenin directly activated Twist1 expression in skull progenitors, conditional Twist1 deletion partially phenocopied absence of β-catenin, and Twist1 deletion partially restored bone formation in the presence of constitutive β-catenin activation. Finally, Twist1 bound robustly to the 3'UTR of Sox9, the central initiator of chondrogenesis, suggesting Twist1 may directly repress cartilage formation through Sox9. These findings provide insight into how β-catenin signaling via Twist1 actively suppresses formation of cartilage, and promotes intramembranous ossification in the skull.
II.B. Introduction

The mammalian skull vault, which protects the brain, is comprised of bones derived from dual embryonic origins. Cells from both cranial neural crest (CNC) and paraxial mesoderm (PM) form skull progenitors, and these distinct populations are already spatially segregated in rostral and caudal domains above the eye in the mouse at E11.5 (Yoshida et al., 2008). Skull bones undergo intramembranous ossification, in which osteoprogenitors are specified and subsequently differentiate into lineage-restricted osteoblasts, moving dorsally before ossifying directly as bone (Yoshida et al., 2008). In the mouse, CNC contributes to the frontal bones and the interparietal bone, and PM gives rise to the parietal bones of the posterior skull vault (Jiang et al., 2002; Yoshida et al., 2008).

Bone formation occurs through two divergent mechanisms, intramembranous and endochondral ossification. The majority of the skeleton undergoes endochondral bone formation, in which a cartilage template first forms, becomes mineralized, and is replaced by osteoblast-made bone. In contrast, intramembranous bones, such as those of the skull, form without a cartilage intermediate. Bone forming osteoblasts of both intramembranous and endochondral bones express the transcription factors Runx2 and Osterix (Osx; Sp7 – Mouse Genome Informatics), which are essential for initial osteoprogenitor specification and osteoblast lineage commitment, respectively (Nakashima et al., 2002; Otto et al., 1997). In these early stages of progenitor formation in endochondral bones, genetic studies demonstrated that the BMP, Wnt, Indian
Hedgehog (Ihh) and Notch signaling pathways are important, however a direct examination of their roles during development of intramembranous bone progenitors is lacking (reviewed in (Long, 2008). Several studies have revealed differences in the molecular signals required for the specification of endochondral and intramembranous bone progenitors. For example, the chondrogenic determinant Sox9 is essential for the formation of the cartilage anlage which precedes endochondral progenitor specification. Consequently, Sox9 mutant cells from chimeric embryos do not contribute to endochondral bone (Bi et al., 1999). One of the major roles of chondrocytes during endochondral bone formation is the paracrine secretion of Ihh that is responsible for specification of endochondral osteoprogenitors and initiation of Runx2 expression (St-Jacques et al., 1999). In contrast, Sox9 and chondrogenesis are dispensable in skull vault morphogenesis, consistent with the finding that intramembranous bone progenitors do not require Ihh for intramembranous osteoprogenitor specification (St-Jacques et al., 1999). Therefore molecular differences in the initiation of osteoprogenitor cell specification between these two ossification programs are evident, and yet the signaling pathways that specify intramembranous bone progenitors remain unidentified. Since skull vault formation occurs in the absence of an intermediary cartilage, it is then plausible that the pathway initiating osteoprogenitor specification in this population may simultaneously inhibit chondrocyte formation in the skull.

Several studies have confirmed that osteoblasts and chondrocytes involved in endochondral bone formation originate from common progenitors
(Akiyama et al., 2005). β-catenin-dependent signaling is crucial in determining the definitive cell fate, and it inhibits chondrocyte differentiation in endochondral osteoprogenitors (Akiyama et al., 2005; Hill et al., 2005; St-Jacques et al., 1999). β-catenin transduces Wnt signaling through the Frizzled and LRP receptors by associating with members of the TCF/LEF transcription factor family in the nucleus and regulating target gene expression (Bhanot et al., 1996; Korinek et al., 1998; Liu et al., 1999; Tamai et al., 2000). β-catenin is also required for intramembranous skull vault mineralization (Day et al., 2005; Hill et al., 2005). Conditional β-catenin deletion in the head results in an absence of the skull vault, with replacement by cartilage. β-catenin is not, however, sufficient for bone formation as gain-of-function genetic studies in osteoprogenitors revealed a global loss of ossification, with reductions in Runx2 and Sox9 mRNA levels observed (Hill et al., 2005). The mechanism underlying the discrepancy between gain- and loss-of-function studies for β-catenin remains unknown.

β-catenin functions by regulating target gene expression. One of its targets, the Twist family, has roles in head morphogenesis, skull osteoblast differentiation and repression of cartilage growth, suggesting a role in intramembranous progenitor specification (Bialek et al., 2004; Logan and Nusse, 2004; Soo et al., 2002). β-catenin activates Twist1 in vitro and Twist2 in vivo making these transcription factors nuclear targets of β-catenin (Howe et al., 2003; Ohtola et al., 2008; Reinhold et al., 2006; Tran et al., 2010). Twist1 is required for early migration and survival of cranial mesenchyme which gives rise to the skull bones (Bildsoe et al., 2009; Soo et al., 2002). Twist1+/− mice have
premature intramembranous osteoblast differentiation and skull suture fusion but
demonstrate no overt endochondral bone phenotype (Bialek et al., 2004; Bildsoe
et al., 2009; Reinhold et al., 2006; Soo et al., 2002). Similarly, Twist2
heterozygosity can restore normal size to the clavicles, which ossify in part via
the intramembranous pathway, in a mouse model of cleidocranial dysplasia
(Bialek et al., 2004). Both results indicate the Twist family could exert specific
effects on osteoblasts in intramembranous bones not observed in endochondral
bones. Furthermore, Twist1 inhibits chondrocytic differentiation in vitro,
suggesting that Twist1 could serve as a chondrogenic repressor in vivo (Reinhold
et al., 2006). Both Twist2Cre-lineage marked descendants and Twist1 mRNA are
present in the cranial mesenchyme between E9.5 and 11.5; therefore the
spatiotemporal expression of the Twist family is consistent with a role in
regulating intramembranous bone progenitor specification (Bialek et al., 2004;
Tran et al., 2010; Yu et al., 2003). In spite of these data, no existing work has
deﬁned the role of the Twist family members as mediators of the Wnt/β-catenin
pathway during cranial bone fate selection in vivo.

Here, we tested whether β-catenin is necessary for fate selection of
intramembranous bone progenitors in the mouse skull. First, we genetically
deleted β-catenin in cranial bone progenitors derived from cranial mesenchyme,
resulting in the near complete transformation of the skull vault into cartilage. β-
catenin was required for cranial osteoblast lineage commitment, and directly
activated Twist1 expression. Twist1 deletion from skull progenitors also resulted
in chondrocyte formation, which was limited to the posterior skull vault. Finally,
we demonstrated genetic interactions of β-catenin with Twist1, and direct
association of Twist1 with the 3'UTR of the central initiator of chondrogenesis, Sox9. Taken together our data show that the Twist family comprises an essential set of effectors of β-catenin-mediated skull progenitor specification.

II.C. Results:

II.C.1. β-catenin is required for cranial osteoblast lineage commitment

First, we characterized Wnt signal transduction via β-catenin in membranous progenitors derived from cranial neural crest (CNC) and from paraxial mesoderm (PM). We used an Engrailed1Cre (En1Cre) knock-in line to conditionally modulate β-catenin activity levels in post-migratory CNC and PM-derived skull progenitors in the supraorbital arch (Kimmel et al., 2000). To distinguish CNC-derived versus PM-derived membranous progenitors we utilized the Wnt1Cre transgenic line (Jiang et al., 2002) to activate the expression of the Rosa26 lacZ reporter (RR) specifically in pre-migratory CNC cells (Soriano, 1999). Compared to E12.5 Wnt1Cre; RR lineage labeled CNC-derivatives (Fig. 2.1A), the En1Cre lineage contributed to CNC-derived frontal and interparietal bone primordia (Fig. 2.1C, Fig. 2.4A) and to PM-derived parietal bone primordia (supplementary material Fig. 2.S1A). En1Cre lineage-marked cells were not present in the dura mater, the surface ectoderm, or the cartilage base of the skull (supplementary material Fig. 2.S2A, E). Alkaline Phosphatase (AP) staining on
Figure 2.1. β-catenin is required for cranial osteoblast lineage commitment. Coronal mouse tissue sections were stained with X-gal for β-galactosidase activity (A-C) or for alkaline phosphatase activity (D-E). The AP+ domain is outlined on serial sections (A, B, F). Section in situ hybridization (F-O). Asterisks indicate where Lef1 and Osx expression was lost in cranial mesenchyme (G, K). Black arrows point to expanded Runx2, Sox9, and Col2a1 domains (I, M, O). Dashed lines indicate brain. Whole mount skeletal preparations stained with alcian blue for cartilage and alizarin red for bone (P, Q). Diagrams to left of figure show plane of section and region of interest (boxed). Tel, telencephalon; se, surface ectoderm; ey, eye; fp, frontal bone progenitors; l, interparietal bone; p, parietal bone; f, frontal bone. Scale bar in (B) is 100 µm, and scale bar for whole mount photographs in (P) is 25 mm.
Figure 2.S1. β-catenin is required for cranial bone fate in paraxial mesoderm osteoprogenitor cells. X-gal (A-C), and AP (D, E) staining, or in situ hybridization on tissue sections (F-K). Dashed lines skull progenitors (A, B, F) or En1Cre lineage (H-K). White arrows indicate absent Lef1 expression (G). Black arrows point to ectopic Sox9 expression (K). Tel, telencephalon. Inset shows plane of section (B). Dm, dura mater; se, surface ectoderm; pp, parietal bone progenitor cells; tel, telencephalon. Scale bars represent 100 µm.

Figure 2.S2. Lack of contribution of En1Cre-lineage to skull base and description of temporal differences in Cre activity. X-gal staining (A, C, E, G) or in situ hybridization (B, D, F, H) on coronal sections. Diagram demonstrates onset of Cre recombinase activity for the lines used in this study (I). Arrows in (D, H) indicate ectopic Sox9 expression. Scale bars represent 100 µm.
serial sections at E12.5 localized intramembranous osteoprogenitors of both embryonic origins to the deepest subset of the En1Cre-lineage (Fig. 2.1D, supplementary material Fig. 2.S1D). Expression of the Wnt/β-catenin target gene, Lef1 (Hovanes et al., 2001) broadly localized to En1Cre-lineage cranial mesenchyme (Fig. 2.1F, supplementary material Fig. 2.S1B,F), including AP+ osteoprogenitors at E12.5. These data suggest that a large portion of En1Cre lineage-marked cranial mesenchyme was transducing β-catenin-dependent Wnt signaling at the onset of skull morphogenesis.

Previous studies left the role of β-catenin undefined in cranial bone progenitors (Brault et al., 2001; Day et al., 2005; Hill et al., 2005). Lineage analysis of En1Cre; RR embryos demonstrated labeling of presumptive intramembranous progenitors at E11-11.5 prior to osteoprogenitor specification in the supraorbital arch (Tran et al., 2010). Compared to En1Cre/+; RR/+ embryos, En1Cre/+; RR/+; β-cateninfl/+ heterozygotes exhibited no demonstrable skull phenotype. Therefore we generated En1Cre/+; RR/+; β-cateninfl/∆ embryos to delete β-catenin in the cranial bone progenitors to pinpoint the role of β-catenin in intramembranous osteoprogenitor fate selection. β-galactosidase and alkaline phosphatase (AP) activity staining of serial sections showed that En1Cre-lineage cells contained AP+ cranial osteoprogenitor cells, in controls and mutants (Fig. 2.1B-E). In conditional β-catenin mutant embryos the AP domain of CNC-derived osteoprogenitors expanded beneath the surface ectoderm (Fig. 2.1D, E), and there were fewer AP+ osteoprogenitor cells of PM origin in the En1Cre-lineage of
mutants than in controls (supplementary material Fig. 2.S1 C,E). Expression of the β-catenin target gene *Lef1* was completely abrogated in both CNC-derived and PM-derived osteoprogenitor cells of mutants (Fig. 2.1G, supplementary Fig. 2.S1G), suggesting efficient deletion of β-catenin by *En1Cre*.

Next, we analyzed the expression of the earliest markers of osteoprogenitor fate in β-catenin-deleted conditional mutants. The osteoprogenitor marker *Runx2* was expressed at E12.5 in *En1Cre* lineage-marked mesenchyme (Fig. 2.1H, supplementary Fig. 2.S1H) at the onset of cranial bone specification. In mutant CNC-derived mesenchyme, *Runx2* expression was present at E12.5, but the domain expanded directly beneath the surface ectoderm and dorsally (Fig. 2.1I). In mutant PM-derived mesenchyme, however, *Runx2* expression was absent by E12.5 (supplementary Fig. 2.S1H,I). In control embryos *Runx2*+ osteoprogenitors had differentiated further, also expressing the osteoblast lineage commitment marker *Osx* (Nakashima et al., 2002) at E12.5 (Fig. 2.1J). However, none of the *Runx2*+ mutant cells in the *En1Cre*-lineage expressed *Osx* (Fig. 2.1K). Expression of the earliest chondrocyte marker, *Sox9* (Bell et al., 1997; Bi et al., 1999), was confined to chondrocyte progenitors which form the skull base, outside of the *En1Cre* lineage-marked domain, and was not detected in intramembranous osteoprogenitor cells of controls at E12.5 and E13.5 (Fig. 2.1L, supplementary Fig. 2.S1J, 2.S2A-B, E-F). In mutant embryos, *Sox9* was still expressed in skull base progenitors outside the *En1Cre* lineage at E12.5 and E13.5 (supplementary Fig. 2.S2C-D), G-H, but we additionally detected *Sox9* mRNA within the *En1Cre*
lineage (Fig. 2.1M, 2.S1K, 2.S2C,D, G,H). Next, we tested if Sox9-expressing
cells in mutants were also positive for other chondrocyte markers. At E12.5
Col2a1 was expressed in the skull base of controls, but not in the En1Cre-
lineage (Fig. 2.1N). However, in mutants we detected Col2a1 in the En1Cre-
lineage in a similar expression pattern to Sox9 (Fig. 2.1O). By E12.5, En1Cre/+;
RR/+; β-cateninfl/Δ cranial osteoprogenitors expressed Runx2 but failed to
express Osx or differentiate further along the osteoblast lineage, instead
expressing Sox9 and Col2a1. These results suggest that β-catenin activity is
crucial during osteoblast lineage commitment for preventing induction of
chondrocytic fate during cranial intramembranous bone development as early as
E12.5 in the mouse embryo.

To determine if ectopic chondrocytes formed cartilage in conditional β-
catenin-deficient embryos, we compared their intact cranial skeletons with those
of controls to determine the affected areas in the skull. At E18.5, the skull bones
of control embryonic heads stained positive for bone (alizarin red), but negative
for cartilage (alcian blue)(Fig. 2.1P). In En1Cre/+; RR/+; β-cateninfl/Δ mutant
embryos, the paired frontal and parietal bones, as well as the interparietal bone
were all absent, and there was an almost uniform replacement of calvarial bone
with alcian blue-stained cartilage at E18.5 (Fig. 2.1Q, 8/8 embryos). These data
indicate that β-catenin is crucial for intramembranous osteoprogenitor
specification.
II.C.2. β-catenin activity is necessary for expression of Twist1 in cranial mesenchyme

Next, we sought to determine how β-catenin regulates cranial osteoprogenitor cell fate specification. Activation of Wnt signal transduction induces expression of the transcription factor Twist1 \textit{in vitro}, which as an inhibitor of chondrogenic differentiation could serve as a mediator of β-catenin in skull progenitor fate selection (Reinhold et al., 2006). We tested if Twist1 expression during cranial progenitor specification (E11.5–E12.5) required β-catenin activity. At E10.5-11.5, Twist1 protein localized to cranial mesenchyme beneath the surface ectoderm above the eye (Fig. 2.2A, B), and at E12.5, we detected Twist1 protein within cranial bone progenitors (Fig. 2.2C). Previously we showed that \textit{En1Cre} was inconsistently active at E10.5 in the cranial mesenchyme, with robust activity by E11.5 (Tran et al., 2010). In β-catenin conditional null mutants Twist1 protein expression was present in cranial mesenchyme at comparable levels to controls at E10.5, but was subsequently absent in β-\textit{catenin} null cranial bone progenitors at E11.5 and E12.5 (Fig. 2.2D-F). β-catenin was also required for Twist1 mRNA expression at E11.5 (data not shown). Therefore, β-catenin is required for the expression of Twist1 in cranial bone progenitors.

II.C.3. β-catenin activity promotes expression of Twist1 in cranial mesenchyme

We next tested whether constitutive activation of β-catenin signaling in the \textit{En1Cre} lineage was sufficient to induce Twist1 expression in cranial
mesenchyme. En1Cre/+; RR; β-catenin $\Delta^{ex3/4}$ mutant embryos were dying by E13.5, but were viable for analysis at E12.5 (Fig. 2.3A, D). En1Cre/+;

Figure 2.2. Requirement for β-catenin in cranial mesenchyme for Twist1 protein expression. Indirect immunofluorescence on axial (A, D) or coronal tissue sections (B, C, E, F). Nuclei were counterstained with DAPI. Ey, eye. Tel, telencephalon (B, C, E, F). Dotted white lines outline the telecephalon (B-F). Dashed lines indicate osteoprogenitors (op, C). White arrows indicate loss of Twist1 expression (E, F). Diagram beneath indicate plane of section, region of interest, and embryonic axes. Scale bars: 100 µm.
RR; \( \beta \)-catenin \( \Delta^{ex3/4} \) embryos revealed a complete absence of Runx2 in cranial bone progenitors (Fig. 2.3B, E). Twist1 was expressed in E12.5 cranial dermal and osteoprogenitor domains of En1Cre-lineage-marked cells of control embryos (Fig. 2.3C). Upon constitutive activation of \( \beta \)-catenin signaling, the Twist1 expression domain expanded into the entire En1Cre lineage (Fig. 2.3D, F, compare arrow). Constitutive \( \beta \)-catenin activation also resulted in an approximately 60% increase in relative Twist1 protein immunofluorescence (Fig. 2.3M, N). Therefore, \( \beta \)-catenin signaling promotes Twist1 expression in the cranial mesenchyme.

In further support of this conclusion, in the mutant we also identified sites of ectopic Twist1 expression in the trigeminal ganglia (supplementary Fig. 2.S3) and in the nasal capsule (Fig. 2.3H, K), which were both restricted to En1Cre lineage-marked cells with forced activation of \( \beta \)-catenin signaling (Fig. 2.3G, J), and, in chondroprogenitors, corresponded with a reduction in endogenous Sox9 expression (Fig. 2.3I, L). Collectively, these experiments demonstrate that \( \beta \)-catenin activity is required and sufficient for Twist1 expression.

II.C.4. \( \beta \)-catenin forms a molecular complex that acts on the Twist1 promoter

Next, we determined whether Twist1 was a direct transcriptional target of \( \beta \)-catenin in vivo. We identified consensus TCF/LEF binding motifs in nine
regions at the *Twist1* locus in addition to one previously identified in the minimal *Twist1* promoter (Howe et al., 2003). Chromatin immunoprecipitation followed by

**Figure 2.S3. β-catenin is sufficient for ectopic Twist1 expression.** Coronal tissue sections through trigeminal ganglia were stained with X-gal (A, C) or hybridized with mRNA probes (B, D). Black arrows point to area of ectopic expression (B, D). Scale bars are 100 µm.
Figure 2.3. β-catenin activates Twist1 expression. Coronal sections were stained with X-gal (A, D, G, J). In situ hybridization on coronal tissue sections through cranial bone progenitors (B-C, E-F) or facial cartilage (H-I, K-L). Inset in (E) is positive control staining on maxilla on the same section. Insets in (G, J) show low magnification for orientation; diagrams to left indicate plane of section. Arrows indicate expansion of Twist1 domain (C, F). Thick dashed lines indicate En1Cre-lineage cells in nasal capsule, while thin dashed black lines detail nasal capsule primordia marked by Sox9 expression (G-L). Indirect immunofluorescence was performed on coronal sections (M-N; inset in M for orientation). Average intensity of relative Twist1 immunofluorescence in cranial bone progenitors outlined in (M-N) was quantified (see Results). Mouse Twist1 locus showing distance of Lef1 binding sites (E1-E10) from the transcription start site (TSS) in kilobases (O). Quantitative real-time PCR was performed on immunoprecipitated chromatin from E12.5 wild-type mouse cranial mesenchyme. H3K4me1, monomethylated lysine four of histone H3 (P, Q). Negative controls for both immunoprecipitation experiments were non-target sequences characterized by low conservation and lack of Lef1 consensus motif near the Twist1 locus. Dashed lines indicate approximate threshold set by non-target sites (P, Q). Tel, telencephalon; se, surface ectoderm; dm, dura mater, mx, maxilla Oe, olfactory epithelium. See also supplementary material Figure 2.S3. Scale bars represent 100 µm.
quantitative PCR (ChIP-qPCR) for mono-methylated lysine 4 of histone H3 (H3K4me1), predictive of active or poised enhancer elements, revealed significant enrichment (greater than 6 fold) for three TCF/LEF consensus sequences (Fig. 2.3P) compared to non-target sites in the Twist1 locus in E12.5 cranial mesenchyme (Creyghton et al., 2010; Heintzman et al., 2007). Next we tested by ChIP-qPCR on E12.5 cranial mesenchyme, whether a transcription complex containing β-catenin directly binds to H3K4me1-marked Twist1 regulatory elements in vivo. β-catenin binding was enriched 2.5 fold at the TCF/LEF consensus site marked by H3K4me1 approximately 1.7 kb upstream of the transcription start site (Fig. 2.3Q). These data indicate that β-catenin binds to a putative 5' enhancer element upstream of the promoter of Twist1 in cranial mesenchyme, which is consistent with the requirement and sufficiency of β-catenin activity for Twist1 expression. However we cannot rule out the possibility that β-catenin acts on enhancers located more distal to those tested here, or even in trans.

II.C.5.Deletion of Twist1 is sufficient to induce chondrogenesis in cranial mesenchyme

β-catenin positively regulates Twist1 expression and negatively regulates chondrogenesis in cranial bone progenitors. We tested the hypothesis that Twist1 is a key mediator of β-catenin and a negative regulator of chondrogenesis in vivo. We used the En1Cre line and the Twist1 conditional null allele to delete Twist1 from cranial mesenchyme prior to fate specification (Chen et al., 2007). β-gal staining on En1Cre; RR embryos at E15.5 revealed En1Cre-lineage cells in
the paired frontal and parietal bones as well as in the interparietal bone (Fig. 2.4A). The lack of Twist1 mRNA confirmed that deletion was effective in Twist1 mutant cranial bone progenitors by E12.5 (Fig. 2.4B). Conditional Twist1 deletion resulted in alcian blue stained cartilage encompassing the posterior half of the skull from the interparietal bones laterally to the parietal bones, and anteriorly to the coronal suture (Fig. 2.4C, n=6/6). Twist1 conditional mutants were also missing a posterior section of the frontal bones, an anterior portion of the parietal bones, and a significant amount of the interparietal bone as judged by alizarin red staining at E16.5 (Fig. 2.4C).

In Twist1 mutants, the frontal bone lacked Osx expression (Fig. 2.4D). However there was no evidence of Sox9 expression in conditional Twist1 mutants, even though we detected subtle domains of Col2a1 expression in Twist1 mutant frontal bone progenitors. Notably we did not detect any cell death of cranial bone progenitors at E12.5 by immunofluorescence for cleaved caspase 3 (data not shown). The parietal and interparietal bones of Twist1 conditional mutants did not express Osx (Fig. 2.4E, F). In place of osteoprogenitors we identified the presence of ectopic chondrocytes by Sox9 and Col2a1 expression (Fig. 2.4E, F). Therefore loss of Twist1 in cranial bone progenitors resulted in replacement of posterior parts of the calvarium with cartilage, partially resembling the transformation of the skull bones to chondrocytes induced by conditional β-catenin deletion.

Next, we tested the effect of Twist1 protein on Sox9 proximal promoter activity. While the regulatory elements that drive tissue-specific expression of
Figure 2.4. Loss of Twist1 is sufficient for chondrogenesis in cranial mesenchyme.
Figure 2.4. Loss of Twist1 is sufficient for chondrogenesis in cranial mesenchyme. Sagittal sections stained with X-gal (A). In situ hybridization was performed on coronal sections (B). Whole mount skeletal preparations (C). Indirect immunofluorescence and in situ hybridization was performed on sagittal sections (D-F). Relative luciferase activity of Sox9luc in C3H10T1/2 cells in transient transfections for 24 hours (G). Data were normalized to negative control (empty PCI-neo vector, Promega) and reported as a percentage. (**) denotes p<0.01 by Student's t-test. Error bars indicate standard deviation. The inset shows western blots to confirm overexpression in C3H10T1/2 cells. Fb or f, frontal bone, pb or p, parietal bone, ip, interparietal bone. Dashed line outlines endogenous cartilage base (F). Black arrows (D-F) point to absent Osx expression domains in mutants. Diagram in (A) provides orientation for subsequent panels. Inset in (B) is for orientation. Scale bars in (A, B, D-F) represent 50 µm and scale bars for whole mounts in (C) represent 25µm.
Sox9 remain elusive, a 530 base pair sequence has been utilized as a proximal promoter in most transgenic assays (Bagheri-Fam et al., 2006; Wunderle et al., 1998). Previous studies indicate the proximal promoter is crucial in driving basal Sox9 expression. A luciferase reporter driven by the Sox9 promoter was active in undifferentiated C3H10T1/2 immortalized mouse mesenchymal cells. However in the presence of over-expressed Twist1 or its ortholog Twist2 (Fig. 2.4G), Sox9-Luciferase activity was significantly down-regulated (Fig. 2.4G, p<0.01). The Twist transcription factor family may therefore negatively regulate Sox9 transcription. Overall, our findings indicate that Twist1 negatively regulates cartilage formation in the skull bones in vivo, at least in part by repressing the chondrogenic initiation program.

II.C.6. β-catenin and the Twist family interact in cranial mesenchyme in vivo.

Since Twist1 conditional mutant skull bones partially phenocopied those of β-catenin conditional mutants, and both family members exhibit similar repressive functions in vitro, we tested whether Twist2 could also function downstream of the Wnt/β-catenin pathway in cranial bone primordia. The Twist2Cre/+; RR/+; β-cateninfl/fl mutant, which is heterozygous for Twist2 (Yu et al., 2003), formed ectopic chondrocytes in the skull at E18.5 (Day et al., 2005). Genetic lineage analysis of Twist2Cre/+; RR/+ embryos revealed expression in cranial mesenchyme at E9.5 and E10.5 (Tran et al., 2010). Consistently, we detected Twist2Cre lineage-marked osteoprogenitor cells at E12.5 in controls and Twist2Cre/+; RR; β-cateninfl/fl mutants (Fig. 2.5A, C). Twist2Cre deleted β-catenin as efficiently as En1Cre line since β-catenin conditional mutants from
Figure 2.5. β-catenin and the Twist family interact in cranial mesenchyme in vivo. X-gal staining (A, C) and in situ hybridization on sections (B, D). Double immunohistochemistry on coronal sections (E, F) or (G, H) axial sections. (E-H) White dashed lines outline surface ectoderm and telencephalon. White arrows indicate Sox9 expression in Runx2+ domain (F, H). Diagrams beneath indicate plane of section, region of interest, and embryonic axes (A-H). Whole mount cranial skeletal preparations, lateral views (I-M). White bracket indicates parietal bone width (L-M). Black dashed line indicates missing portions of frontal and parietal bones (J). Black asterisk indicates notch of posterior frontal bone missing (K, inset), white asterisk (M, inset) indicates posterior frontal ossification. Tel, telencephalon, se, surface ectoderm, f, frontal bone, p, parietal bone, i, interparietal bone. Diagrams below (A-H) are for orientation to plane of section. Scale bars represent 100 μm for sections (A-H) and 25mm for whole mounts (I-M); all control/mutant pairs were photographed at the same magnification. Embryonic axes are indicated.
both lines lacked *Lef1* expression in cranial osteoprogenitors by E12.5 (Fig. 2.5B, D; Fig. 2.1F-G). At E12.5, *En1Cre/+; RR; β-catenin*^{Δex3/+} mutant osteoprogenitors produced both Runx2 and Sox9 protein, whereas heterozygosity for *Twist2* in *Twist2Cre/+; RR; β-catenin*^{fl/fl} mutants resulted in substantially more Sox9 expression within the cranial osteoprogenitors (Fig. 2.5E-H, n=3/3). Therefore deletion of a single allele of *Twist2* in CNC cells exacerbated the conversion of Runx2\(^{+}\) osteoprogenitors to Sox9\(^{+}\) chondroprogenitors in the absence of β-catenin.

To further characterize the genetic interaction between β-catenin and the Twist family we next tested if *Twist1* loss-of-function could rescue inhibition of skull osteoprogenitor fate induced by β-catenin gain-of-function (Fig. 2.3E, (Hill et al., 2005)). The *En1Cre; β-catenin*^{Δex3/+} mutants died by E12.5, so we used an inducible *Prx1CreER* driver (Kawanami et al., 2009) (*Prx1* is also known as *Prrx1*—Mouse Genome Informatics). In the head, the *Prx1Cre* transgene labelled cranial mesenchyme at E11 (Hill et al., 2005), and induction of *Prx1CreER* at E9.5 and E10.5 resulted in *Prx1CreER* lineage cells contributing to the frontal, parietal, and interparietal bones by E16.5 (supplementary Fig. 2.S4A). Constitutive β-catenin activation induced expression of Lef1 but not Sox9 in the frontal bone (supplementary Fig. 2.S4B-G). *Prx1CreER; β-catenin*^{Δex3/+} embryos had an almost complete loss of the posterior frontal bones and greatly reduced
parietal bones at E16.5 compared to controls (Fig. 2.5I-J). At E18.5, Prx1CreER; Twist1<sup>fl/fl</sup> embryos exhibited decreased bone mineralization in the posterior frontal
Figure 2.S4. Activation of β-catenin results in ectopic Lef1 expression; deletion of Twist1 restores AP in Lef1 expressing cells.

Figure 2.S4. **Activation of β-catenin results in Lef1 expression; deletion of Twist1 restores AP in Lef1 expressing cells.** Whole mount X-gal staining performed with skin removed, and sagittal (A) or coronal sections (B, E) counterstained with eosin. Immunofluorescence on sections (C-D, F-G, I-J). AP staining on sections (H, K). Black brackets indicating missing bone, black dashed lines indicating AP+ bone, and asterisk indicating the coronal suture (K). Scale bars represent 100 μm for tissue sections and 25 mm for whole mount pictures.
bone and anterior parietal bones compared to controls (Fig. 2.5K, L). At E18.5, 
Prx1CreER; $\beta$-catenin$^{\Delta ex_{3/+}}$; Twist1$^{fl/fl}$ mutants rescued a substantial amount of 
mineralization in frontal and parietal bones, compared to Prx1CreER; $\beta$-
catenin$^{\Delta ex_{3/+}}$ mutants (Fig. 2.5M). Prx1CreER; $\beta$-catenin$^{\Delta ex_{3/+}}$; Twist1$^{fl/fl}$ mutants 
had AP activity indicative of ossification in both anterior and posterior portions of 
the frontal bone that was missing from Prx1CreER; $\beta$-catenin$^{\Delta ex_{3/+}}$ mutants (supplementary Fig. 2.S4K, n=3/5). AP expression in the frontal bone of 
Prx1CreER; $\beta$-catenin$^{\Delta ex_{3/+}}$; Twist1$^{fl/fl}$ mutants coincided with Lef1 expressing, $\beta$-
catenin-stabilized cells (supplementary Fig. 2.S4H-J), whereas neither Lef1 nor 
Sox9 was detected in frontal bones of controls with wild-type $\beta$-catenin levels (supplementary Fig. 2.S4C,D; data not shown). Therefore, deletion of Twist1 
restored bone formation inhibited by constitutively active $\beta$-catenin. Our data 
suggest that an exquisitely dose-sensitive genetic interaction in vivo between $\beta$-
catenin and Twist1/2 is required to ensure proper osteoprogenitor fate in 
intramembranous bones of the skull.

II.C.7.Twist1 binds to Sox9 3’UTR in vivo in cranial mesenchyme

Next we tested whether Twist1, which is induced in response to $\beta$-catenin, 
could bind to the Sox9 locus in vivo. In Drosophila, Twist consensus sites differ 
substantially from canonical E-box sites (Ozdemir et al., 2011). We took an 
unbiased approach to identifying binding sites for Twist1 on the Sox9 locus and 
mined ChIP-seq (ChIP followed by sequencing) data from a human mammary
epithelial cell line (Casas et al., 2011). Twist1 was enriched by nine fold at a site within the 3'UTR of SOX9 (Fig. 2.6A; chr17:67633050-67633250, hg18 assembly). Next we tested whether Twist1 bound to the orthologous region of mouse Sox9 in skeletogenic and mesenchymal murine cell lines (Fig. 2.6B-C). We observed Twist1 enrichment at the same site in Sox9 3'UTR in three different cell contexts: in vivo in cranial mesenchyme of wild-type E12.5 embryos, in C3H10T1/2 cells transiently over-expressing Twist1, and in an ATDC5 chondrosarcoma cell line stably expressing a tamoxifen inducible form of Twist1 (Yang et al., 2004). Notably, we did not detect enrichment for Twist1 at the Sox9 promoter (Fig. 2.6B). Therefore, Twist1 binds robustly in vivo to a genomic element downstream of the Sox9 coding region, suggesting Twist1 may regulate Sox9 in skull progenitor cells.

In summary, we identified requirements for β-catenin in skull osteoblast lineage commitment and a mechanism for suppression of chondrogenesis. We identified Twist1 as a target of β-catenin, and that Twist1 deletion both phenocopies and genetically compensates for β-catenin function in determining cell fate in the skull. In early cranial bone development direct differentiation of cranial bone progenitors (Runx2+) into osteoblasts (Osx+) occurs (Fig. 2.6D). In our model, β-catenin activates and functions through Twist1 to promote a bypass of chondrocyte fate (Sox9+; Fig. 2.6D). Our data suggest that β-catenin directly activates Twist1 transcription (Fig. 2.6E). While the precise mode of repression of chondrocyte fate is less clear, genetic deletion of β-catenin or Twist1 results in ectopic expression of Sox9. Since Twist1 can associate with Sox9 chromatin, it is
Figure 2.6. **Twist1 binds to Sox9 3’UTR in vivo in cranial mesenchyme.** Alignment of a ChIP-seq performed on human mammary epithelial cell line expressing TwistER at the SOX9 locus using the UCSC genome browser, 2006 assembly (Casas et al., 2011). ChIP-qPCR was performed using anti-Flag (C3H10T1/2 cells) or anti-Twist1 (E12.5 cranial mesenchyme, ATDC5-TwistER cells) antibodies and primers for non-target sites on mouse chromosome 12, the Sox9 promoter, or the Sox9 3’UTR (B). Dashed lines indicate approximate threshold set by non-target sites (B). Western blot for Twist1 was performed on E12.5 cranial mesenchyme input or anti-Twist1 immunoprecipitate (C). Model for the role of β-catenin and Twist1 in cranial bone progenitor specification. Thick black, green, or purple arrows represent steps of differentiation. Thin arrows denote transcriptional activation or inhibition, respectively. Model for the direct activation of Twist1 by β-catenin, and for binding of Twist1 to Sox9 in cranial bone progenitors (E). Green and black promoter arrows represent active and inactive transcription, respectively. CNC, cranial neural crest. PM, paraxial mesoderm.
TABLE S4. Primer sequences used for ChIP-qPCR

H3K4me1, β-catenin ChIP- Twist1qPCR

<table>
<thead>
<tr>
<th>Twist</th>
<th>ChIP-Sox9qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twist1-62</td>
<td>ATGGTGGTGCAGAGGTGTATTA CATGGGCTCCATTATATGC</td>
</tr>
<tr>
<td>Twist1-60</td>
<td>TTAGCCAGAGATCCGCCGATT CTTTGTGCCATCCCATCATA</td>
</tr>
<tr>
<td>Twist1-22</td>
<td>CCTCCTCCCCATCATTTGAAA ACCATGCGCACCTTTGTAT</td>
</tr>
<tr>
<td>Twist1-18</td>
<td>TGACTTTGCGAAGAGAAA ACCTCCCGGTCTCTATTGAG</td>
</tr>
<tr>
<td>Twist1-4.5</td>
<td>CTCTTGTGGTCCTGCACCAA TCCTACTCTCAAGGCTCCAAG</td>
</tr>
<tr>
<td>Twist1-3</td>
<td>GAAAAAACAGCCCCTTTGAA AGGCTTCTGCAACTCTGTGGA</td>
</tr>
<tr>
<td>Twist1+5</td>
<td>AACCCTCGTTATGCTTCTT ACCGCGCATAGGCTCTCCTT</td>
</tr>
<tr>
<td>Twist1+10</td>
<td>TACACCACAAAATCTTTGAG GACACTCCACCCCTCAAATC</td>
</tr>
<tr>
<td>Twist1+16</td>
<td>TGAAATGTACACCCTGTGCCAAG TCAGCATTTCAAGAGAGCCAA</td>
</tr>
<tr>
<td>Twist1-1.7</td>
<td>GCCAAGCCTGTGCCATTTGTC AAACCTGGGCTGGAGATG</td>
</tr>
<tr>
<td>Twist1-1.8</td>
<td>GGGTCTACGGGCTCCAAGGA CTGGTCTGCTGAGACCTCT</td>
</tr>
<tr>
<td>Twist1-1.9</td>
<td>CTTGTGTTCCAAACCAGAAAA GCTGAGCCCTGCTAAACG</td>
</tr>
<tr>
<td>Twist1-37</td>
<td>TGATGTTAAGGCTCACCATCA GCTTTGCTCTCTAAACTCAG</td>
</tr>
<tr>
<td>Twist1-37</td>
<td>CAGGAAAGAGGAAGAAGAATC GAACAAACAGAGCCCAACCAT</td>
</tr>
<tr>
<td>Twist1-26</td>
<td>CTGCATATTGACCTCTTCTT TCTGAAACCGGACGGTTTTG</td>
</tr>
<tr>
<td>Twist1-8</td>
<td>TGGCAATACTTGCTGTTTCT CTGCACAAATGGAATGAG</td>
</tr>
</tbody>
</table>

Figure 2.5. Primer sequences used for ChIP-qPCR
tempting to speculate that Twist1 mediates negative regulation of Sox9 transcription by β-catenin (Fig. 2.6E).

II.D. Discussion:

Previous studies have implicated a role for β-catenin in intramembranous bone formation-related osteoblast differentiation. We used multiple Cre lines to functionally manipulate mouse cranial bone progenitors contributing to the frontal, parietal, and interparietal bones (Fig. 2.1B, 2.4A, 2.5A, supplementary Fig. 2.S2I). Our data provide mechanistic insight into the molecular basis of cranial bone progenitor specification during intramembranous bone formation in the skull.

By combining genetic lineage analysis with a spatially restricted En1Cre line, this study provides key in vivo evidence that β-catenin-deficient skull bone progenitors become cartilage cells early at the onset of osteoprogenitor fate selection at E11.5-12.5 (Fig. 2.1). Deletion of β-catenin from skull bone osteoprogenitors results in the near complete replacement of the bony skull plates with cartilage arising from En1Cre-lineage (Fig. 2.1Q). Currently no single or compound Wnt ligand knockout mouse phenocopies the β-catenin conditional knockout; although the Wnt9a\(^{−/−}\) mouse has a small amount of ectopic cartilage, skull ossification is mostly intact (Spater et al., 2006). Therefore future studies should identify the specific ligands required to prevent chondrogenesis in the skull. Although Sox9 expression localized to β-catenin conditional mutant skull osteoprogenitors (Fig. 2.1), the cranial En1Cre-lineage is comprised of not only
osteogenic mesenchyme but also non-osteogenic, dermal mesenchyme. The
dermal progenitors form first, and the osteogenic mesenchyme grows dorsally
through the non-osteogenic layer (Roybal et al., 2010). Therefore, the more
dorsal population of Sox9- or Runx2-expressing cells in β-catenin mutants may
be converted dermal fibroblasts (Fig. 2.1I, M). We previously demonstrated that
cranial dermal progenitors convert to cartilage upon deletion of β-catenin, and
others showed that non-osteogenic mesenchyme progenitors can ossify (Roybal
et al., 2010; Tran et al., 2010). Multiple cranial lineages, therefore, may form
chondrocytes upon ectopic activation of Sox9, which can be sufficient for
chondrogenesis (Eames et al., 2004).

We also demonstrate that constitutive activation of β-catenin resulted in an
absence of Runx2 expression in the skull in vivo, and a previous study
demonstrated similar results in limb bud culture (Hill et al., 2005). The
incongruous results between loss- and gain-of-function studies for β-catenin
could be explained by a sequential, biphasic role for β-catenin in regulating
osteoprogenitor fate that is too subtle for resolution by existing Cre lines.
Alternatively, it has been speculated that at high levels β-catenin represses
Runx2 as an extension of the general mechanism through which it inhibits Sox9
in skull progenitors (Hill et al., 2005). The mechanism by which β-catenin controls
cranial osteoprogenitor fate remains incompletely defined, but we show that β-
catenin directly activates Twist1, which results in increased Twist1 protein levels
(Fig. 2.3O). Since Twist1 is an inhibitor of differentiation of both the osteoblast
and chondrocyte lineages (Bialek et al., 2004; Gu et al., 2012), constitutive
activation of β-catenin may inhibit differentiation through high levels of Twist proteins.

Previous studies suggest Twist1 is a target of Wnt/β-catenin pathway activation in vitro, and that Twist1 inhibits the differentiation of chondrocytes in cell lines (Howe et al., 2003; Reinhold et al., 2006). In vivo, Twist1 haplo-insufficiency inhibits the differentiation of cranial bone osteoblasts (Bialek et al., 2004), however genetic evidence for the protein as a chondrogenic repressor was lacking. Here we extend previous knowledge by first showing that β-catenin activates Twist1 expression and associates with an upstream putative enhancer element of Twist1, providing evidence that Twist1 is a direct target of the Wnt/β-catenin pathway in vivo (Fig. 2.3). We tested Twist1 function through conditional deletion in cranial osteoprogenitors, and detected the presence of chondrocytes in the posterior skull (Fig. 2.4). We did not detect chondrocytes in the anterior frontal bones. There was, however, a loss of ossification at the prospective coronal suture boundary between the frontal and parietal bones (Fig. 2.4). Twist1 promotes survival of pre-migratory CNC cells (Bildsoe et al., 2009), although we did not detect an increase in cell death in Twist1 conditional null osteoblast progenitors. Our finding that Twist1 represses chondrogenesis in embryonic development is significant, although deletion of Twist1 from mesenchymal progenitors of the appendicular skeleton did not result in formation of ectopic chondrocytes (Krawchuk et al., 2010; Loebel et al., 2012; Zhang et al., 2010). Our data could therefore provide insight into basic mechanistic differences between the two modes of vertebrate ossification. However in Twist1 conditional
null mutants, instead of the entire skull, it was the interparietal bone that was most completely replaced by cartilage (Fig. 2.4). Our data are consistent with observations that Twist1 heterozygosity in mice, and human TWIST1 mutations in Saethre-Chotzen syndrome, disproportionately affected the interparietal bone (el Ghouzzi et al., 1997). One possibility is that Twist1 shares a role with other factors during skull progenitor fate selection, and the posterior skull may represent the least intrinsic redundancy.

We provide mechanistic insight into how β-catenin regulates intramembranous bone formation and suppresses cartilage fate. Thus, if β-catenin inhibits cartilage fate in skeletal progenitors, it may do so through recruitment of Twist1 as a repressor. Here, Twist1 deletion rescued inhibition of bone formation by constitutive β-catenin activation (Fig. 2.5). In addition to Twist1, Twist2 also contributes to repression of chondrocytic gene expression in the skull, which is consistent with previous reports that Twist family members can have overlapping functions (Sosic et al., 2003).

How does β-catenin promote cranial osteoblast formation? We show that β-catenin activates Twist1 in cranial osteoprogenitor cells (Fig. 2.2, 2.3), which is required to inhibit chondrocyte formation (Fig. 2.4). Sox9 is the central regulator of chondrogenesis, but it also triggers Runx2 degradation and negatively regulates Runx2 transcription (Cheng and Genever, 2010; Yamashita et al., 2009). Further, Sox9 over-expression is sufficient at high levels to inhibit bone formation in vivo (Eames et al., 2004; Zhou et al., 2006). Therefore the role of β-catenin and Twist may be largely permissive and represses the dominant
molecular program, i.e. chondrogenesis, in order to promote osteogenesis. The exact mechanism of repression of chondrogenesis remains elusive, but Sox9 protein and mRNA were undetectable in cranial bone progenitors (Fig. 2.1), and deletion of β-catenin or Twist1 results in ectopic Sox9 expression (Fig. 2.1, 2.4). Additionally, genome-wide mapping of Twist1 binding sites revealed that Twist1 bound immediately downstream of Sox9 in robust fashion (Fig. 2.6). This is consistent with studies that showed that regulatory elements frequently reside in the 3′ region of genes and can form chromatin loops that interact with 5′ enhancers or the promoter (Creighton et al., 2010; Heintzman et al., 2007; Palmer et al., 2007; Yochum et al., 2008; Yochum et al., 2010). We found that Twist1 could bind SOX9 chromatin in non-chondrogenic cells such as human mammary epithelial cell lines (HMLEs), even in the absence of co-factors normally found in chondrogenic cells (Fig. 2.6). However, HMLEs also express TWIST1 in response to Wnt pathway activation (Howe et al., 2003), so canonical Wnt signaling could play a role in targeting Twist1 binding to Sox9. Although future studies are clearly required, our results suggest that the mechanism of chondrogenic inhibition could involve Twist1 binding to Sox9 (a model is shown in Fig. 2.6E). Future experiments will test the requirement and role of Twist1 binding to Sox9 for β-catenin function in cranial bone progenitors.

The vertebrate skeleton is diversely patterned across species; indeed the study of the phylogeny of the skeleton is one of the underpinnings of vertebrate evolutionary theory. Biologists have postulated that the phylogenetic changes which yielded various skeletal patterns and elements in chordates must have
been complex in nature (DeBeer, 1937). However our data provide proof-of-principle for a model in which a single cue is required to switch between chondrogenic precursors of endochondral bone formation and the mesenchymal condensation of intramembranous bone formation. In our model the morphogenesis of the skull remains intact in β-catenin loss-of-function mutants, but the underlying fate of the skeletal tissue transforms nearly completely from dermatocranium to chondrocranium. The mutant phenotype resembled the skull plan of chondrichthyes fish, where cartilage elements constitute not only the skull base but also the skull vault. Whether different β-catenin levels account for species-specific differences in skull plans is the subject of future work. Additional studies further elucidating how the β-catenin-Twist1/2 axis interacts to prevent chondrogenesis in intramembranous ossification may also begin to explain the evolutionary basis for intramembranous bone formation.

II.E. Materials and Methods

II.E.1. Mice and Genotyping:

Conditional functional studies were conducted using En1Cre mice (Kimmel et al., 2000), Twist2Cre mice (Yu et al., 2003), and Prx1CreERGFP mice (Kawanami et al., 2009). The conventional null, conditional loss- and gain-of-function floxed alleles for β-catenin (Ctnnb1) \([β\text{-}\text{catenin}^{Δ+/+}, β\text{-}\text{catenin}^{fl/fl}, β\text{-}\text{catenin}^{Δex3/+}; (Brault et al., 2001; Haegel et al., 1995; Harada et al., 1999)]\), the conditional floxed Twist1 allele (Chen et al., 2007), and R26R/R26R mice
(Soriano 1999) were described previously. Mice and embryos were genotyped as described previously (Atit et al., 2006). For induction of CreER activity, 4-hydroxytamoxifen was dissolved in corn oil (10mg/ml, Sigma Aldrich, St. Louis, MO) and delivered at 1mg/40g bodyweight by oral gavage to pregnant mice carrying E9.5+E10.5 embryos.

For timed matings the vaginal plug day was assigned as E0.5. At desired time points, embryos were harvested and processed for frozen sections as previously described (Atit et al., 2006). For each experiment, at least three different mutants with littermate controls were analyzed. At least two to four litters were used for each functional analysis.

Case Western Reserve Institutional Animal Care and Use Committee approved all animal procedures.

II.E.2. In situ hybridization, immunohistochemistry, and histology:

Embryos were fixed in 4% paraformaldehyde, cryopreserved, and sectioned at 8-12 mm. In situ hybridization, β-galactosidase with eosin counter-staining, and immunohistochemistry were performed essentially as described (Atit et al., 2006; Ohtola et al., 2008). For skeletal preparations embryos were stained in 0.03% Alcian blue and 0.005% Alizarin red. The in situ probe for Lef1 was a gift from Fanxin Long (Hu et al., 2005), Runx2 (Enomoto et al., 2000) was a gift from Matthew Warmann, Sox9 (Wright et al., 1995) was obtained from Veronique Lefebvre (Cleveland Clinic Foundation, Cleveland, OH), and Twist1 was shared by Richard R. Behringer (Baylor College of Medicine, Houston, TX).
Primary antibodies used for indirect immunofluorescence were: goat anti-Runx2 (1:20, R&D Biosystems), rabbit anti-Sox9 (1:100, Millipore), mouse anti-Twist1 (1:500, Santa Cruz), and rabbit anti-Lef1 (1:100, Cell Signaling). All control/mutant pairs were photographed at the same magnification. To measure immunofluorescence intensity, stained tissue sections were photographed for DAPI (blue) and Twist1 (red) channel. Three sections were photographed per embryo, and n=4 for control and mutant embryos. Cranial bone progenitor regions (Figure 2.3 M,N) were selected (about 400 nuclei per embryo). Nuclei were set as counting regions, and average Twist1 intensity was measured using MetaMorph software (Molecular Devices).

II.E.3. Cell lines, plasmids, transfection, and luciferase assays:

Mouse C3H10T1/2 cells were cultured in DMEM GlutaMAX-I (Gibco) with 10% fetal bovine serum (FBS) and 50mg/ml Gentamicin (Gibco). ATDC5-TwistER cells were infected with TwistER virus as described (Yang et al., 2004), then selected with 10 mg/ml Blasticidin (Invitrogen) in 1:1 DMEM:F12, 5% FBS (Gibco). Individual clones were selected for chromatin immunoprecipitation (ChIP) according to Twist1 expression as determined by Western Blot. Transfections for the luciferase assay were performed in a 12-well plate seeded with 2.5x10^5 cells per well in the absence of antibiotics the day before transfection. The plasmids pcDNA3-myc-Twist1, pcDNA3-flag-Twist2 were gifts from Drazen Sosic (UT-Southwestern Medical Center, Dallas, TX) and PCIneo
(Promega), as a negative control, or PEGFP (Clontech) and P-TK-Renilla (Promega) as transfection efficiency controls were used for transfection. Sox9luc, consisting of 530 base pairs proximal to the mouse Sox9 transcription start site cloned into PGL4.10 (Promega), was a kind gift from Cynthia Bartels (Case Western Reserve University School of Medicine, Cleveland, OH). Transient transfections were performed at 80-90% confluence using Lipofectamine 2000 (Invitrogen, for C3H10T1/2 cells), according to the manufacturer’s instructions. Cells were harvested twenty-four hours after transfection for Western blot, luciferase assay, and quantitative real-time PCR. 25 micrograms total protein was separated by SDS-PAGE, and western blots were performed with the following antibodies: mouse anti-FLAG (1:1000, Sigma), anti-c-myc (1:200, Santa Cruz), mouse anti-Twist1 (1:500, Santa Cruz), rabbit anti-tubulin (1:5000, ICN BioMedicals), and HRP—conjugated goat anti-mouse or anti-rabbit (1:10000, Thermo). 24 hours after transfection, luciferase assay was performed as described ((Tran et al., 2010); Promega). Light units were normalized to protein content (see (Bialek et al., 2004; Sosic et al., 2003)) determined by the Pierce BCA Protein Assay Kit. A paired, single tail, Student’s t-test was performed on Microsoft Excel.

II.E.4. Chromatin Immunoprecipitation (ChIP) and real-time PCR:

E12.5 cranial mesenchyme was harvested and chromatin immunoprecipitation (ChIP) protocols were modified from previously published
procedures (Schnetz et al., 2009; Zhang et al., 2009). ATDC5 TwistER cells were treated with 20 nM 4-hydroxytamoxifen (Sigma) for 24 hours before ChIP. Cells from tissues, as well as from C3H10T1/2 and ATDC5 cell lines were dissociated with 0.05% Trypsin-EDTA (Invitrogen) followed by DMEM with 10% Fetal Bovine Serum (FBS, Gibco). The basic ChIP protocol was carried out as previously described (Schnetz et al., 2009), and target or non-target control sites were PCR amplified from immunoprecipitated samples and total genomic DNA (input) in triplicate. Quantitative Real-time PCR was performed using Sybr Green chemistry (ABI) as described previously (Livak and Schmittgen, 2001; Tran et al., 2010). Primer sequences are listed supplemental material Table S1. Results for target sequences were normalized to non-target values, and reported as fold enrichment versus non-target sequences.

II.F. Acknowledgements:


We thank Diego Correa, Emily Hamburg, Veronique Lefebvre and Peggy Myung for critical reading of the manuscript and R.P.A. lab members for technical assistance. We thank Scott Howell for immunofluorescence intensity measurements. We thank Drazen Sosic and Eric Olson for the myc-Twist1 and flag-Twist2 expression vectors. We thank Cynthia Bartels, Andrew Jarrell, Gregg
DiNuoscio, Jeremy Rich, Kumar Sukhdeo, Makoto M. Taketo, Adrienne Welsh, Stephen Haynesworth, and Gabe Zentner for their contribution. This work was supported in part by Case Startup funds from Case Western Reserve University (R.P.A.), by the National Institutes of Dental and Craniofacial Research [grants F31 DE020220-02 to L.H.G. and R01-DEO1870 to R.P.A.], a Pilot and Feasibility grant from the Case Skin Disease Research Center (R.A.); an Howard Hughes Medical Institute-SPUR fellowship (C.T.); by the National Institute of Child Health and Development [R01 HD056369 to P.C.S.], by the National Human Genome Research Institute [R01HG004722 to P.C.S.] by the National Institutes of Health Director’s New Innovator award [1 DP2 OD002420-01 to J.Y], and by The Mary Kay Ash Foundation Cancer Research Grant [096-09 to J.Y.]. Deposited in PMC for release after 12 months.

**II.H.Conflict of Interest:** The authors report no conflict of interest.
Chapter III. Sequential requirements for cranial ectoderm and mesenchyme-derived Wnts in specification and differentiation of osteoblast and dermal progenitors

L. Henry Goodnough¹, Gregg DiNuoscio², Trevor Williams⁵, Richard Lang⁶, and Radhika P. Atit²,³,⁴
Department of Pathology¹, Department of Biology², Department of Genetics³, and Department of Dermatology⁴
Case Western Reserve University School of Medicine, Cleveland, OH, 44106, USA
Department of Craniofacial Biology⁵
University of Colorado School of Dental Medicine, Aurora, CO, 80045, USA
Visual Systems Group⁵,
Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229, USA⁶.

III.A

Abstract: The cranial bones and dermis differentiate from mesenchyme beneath the surface ectoderm. Fate selection in cranial mesenchyme requires the canonical Wnt effector molecule β-catenin, but the relative contribution of Wnt ligand sources in this process remains unknown. Wnt ligands are expressed in cranial surface ectoderm and underlying supraorbital mesenchyme during dermal and osteoblast fate selection. Using conditional genetics, we eliminate secretion of all Wnt ligands from cranial surface ectoderm or undifferentiated mesenchyme and uncover distinct roles for ectoderm- and mesenchyme-derived Wnts. Ectoderm Wnt ligands induce osteoblast and dermal fibroblast progenitor specification while initiating expression of a subset of mesenchymal Wnts. Mesenchyme Wnt ligands are subsequently essential during differentiation of dermal and osteoblast progenitors. Finally, ectoderm-derived Wnt ligands generate graded expression of differentially responsive β-catenin target genes in
cranial mesenchyme, suggesting threshold-dependent fate selection of the dermal fibroblast and osteoblast lineages. Thus two sources of Wnt ligands function sequentially during osteoblast and dermal fibroblast formation.

### III.B

**Introduction:**

The bones of the skull vault develop in close contact with the embryonic skin. A layer of dermal soft connective tissue ensheaths the hard connective tissue of the bones to enclose the brain. In the mouse embryo, bone-forming osteoblasts and skin-forming dermal fibroblasts are derived from cranial neural crest and paraxial mesoderm (Jiang et al., 2002). At E11.5, cranial dermal fibroblast progenitors first undergo specification beneath the surface ectoderm. Osteoblast progenitors are specified in a deeper layer above the eye (Tran et al., 2010). Subsequently osteoblast progenitors proliferate and migrate apically beneath the dermal progenitors (Yoshida et al., 2008). Both cell types secrete collagen as extracellular matrix, but skull bones provide physical protection for the brain, while the overlying dermis lends integrity to the skin and houses the epidermal appendages (Hardy, 1992).

Both paracrine and autocrine intercellular signals function in early bone and skin development. In craniofacial bone formation the mesenchyme sets the timing of ossification (Merrill et al., 2008; Schneider and Helms, 2003), while the surface ectoderm functions in a permissive manner (Hall, 1981). Likewise during skin formation ectodermal signals are essential for formation of the hair-follicle forming dermis (Chen et al., 2012), but the dermal mesenchyme determines
epidermal appendage identity such as hair or feather (Eames and Schneider, 2005). To overcome challenges in engineering replacement connective tissues further delineation of specific ectoderm-mesenchyme signaling during early development of the bone and dermis is required.

Mesenchymal canonical Wnt/β-catenin signal transduction is essential in the specification and morphogenesis of both craniofacial dermis and bone (Goodnough et al., 2012; Tran et al., 2010), and dysregulation in components of such signaling pathways is associated with diseases of bone and skin (Hsu et al., 2010; Richards et al., 2008; Rivadeneira et al., 2009; Wang et al., 2007). All Wnt ligands are secreted through a conserved pathway requiring a regulator, Wntless (Wls), which functions specifically in trafficking of Wnt ligands (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009). Wnt ligand binding to target cell surface receptors (Fzd and LRP5/6) results in nuclear translocation of β-catenin, which binds to TCF/LEF transcription factors and activates expression of downstream targets. Wnt ligands also activate the non-canonical Wnt/Planar Cell Polarity (PCP) pathway, which influences cellular movements (Clevers and Nusse, 2012; van Amerongen and Nusse, 2009). β-catenin is essential in osteoblast differentiation and inhibition of chondrogenesis (Goodnough et al., 2012). However, deletion of individual Wnt ligands resulted only in mild effects on bone differentiation (Bennett et al., 2005; Spater et al., 2006). β-catenin is also a central regulator of early dermal specification (Chen et al., 2012; Ohtola et al., 2008; Tran et al., 2010), while roles for Wnt ligands so far have only been directly shown later during hair follicle initiation (Chen et al., 2012; Hu et al., 2010). In
bone and skin development, redundant functions of multiple Wnts may compensate for deletion of individual ligands. Conventional knockouts of individual ligands removed Wnt expression from all cells in the embryo, and have confounded the identification of tissue sources of Wnt ligands in bone and skin development. Thus the relative contributions from different sources of Wnt ligands for fate selection in cranial mesenchyme remain unknown.

Previous limitations were the lack of genetic tools to spatiotemporally manipulate early surface ectoderm and mesenchyme, and an inability to circumvent the intrinsic redundancy of Wnt ligands. We took a conditional approach to ablate the secretion of all Wnt ligands from either surface ectoderm or cranial mesenchyme prior to fate selection of the cranial bone and dermal lineages. Our findings provide key insights into how local developmental signals are utilized during morphogenesis to generate the cranial bone and dermal lineages.

III.C
Results:

We found that the genes for most Wnt ligands are expressed in the cranial surface ectoderm and mesenchyme during the specification of cranial osteoblast and dermal lineages in E11.5-E12.5 mouse embryos (Fig. 3.1A). To identify the cells with the potential to secrete Wnt ligands, we examined the spatiotemporal expression of Wls, the Wnt ligand trafficking regulator. We detected Wls protein expression from E11.5-E12.5 in the cranial surface ectoderm and in the
Figure 3.1. (A) RT-PCR for individual Wnt ligands was performed on cDNA from total mouse embryonic cranial mesenchyme and surface ectoderm. (B-C, E-F) Indirect immunofluorescence, (D) in situ hybridization, or immunohistochemistry (G) was performed on coronal mouse embryonic head sections. (E, F, G) Boxes indicate region in insets. White arrowheads indicate co-expression of (E) Wls/Runx2 or (C,F) Lef1/Runx2, (G) red arrowheads indicate osteoblast progenitors, and blue arrowheads indicate dermal progenitors. For indirect immunofluorescence, nuclei were stained blue with DAPI (B-C,E-F). Summary scheme of E11.5 supraorbital cranial mesenchyme (H) and embryonic axes and region of interest in sections used in figures are shown. Osteoblast pro., osteoblast progenitor; dermal pro., dermal progenitor; mes, mesenchyme. Scale bars represent 100 μm. Diagram inset in (D) figure depicts lateral view of embryonic head with box around region of interest.
underlying mesenchyme (Fig. 3.1B, E). Both the Runx2-expressing cranial bone progenitor domain and the Twist2-expressing dermal progenitor domain expressed Wls (Fig. 3.1C-D, F). The expression of the Wnt target gene Lef1 was expressed in osteoblast and dermal progenitors (Fig. 3.1 C, F), and the Wnt signal transducer β-catenin localized to the nucleus (Fig. 3.1G). Thus during specification of cranial bone and dermis, both ectodermal and mesenchymal sources secrete Wnt ligands, and the dermal and bone progenitors actively transduce Wnt signaling via β-catenin (Fig. 3.1H).

To dissect the requirements of ectodermal and mesenchymal Wnt signals, we generated mutant mice with conditional deletion of Wls in the early surface ectoderm using Crect (Reid et al., 2010) and in the cranial mesenchyme using Dermo1Cre (Yu et al., 2003). Crect efficiently recombined the Rosa26 LacZ Reporter (RR) in the ectoderm by E12.5 but left Wls expression intact in the mesenchyme (Fig. 3.2A, E, B, F) (Soriano, 1999), and the Crect-lineage remained ectoderm-restricted at E15.5 (Fig. 3.3 A, B). Dermo1Cre recombination showed β-galactosidase activity and Wls deletion restricted to the cranial mesenchyme and meningeal progenitors, and Wls was still expressed in the ectoderm in mutants (Fig. 3.2C, D, G, H).

First, we compared the extent to which Wls deletion from ectoderm or mesenchyme affected formation of the craniofacial skeleton. E18.5 Crect; RR; Wls floxed mutant embryos, which experienced perinatal lethality, demonstrated a hypoplastic face with no recognizable upper or lower jaw, and exhibited no sign of mineralization in the skull vault (Fig. 3.2I-L). Dermo1Cre; RR; Wls floxed mutant
Figure 3.2. (A, C, E, G) β-galactosidase staining with eosin counterstain or (B, D, F, H) indirect immunofluorescence with blue, DAPI-stained nuclei was performed on coronal mouse embryonic head sections. (A-H) Box outlines indicate region in inset. (I-L) Lateral view of whole-mount skeletal preps or gray-scaled brightfield images of embryonic mouse heads. (M-N) Lateral view, bright field images of embryonic heads. (M, I) and (N, J) are corresponding bright field and skeletal prep. Ey, eye. Scale bars for sections represent 100 μm. Scale bar (M) for whole mount pictures (I-L) represents 5mm. Inset to (D) represents lateral or of E12.5 with box outlining region of interest. Blue domain in bottom diagram depicts cranial bone progenitors.
embryos exhibited lethality after E15.5, which precluded assessment of skeletogenesis by whole-mount. We generated En1Cre/+; RR; Wls flo/mutants, using a Cre that recombines in early cranial mesenchyme but lacks activity in meningeal progenitors (Tran et al., 2010). En1Cre/+; RR; Wls flo/mutants survived until birth, and demonstrated reduced bone mineralization as well as intact dermis with hair follicles (Fig. 3.S1-S2). The earlier arrest in Crect; RR; Wls flo/mutants (Fig. 3.2) suggested ectoderm Wls appears to play an earlier role than mesenchymal Wls in cranial development.

We next examined the effects of ectoderm or mesenchyme Wls deletion on cranial bone and dermal development by histology. Von Kossa staining for bone mineral was absent in Crect; RR; Wls flo/mutants (Fig. 3.3E,F). The thin domain of mesenchyme above the eye in mutants appeared undifferentiated and showed no condensing dermal cells or early stage hair follicles. Additionally, the baso-apical expansion of both dermis and bone was evident by E15.5 in controls, but not in the thin cranial mesenchyme of mutants (Fig. 3.3A-B, white arrowheads). Although ossification was absent, we observed the presence of thin nodules of ectopic, alcian blue-stained cartilage (Fig. 3.3I, J). Therefore the result of Wls deletion in the ectoderm was an absence of skull ossification and hair-inducing dermis, a failure of baso-apical expansion of mesenchyme, and the presence of ectopic chondrocyte differentiation. By comparison, in Dermo1Cre; RR; Wls flo/mutants, lineage-marked mesenchymal cells (Fig. 3.3C, D) lacked mineralized bone and cartilage (Fig. 3.3 G, H, K, L). The mutant mesenchyme also failed to condense and form sufficient hair-follicle generating dermis (Fig.
Figure 3.S1. Whole-mount skeletal preps of embryonic mouse heads. P, parietal bone, f, frontal bone, n, nasal bone, ey, eye, mx, maxilla. Scale bar represents 5mm.

Figure 3.S2. b-galactosidase staining (A, D), Von Kossa staining (B, E), or alcian blue staining (C, F) was performed on coronal embryonic head sections and counterstained with eosin. fb, forebrain, mn, meningeal progenitors. Black arrowheads indicate meningeal progenitors. High magnification images with accompanying low magnification and box depicting inset (A, D). Red arrowheads indicate early hair follicles (E, F). Represent 100 µm.
Figure 3.3. (A-D) β-galactosidase staining, (E-H) Von Kossa staining, (I-L) or alcian blue staining was performed on coronal mouse embryonic head sections and counterstained with eosin. Br, brain; fb, frontal bone; de, dermis; hf, hair follicle; mn, meningeal progenitors. Black arrowheads indicate hair follicles, white arrowheads indicate dorsal dermis and bone. Diagrams inset (A) figure depicts lateral view of E15.5 embryonic head with plane of section and region of interest. Red regions in diagram represent bone primordia. Scale bars represent
3.3C, D), particularly at the apex of the head. Instead of extending from the base of the skull to the cranial apex, mutant mesenchyme accumulated above the eye (Fig. 3.3C, D, white arrowheads). However, the mesenchyme was thicker than in ectoderm Wnt-deficient mutants (Fig. 3.3B, D, white arrowheads). Our data suggest that Wls deletion using the DermolCre resulted in lack of bone mineralization as well as failure of dermis and hair follicle formation.

Deletion of Wls from ectoderm or mesenchyme resulted in complete absence of skull vault mineralization with failure of dermis formation, pointing to early defects in formation of the two lineages. Thus we tested if cranial mesenchyme undergoes proper dermal and osteoblast lineage fate selection and differentiation in the absence of Wls. Few Runx2+ osteoblast progenitors formed in Crect; RR; Wls fl/fl mutant embryos, and expression shifted directly beneath the surface ectoderm (Fig. 3.4A, E). During subsequent differentiation, condensing osteoblast progenitors express alkaline phosphatase (AP; Fig. 3.4B), but ectoderm Wnt-secretion deficient embryos lacked AP activity entirely (Fig. 3.4F). Thus an arrest in osteoblast progenitor differentiation occurred between the onset of Runx2 expression and AP activation, and the block was persistent as committed osteoblast progenitors expressing Osx were present in controls but not mutants (Fig. 3.4C, G). Cell survival was not affected in the cranial mesenchyme prior to changes in marker expression (data not shown). The altered cell fate marker expression immediately after deletion of Wls was suggestive of defects in cell fate selection, not in cell proliferation. Whereas chondrocytes expressed Sox9 only at the skull base in controls (Fig. 3.4D), in
Figure 3.4. Indirect immunofluorescence with DAPI-stained (blue) nuclei was performed on coronal mouse embryonic head sections (A, C-E, G-H, J, N, L, P). Alkaline Phosphatase staining (B, F), in situ hybridization (I, M), or β-galactosidase staining with eosin counterstain (K, O) was performed on coronal tissue sections. Diagram in (B) demonstrates plane of section and region of interest for E12.5-E13.5 (A-N). Box and dashed lines in (K, O) demonstrate the region of high magnification, and β-galactosidase stained sections were included for perspective for (L, P). Black arrows in (O) inset indicate ectopic cartilage. Diagram inset in high magnification photograph from (K) shows plane of section and region of interest for E15.5. Summary scheme (Q) depicts supraorbital ectoderm and mesenchyme at E11.5 graded level of ectodermal Wnts. Scale bars represent 100 µm.
mutants ectopic Sox9-expressing chondrocyte progenitors formed within the frontal bone domain (Fig. 3.4H). Next, we examined formation of dermal fibroblast progenitors in Crect; RR; Wls \textsuperscript{+/--} mutant embryos. Cranial dermal fibroblast progenitors expressed the marker Twist2 by E12.5 in supraorbital mesenchyme (Fig. 3.4I), but mutant embryos lacked Twist2 expression (Fig. 3.4M). Twist2 expression became more progressively restricted to upper dermal fibroblasts from E13.5-E15.5 in controls, but was completely absent from cranial mesenchyme of mutants (Fig. 3.4J-L, N-P). Thus ectoderm Wls is required for the induction of dermal fibroblast progenitors, and deletion leads to a lack of dermal fibroblasts, which contributes to the lack of hair follicle formation. Together our data suggest ectoderm Wnts form an inductive signal to the underlying mesenchyme for specification of osteoblast and dermal fibroblast progenitors, and for repression of chondrogenesis (Fig. 3.4Q).

Next, we tested if mesenchyme Wls deletion resulted in a later defect in differentiation of cranial bone and dermal fibroblast progenitors. En1Cre; RR; Wls \textsuperscript{+/--} mutants expressed diminished levels of the skeletal fate and differentiation markers Runx2 and Osx, but had intact Axin2 expression and thus normal levels of Wnt signaling pathway activity and dermal differentiation (Fig. 3.S3A-H). In Dermo1Cre; RR; Wls \textsuperscript{+/--} mutants, Runx2 expression was normal during fate selection stages (E12.5; Fig. 3.5A,E), and cell proliferation by Ki67 immunofluorescence was comparable to controls at the same stage (data not shown). However, during later dermal and osteoblast progenitor differentiation (E15.5), Axin2 was completely abrogated in mutants (Fig. 3.5B, F), and Runx2-
Figure 3.53. Indirect immunofluorescence with DAPI-stained (blue) nuclei (A-B, E-F) or in situ hybridization (C-D, G-H) was performed on coronal embryonic head sections. Scale bars represent 100 µm.
Figure 3.5. Indirect immunofluorescence with DAPI-stained (blue) nuclei was performed on coronal mouse embryonic head sections (A, E, D, H, I, K). In situ hybridization (B-C, F-G) or b-galactosidase staining with eosin counterstain (J, L), was performed on coronal tissue sections of embryonic murine heads. Diagram in (E) demonstrates plane of section and region of interest for E11.5-E12.5. Box in (I, K) demonstrate the region of high magnification. Diagram inset in high magnification photograph from (K) shows plane of section and region of interest for E15.5. (J, L) Low magnification photographs in lower left. Black arrowheads (L) indicate rounded cell morphology. (M) Summary scheme depicts supraorbital ectoderm and mesenchyme at E11.5. Gray arrows (M) represent mesenchymal Wnt ligand secretion functioning in an autocrine manner. Scale bars represent 100 µm.
expressing cells failed to differentiate further to Osx-expression (Fig. 3.5C, G). Nonetheless, no ectopic expression of Sox9 was observed in mesenchyme Wls-deficient mutants (Fig. 3.5D,H). In dermal progenitors undergoing specification, Twist2 expression was comparable to controls in mutant embryos (Fig. 3.5I,K), and yet at later stages the dermal progenitors had a rounded cell morphology, and failed to condense as well as support hair follicle formation (Fig. 3.5J,L, black arrowheads). Based on Dermo1Cre deletion of Wls, differentiation of dermal and osteoblast progenitors, not cell fate selection, requires mesenchyme-derived Wnt ligands (Fig. 3.5M).

Next, we tested how ectoderm Wnt ligands induce dermal and osteoblast progenitor cell fate selection. Wnt ligands can induce nuclear translocation of β-catenin in a dose-dependent manner (Willert et al., 2003). A gradient of active β-catenin accumulated in nuclei of dermal and osteoblast progenitors; the highest levels of nuclear localization occurred closest to the ectoderm while nuclear staining diminished further from the ectoderm (Fig. 3.6A). Different levels of active β-catenin promote expression of distinct target genes, and high levels induce target genes such as Lef1, while low levels induce expression of Tcf4 (Rudloff and Kemler, 2012). Tcf4 was expressed in a gradient increasing in intensity with distance from the ectoderm (Fig. 3.6B). In contrast, Lef1 was expressed in a complementary domain with the highest levels beneath the ectoderm (Fig. 3.6C). Tcf4 expression expanded into the mesenchyme under the ectoderm in ectoderm Wls-deficient mutants (Fig. 3.6D, F) but was unaffected in mesenchyme Wls-deficient mutants compared to controls (Fig. 3.6E, G). In
Figure 3.6.

Immunohistochemistry (A) or indirect immunofluorescence with blue, DAPI-stained nuclei was performed on coronal mouse embryonic head sections (B-K). (A-G) Boxes in lower right picture indicate regions shown in upper left. Embryonic head diagram depicts region of interest and plane of section. Embryonic axes are presented. Scale bars represent 100 µm.
Figure 3.7. (A-N) *In situ* hybridization was performed on coronal mouse embryonic head sections. Diagram of embryonic head in (A) inset depicts region of interest and plane of section. Insets in (D,K) show β-galactosidase staining and eosin counterstaining on serial sections. (O) A model for role of tissue sources of Wnt ligands during cranial mesenchymal lineage fate selection. Scale bars represent 100 µm.
addition, Lef1 expression was completely abolished in the mesenchyme of ectoderm-\textit{Wls} mutants (Fig. 3.6H, J), but was comparable to controls in the absence of mesenchyme-\textit{Wls} (Fig. 3.6I, K). Expression of Lef1, the highest level β-catenin target gene, requires ectoderm \textit{Wls} in cells close to the ectoderm, consistent with Wnt signaling reporter activity and soluble Wnt inhibitor expression (Fjeld et al., 2005; Mani et al., 2010). By contrast, no single source of \textit{Wls} was required for expression of the lower level response gene Tcf4 in the mesenchyme.

Next, we examined whether surface ectoderm Wnt ligands are required for expression of Wnt ligand mRNA in the mesenchyme (Fig. 3.7). The non-canonical ligands Wnt5a and Wnt11 were expressed in cranial mesenchyme, with the highest levels corresponding to dermal progenitors. Wnt4, which signals in canonical or non-canonical pathways, was expressed strongly in dermal progenitors, as well as in osteoblast progenitors and in the skull base (Fig. 3.7A-G). Expression of both \textit{Wnt5a} and \textit{Wnt11} mRNA were absent from the mesenchyme of \textit{Crect}; \textit{RR}; \textit{Wls} \textit{fl/fl} mutants, whereas \textit{Wnt4} expression was maintained (Fig 3.7H-J). Next we tested whether ectodermal Wnts signal through β-catenin directly in the underlying mesenchyme to regulate expression of Wnt ligands. \textit{En1Cre} deletion of β-catenin in the cranial mesenchyme (Goodnough et al., 2012) resulted in an absence of \textit{Wnt11} expression, except in a small portion of supraorbital lineage-labelled mesenchyme (Fig. 3.7D, K). By comparison \textit{Wnt5a}, \textit{Wnt11}, and \textit{Wnt4} expression were present in the \textit{Dermo1Cre}; \textit{RR}; \textit{Wls} \textit{fl/fl} mutants (Fig. 3.7L-N). The Wnt-expressing domains were smaller and only
located close to the surface ectoderm but nonetheless were lineage-labelled (Fig. 3.7E-G, L-N; not shown). Thus, consistent with their role as initiating factors, ectoderm Wnt ligands signal to mesenchyme in a paracrine manner, functioning in the mesenchyme via β-catenin to drive transcription of some Wnt ligands. Mesenchymal cells then propagate Wnt expression away from the ectoderm (Fig. 3.7O).

III.D

Discussion:

Here we obtained data suggesting that ectodermal and mesenchymal Wnts function sequentially in early dermal and osteoblast progenitor specification and differentiation. Wnt signals originate in the surface ectoderm and orchestrate transcription of mesenchymal Wnts, which in turn signal through an autocrine mechanism. The dermal progenitors and osteoblast progenitors closest to the ectoderm experience the highest concentrations of nuclear β-catenin, in response to Wnt ligands from overlying ectoderm. Subsequent differentiation of osteoblast and dermal fibroblast progenitors requires Wls from the mesenchyme. Thus our study demonstrates that two different sources of Wnt signals coordinate to form two separate lineages, bone and dermis.

We present evidence for initiation of mesenchyme Wnt expression by ectoderm Wnts, and the generation of a gradient of nuclear β-catenin and Wnt target gene expression diminishing with distance from surface ectoderm. The gradient is present at specification of osteoblast and dermal fibroblast
progenitors, which express distinct Wnt target genes. Dermal progenitors exhibit the same target gene expression and reporter activity as the markers induced by constitutive activation of β-catenin in mesenchyme, and have the highest levels of nuclear β-catenin (Fig. 3.6) (Chen et al., 2012; Mani et al., 2010; Tran et al., 2010). Forcing such high levels of Wnt pathway activity precludes osteoblast marker expression in the mesenchyme (Goodnough et al., 2012). Osteoblast progenitors predominantly have lower levels of β-catenin (Fig. 3.6) and express more sensitive Wnt target genes (Fig. 3.6; (Rudloff and Kemler, 2012)) as well as at least one Wnt inhibitor, Dkk2, consistent with a low threshold in a morphogen gradient (Fjeld et al., 2005). Our data provide evidence that the highest threshold Wnt response, but not low-level target gene expression, requires ectoderm Wnt ligands. Osteoblast progenitors may directly respond to high levels of Wnt ligands when closest to the ectoderm at early stages (Fig. 3.1). Alternatively, the osteoblast response to high levels of Wnts is indirect; osteoblast progenitors may require a separate signal from dermal progenitors. Future experiments will test direct or indirect requirements of Wnt sources in osteoblast formation.

During fate selection of cranial dermal and osteoblast progenitors, upstream ectoderm Wnt ligands initiate expression of a subset of mesenchymal Wnt ligands via β-catenin. Ectoderm Wnts also act upstream of mesenchyme Wnts in mouse limb development (Zhu et al., 2012). Here ectoderm Wnts act in a temporally earlier role than mesenchyme Wnts, and other studies support a direct relationship. In at least one instance, mesenchyme Wnt ligands are direct targets of canonical Wnt signaling (Zhou et al., 2007). Alternatively, ectoderm
and mesenchyme Wnts may signal in parallel pathways to the mesenchyme. The
signal that acts upstream to initiate Wnt ligand expression in the cranial ectoderm
remains unknown.

We report here that osteoblast differentiation requires sequential Wnt
signals from surface ectoderm and mesenchyme. β-catenin deletion in the
ectoderm did not inhibit skull bone mineralization (Reid et al., 2010), so autocrine
effects of Wls deletion on the ectoderm were unlikely to contribute to the skull
phenotype. Removal of surface ectoderm Wls did, however, result in ectopic
chondrogenesis (Fig. 3.3) which phenocopied mesenchymal β-catenin deletion
(Goodnough et al., 2012). In contrast, mesenchymal Wls deletion did not result in
ectopic cartilage formation, suggesting repression of chondrogenesis in cranial
mesenchyme requires an early, ectoderm Wnt signal. Our results thus implicate
β-catenin here as a Wnt pathway transcription factor that acts to repress
chondrogenesis and functions downstream of ectoderm ligands. Ectoderm Wnt
ligands thus may act as diffusing morphogens acting directly on osteoblast
progenitors while the cells are closest to the ectoderm. Indeed, later deletion of
Wls from the ectoderm using the K14Cre line resulted in no skull bone phenotype
(data not shown). During osteoblast progenitor differentiation, Wls deletion with
Dermo1Cre resulted in a similar but more severe differentiation arrest than the
more restricted En1Cre. Consistently, using a different Wls allele, deletion of
mesenchymal Wnts led to absence of osteoblast differentiation expression and
reduced cell proliferation (Maruyama et al., 2012). We show that the
mesenchyme Wnts maintain the differentiation process but require an inductive ectoderm Wnt signal.

We demonstrate that dermal progenitors require sequential activity of ectoderm and mesenchyme Wls for specification and differentiation (Fig. 3.4). Cranial dermal progenitors located beneath the ectoderm require β-catenin for specification (Tran et al., 2010), but the tissue contribution of Wnt sources remained previously undetermined. Here a mesenchymal Wls source is indispensable in the dermal lineage for differentiation and hair follicle formation. Previous reports in murine trunk skin development suggested that ectoderm Wnts alone are essential in hair follicle induction (Chen et al., 2012; Fu and Hsu, 2012). Differential requirements may exist for mesoderm-derived trunk dermal progenitors and cranial neural crest-derived dermal progenitors. Future studies will be needed to uncover the requirements for a mesenchymal Wnt signal in dermal fibroblast differentiation in different parts of the embryo.

Conditional Wls deletion resulted in a failure of cranial dermal and osteoblast progenitors to undergo baso-apical extension and altered mesenchymal cell morphology (Fig. 3.3), a process that occurs independently of β-catenin (Goodnough et al., 2012). Since Wls deletion blocked secretion of canonical and non-canonical Wnt ligands, extension defects in the mesenchyme are consistent with known roles for non-canonical Wnt ligands in orienting cell movements (Gros et al., 2009). Homozygous null mutants of core planar cell polarity (PCP) components lacked proper skull tissue development and neural tube closure (Gao et al., 2011). However, mutants for individual non-canonical
Wnt ligands lack a cranial PCP phenotype, and non-canonical Wnt5a or Wnt11 were present in cranial mesenchyme, suggesting the ligands function redundantly (Yamaguchi et al., 1999). Therefore the role of PCP signaling remains to be rigorously tested in conditional mutant mice. The branches of non-canonical and canonical Wnt signaling interact extensively, and in our model canonical β-catenin transduction of an early ectoderm signal initiates non-canonical Wnt expression (Fig. 3.6), consistent with reports from other systems (Gros et al., 2009; van Amerongen and Nusse, 2009; Zhou et al., 2007). Our results reinforce the role of non-canonical Wnt ligands in the pathogenesis of craniofacial anomalies (Kibar et al., 2007; Lei et al., 2010). The ability of exogenous non-canonical Wnts to compensate for Wls deletion in the baso-apical extension of dermal and osteoblast progenitors remains to be tested.

Our results from tissue-specific deletion of Wls have implications in diseases with dys-regulation of dermal fibroblasts or osteoblasts, and in understanding the pathogenesis of craniofacial birth defects. Removal of Wls from the ectoderm by E12.5 of mouse development reveals a default state for formation of cartilage in the cranial skeleton and dermis if all Wnt secretion were absent from the ectoderm. This forms an important baseline state that can be used to interpret less severe genetic conditions resulting from loss or mutation of individual Wnt ligands. In this respect, we hypothesize that mutations in the Wnt secretory pathway may underlie diseases of osteoblasts, and dermal fibroblasts, warranting continued investigation into the role of Wnt production in bone and skin formation and homeostasis (Grzeschik et al., 2007; Petti et al., 2011;
Rivadeneira et al., 2009; Zheng et al., 2012). Understanding the signals surrounding osteoblast and dermal fibroblast formation is crucial to meet the demands of engineering appropriate connective tissues.

III.E

Materials and Methods:

1. Mice and Genotyping:

Conditional functional studies were conducted using Dermo1Cre, En1Cre, and Crect mice (Kimmel et al., 2000; Reid et al., 2010; Yu et al., 2003). The conditional loss-of-function floxed allele for Wls (Wlsfl/fl) was described previously (Carpenter et al., 2010). RR/RR mice harboring a LacZ transgene downstream of a floxed stop transcription signal in the ubiquitous Rosa26 locus were obtained for lineage tracing (Soriano, 1999). Mice and embryos were genotyped as described previously. For timed matings the vaginal plug day was assigned as E0.5. At desired time points, embryos were harvested and processed for frozen sections as previously described (Atit et al., 2006). For each experiment, at least three different mutants with littermate controls were analyzed. At least three to five litters were used for each functional analysis. Case Western Reserve Institutional Animal Care and Use Committee approved all animal procedures.

2. In situ hybridization, immunohistochemistry, and histology:

Embryos were fixed in 4%PFA, cryopreserved, and sectioned at 8-12 mm. In situ hybridization, β-galactosidase with eosin counter-staining, and
immunohistochemistry were performed essentially as described (Atit et al., 2006; Hamburg and Atit, 2012; Ohtola et al., 2008). Alcian blue staining of sections was performed as described. For Von Kossa staining of frozen sections, slides were fixed with 4% PFA, incubated in the dark with 2% silver nitrate, rinsed, exposed to light, and counterstained with eosin. In situ probes for Twist2 (Drazen Sosic, Dallas, TX), Wnt4 (V. Lefebvre, Cleveland, OH), Wnt5a (Andrew McMahon, Boston, MA), Wnt11 (Steve Potter, Cincinnati, OH), Axin2 (Brian Bai), and Osx (Matthew Warmann, Boston, MA) were gifts. Primary antibodies for Runx2, Sox9, Twist2, Lef1, Osx, Wls, and β-catenin (goat anti-Runx2; 1:20, R&D Biosystems; rabbit anti-Sox9; 1:100; Millipore; mouse anti-Twist2, 1:500, Santa Cruz; rabbit anti-Lef1, 1:100, Abcam; rabbit anti-Osx, 1:400, Abcam; rabbit anti-Wls, 1:2000, gift from Richard Lang; 1:100 BD Biosciences) were used for indirect immunofluorescence and immunohistochemistry. All control/mutant pairs were photographed at the same magnification.

3. Alcian blue and Alizarin red staining:

Embryos were sacrificed, skinned and eviscerated, fixed in 95% ethanol, then stained for 24 hours each in 0.03% Alcian blue and 0.005% Alizarin red. Stained embryos were subsequently cleared in graded series of potassium hydroxide and glycerol until photography, after which they were stored in 0.02% Sodium Azide in glycerol.

4. RT-PCR

Cranial mesenchyme and surface ectoderm was micro-dissected from E12.5
embryos and flash frozen in liquid nitrogen. Total RNA was isolated using the Qiagen RNEasy micro kit, and cDNA was reverse transcribed using the ABI kit. RT-PCR for the Wnt ligands was amplified for 35 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 60 seconds and the products were resolved on a 2% agarose gel. Primer sequences for RT-PCR are listed in Table 3.S4.

**Table 3.S4.** RT-PCR primers for Wnt ligands

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Wnt Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGAACCCTTCACAAACAACGAG</td>
<td>Wnt 1 Forward</td>
</tr>
<tr>
<td>GGTGCTGCTCCGCAGTTTG</td>
<td>Wnt 1 Reverse</td>
</tr>
<tr>
<td>CTGGCTCTGGCTCCCTCTG</td>
<td>Wnt 2 Forward</td>
</tr>
<tr>
<td>GGAACCTGGTGTTGGCAGCTCTG</td>
<td>Wnt 2 Reverse</td>
</tr>
<tr>
<td>CTTCGCTCATGCTATCTCGTCAG</td>
<td>Wnt 2b Forward</td>
</tr>
<tr>
<td>ACACCTTGTTGCTGTCACCTTC</td>
<td>Wnt 2b Reverse</td>
</tr>
<tr>
<td>CAAACACCAATGAAGCAAGCAG</td>
<td>Wnt 3 Forward</td>
</tr>
<tr>
<td>TCGGGACTCAGGGTGTTTCTC</td>
<td>Wnt 3 Reverse</td>
</tr>
<tr>
<td>CACCCAGTCAGCAACAGCC</td>
<td>Wnt 3a Forward</td>
</tr>
<tr>
<td>AGGAGCCTGTCACGGCAAAG</td>
<td>Wnt 3a Reverse</td>
</tr>
<tr>
<td>GAGAAGTGGTGGCTGACCGG</td>
<td>Wnt 4 Forward</td>
</tr>
<tr>
<td>ATTTTGTCGAGGCTACTGACC</td>
<td>Wnt 4 Reverse</td>
</tr>
<tr>
<td>CTCCTTCGCCCAGGTTGGTTAG</td>
<td>Wnt 5a Forward</td>
</tr>
<tr>
<td>TGTCTTCGACCTTCTCCAATG</td>
<td>Wnt 5a Reverse</td>
</tr>
<tr>
<td>ATGCCCGAGCGTGAGGAGAAG</td>
<td>Wnt 5b Forward</td>
</tr>
<tr>
<td>ACATTTCAGGCAAGATCAGC</td>
<td>Wnt 5b Reverse</td>
</tr>
<tr>
<td>TGCCCGAGGCGCAAGACTG</td>
<td>Wnt 6 Forward</td>
</tr>
<tr>
<td>ATGCAACACGAAAGCTGCTCTC</td>
<td>Wnt 6 Reverse</td>
</tr>
<tr>
<td>CGACTGTGGCTGCGACAGAAG</td>
<td>Wnt 7a Forward</td>
</tr>
<tr>
<td>CTTCTGTTTCTCCAGGATCTTC</td>
<td>Wnt 7a Reverse</td>
</tr>
<tr>
<td>TCTCTGCTTGGCGTCCTCTAC</td>
<td>Wnt 7b Forward</td>
</tr>
<tr>
<td>Wnt 7b Reverse</td>
<td>GCCAGGCCAGGAATCTTGTTG</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Wnt 8a Forward</td>
<td>ACGTGGGAATTGCTCTGAGCATG</td>
</tr>
<tr>
<td>Wnt 8a Reverse</td>
<td>GATGGCAGCAGACGGATGG</td>
</tr>
<tr>
<td>Wnt 8b Forward</td>
<td>TTGGGACCGTTGAATTTG</td>
</tr>
<tr>
<td>Wnt 8b Reverse</td>
<td>AGTCATCAGCCAGCAAGT</td>
</tr>
<tr>
<td>Wnt 9a Forward</td>
<td>GCAGCAAGTTTGTCAAGGAAGT</td>
</tr>
<tr>
<td>Wnt 9a Reverse</td>
<td>GCAGGAGCCAGACACACCAG</td>
</tr>
<tr>
<td>Wnt 9b Forward</td>
<td>AAGTACACAGCAAGTGATCTAG</td>
</tr>
<tr>
<td>Wnt 9b Reverse</td>
<td>GAACACGACAGGAGCTGACAC</td>
</tr>
<tr>
<td>Wnt 10a Forward</td>
<td>CCTGTCTCTCTACTCTGCTGG</td>
</tr>
<tr>
<td>Wnt 10a Reverse</td>
<td>CGATCTGGATGCGCCTGATAGC</td>
</tr>
<tr>
<td>Wnt 10b Forward</td>
<td>TTCTCTGGGATTCTTGGATTC</td>
</tr>
<tr>
<td>Wnt 10b Reverse</td>
<td>TGCACTTCCGGCTTCAGGCTTTC</td>
</tr>
<tr>
<td>Wnt 11 Forward</td>
<td>CTCATCGAGCAACACTGTAAC</td>
</tr>
<tr>
<td>Wnt 11 Reverse</td>
<td>CTCTCTCCAGGTCAAGCAGGTAAG</td>
</tr>
<tr>
<td>Wnt 16 Forward</td>
<td>AGTAGCAGGCACCAAGGAGAC</td>
</tr>
<tr>
<td>Wnt 16 Reverse</td>
<td>GAAACTTTTCTGCTGAACCACATGC</td>
</tr>
</tbody>
</table>

### III.F

**Acknowledgements:**

We thank R.P.A. lab members for technical assistance and discussion. We thank Samantha Brugmann and Veronique Lefebvre for critical reading of the manuscript.
IV. Future Directions:

A. Introduction:
During development, proper spatiotemporal patterning and specification of tissues requires precisely orchestrated changes in gene expression, which are regulated by transcription factors in response to instructive cues from cell signaling pathways. My previous studies focused on the transcription factors that act during specification of the precursor cells to the cranial bones, and the cell-signaling pathways that control transcription factor expression. I demonstrated that the transcription factor, $\beta$-catenin, acts as a molecular switch in cranial bone progenitors, simultaneously effecting specification and repressing cartilage cell fate. $\beta$-catenin directly induces expression of another transcription factor, Twist1. *Twist1* deletion in cranial mesenchyme results in regional loss of ossification and ectopic chondrogenesis in the posterior skull. In subsequent studies I showed that the cranial bone progenitors receive an inductive Wnt signaling cue from nearby surface ectoderm during specification. These studies have provided key insights into how cell signaling pathways and transcription factors instruct the patterning of the cranial bones, and bring us closer to a set of signals and factors that together can specify bone progenitor cell fate in a tissue engineering setting. However, whether $\beta$-catenin target genes such as Twist1 are part of a group of transcription factors that specifies cranial bone progenitor cell fate is unknown. Identifying how Twist1 functions at the genome level during cell fate specification may help identify additional cooperating factors.
B. Future Direction #1 Role of Twist 1 in cranial bone progenitor specification

1. Rationale

Twist1 is regionally required for skull bone ossification. In posterior skull progenitors, Twist1 is required for ossification and repression of chondrocyte formation at the interparietal bone (Fig. 4.1). In contrast, in frontal and parietal bone progenitors, Twist1 deletion results in substantial loss of ossification abutting the coronal suture at the CNC-mesoderm boundary. Since En1Cre first specifically labels a population of coronal suture progenitors, Twist1 deletion may have occurred only in those cranial bone progenitors, leading to restricted loss of ossification in the skull (Deckelbaum et al., 2012). Therefore, I propose that Twist1 is strictly required in early cranial bone progenitor fate selection. We deleted Twist1 using an earlier, broader, cranial bone progenitor Cre, Twist2Cre (Yu et al., 2003). Next, we analyzed the effect of Twist1 deletion on cranial skeletal formation. Twist2Cre; Twist1 fl/fl mutants lack any evidence of skull formation, and exhibit a complete absence of frontal and parietal bone mineralization (Fig. 4.2). Thus, an earlier, broader mesenchymal deletion of Twist1 during cranial bone progenitor specification results in no skull formation, whereas even a slightly later Cre
results only in restricted loss of ossification. Collectively, these data suggest a transient requirement for Twist1 during cranial bone progenitor specification.

1. Experimental strategy

In support of a role for Twist1 in cranial bone and suture progenitor specification, I will test the requirement for Twist1 for expression of osteoblast progenitor cell fate markers, as well as in cell survival.

a. First, I will rule out altered cell survival in cranial bone mesenchyme I will test for cell death in Twist2Cre;Twist1 fl/fl mutant embryos. Our preliminary data using TUNEL-staining (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) for apoptosis suggests that there is no observable increase in cell death in the cranial mesenchyme of Twist2Cre;Twist1 fl/fl mutant embryos. I will perform indirect immunofluorescence for markers of apoptosis, TUNEL and cleaved-caspase3, on a sufficient number of embryos (between E10.5-E12.5) to obtain statistical significance.

b. Next I will determine the requirement of Twist1 for initiation of expression of cranial bone fate selection markers. I will first test if Twist2Cre;Twist1 fl/fl mutant cranial bone
progenitors are regionally patterned and express the early cranial bone marker, *Dlx5*. I will analyze osteoblast progenitor specification with expression of *Runx2*, *AP*, and *Osx*.

3. Interpretation

I expect to see diminished or absent expression of *Runx2*, *AP*, and *Osx* in *Twist2cre; Twist1 fl/fl* mutants for osteoblast specific markers. I also expect to detect no significant difference in cell death in the absence of Twist1 in cranial mesenchyme. Diminished expression of *Runx2*, *Osx*, or *Dlx5* mRNA would suggest that Twist1 directly or indirectly regulates expression of the transcription factors that are required for cranial bone progenitor specification. I would then propose further studies to test whether the Twist1 transcription factor directly regulates expression of *Runx2/Osx/Dlx5*. Specifically, I would begin by testing if Twist1 binds to the gene promoters of *Runx2/Osx/Dlx5* by ChIP-qPCR (Goodnough et al., 2012). Collectively these findings will implicate Twist1 as an upstream regulator of cranial bone progenitor specification, broadening our understanding of the factors that are may be important in engineering of bone-forming osteoblasts for clinical application.

C. Future Direction #2 Genome-wide role of Twist1 in regulating cranial bone progenitor specification

1. Rationale

The first attempts at determining the genome-wide targets of Twist transcription factors came from Twist ChIP-chip (chromatin-immunoprecipitation followed by microarray) experiments in Drosophila, which demonstrated that Twist bound to
promoters of up to 25% of known transcription factors in the Drosophila genome. Many Twist targets are transcription factors that also bind to DNA near Twist-DNA binding, and so an early model of Twist genome-wide binding was the induction of transcription factors involved in cooperative binding ((Sandmann et al., 2007; Zeitlinger et al., 2007), Figure 4.3 schematic). This model of molecular control of cell fate suggests that interconnected transcription factors frequently function together (Holmberg and Perlmann, 2012). Therefore, if Twist1 functions to directly control cranial bone progenitor specification, studies of transcription factors controlled by Twist1 may yield other candidate regulators of skeletal cell fate. Therefore, we hypothesize that Twist1 promotes cranial bone progenitor specification by direct binding and induction of other transcription factors in early cranial mesenchyme.

2. Experimental strategy

a. RNA-seq To gain insight into how Twist1 is required for specification of cranial bone progenitor specification, I will identify the genes that are regulated by Twist1. I will perform deep RNA-sequencing of poly-adenylated RNA isolated from cranial mesenchyme of Twist2Cre; Twist1 floxed heterozygote controls and homozygous mutant embryos. I will purify cranial mesenchyme from control and mutant Twist2Cre; Twist1 fl/+ or fl/fl embryos expressing a genetic β-galactosidase lineage tracer or YFP fluorescence reporter from the R26 locus. I will use the fluorogenic substrate β-galactosidase, CMFDG, to sort lineage-labeled cells. I have isolated 4X10^4-1.5X10^5 cells per embryo, which represents between 45-70% of live cells sorted, and which yield 150-600 ng total RNA per
sample. We will proceed with RNA-sequencing using the sequencing core in the Department of Genetics at Case Western Reserve University, which will perform library construction as well as sequencing and preliminary bioinformatics.

b. Chip-seq Next, we will profile the genome-wide binding sites of Twist1, which, when correlated and annotated with Twist1-dependent gene expression, will allow us to determine the direct transcriptional targets of Twist1 in cranial mesenchyme. I will perform Twist1 ChIP-seq on microdissected E12.5, wild-type cranial mesenchyme. I will use 2X10^7 cells, harvested from approximately 3 litters of embryos. I will collect cells for Twist1 ChIP by microdissection without FACS in order to avoid cell loss, since ChIP typically requires a higher starting cell number. I will perform the Twist1 ChIP as described (Chapter II), and confirm that the ChIP worked by western blot for Twist1 and qPCR for the Sox9

Figure 4.3. Proposed workflow to analyze Twist1 target genes and define a role of Twist1 in cranial bone progenitor specification
3'UTR as a positive control (Goodnough et al., 2012). Then I will proceed with the sequencing as in future direction (A).

c. Identification of candidates for functional analysis. Following ChIP- and RNA-sequencing, I will use state-of-the-art bioinformatics approaches to integrate the two data sets and identify Twist1-regulated target genes.

   i. Identification of Twist1 target genes. First, I will determine the genes whose transcripts are up- or down-regulated in the absence of Twist1 in cranial mesenchyme. We will compare the RNA-sequencing results from Twist2Cre; Twist1 fl/+ heterozygote control embryos and Twist2Cre; Twist1 fl/fl homozygous mutant embryos. For Twist1-target genes, we will first consider only consider genes that are 2 fold or more differentially expressed between control and mutant embryos, with a significance of p-value<0.05.

   ii. Direct Twist1 target genes. Transcription factors directly regulate gene expression in the nucleus by binding to non-coding regions of genomic DNA such as promoters or enhancers (regulatory elements) and initiating, inhibiting, or altering mRNA transcription by RNA polymerase II (Bulger and Groudine, 2010). Therefore, to identify genes directly regulated by Twist1 binding, we will consider Twist1 target genes (from RNA-seq), for which we also observe Twist1 binding in close proximity to putative/known regulatory elements. Many recent tools have overcome the challenge of identifying putative regulatory elements within the vast amount of non-coding DNA in the genome. For example, transcription factor binding to regulatory elements causes stereotypical changes to the way histone proteins bind genomic DNA, and specific covalent histone modifications that are
predictive of regulatory elements have been mapped across the human/mouse genomes for public use (Guenther et al., 2007; Heintzman et al., 2007; Jenuwein and Allis, 2001). First, I will use computational tools to assign each Twist1 ChIP-seq binding event to the nearest gene. I will select significantly altered Twist1 target genes identified from (i). Then, I will compare my observed Twist1-binding events to existing genome-wide maps for the promoter-specific H3K4me3 (trimethylation of lysine four on histone H3) modification in multiple cell types (Heintzman et al., 2007), as a way to easily identify putative promoter sequences. By selecting differentially expressed Twist1 target genes for which a Twist1 binding event occurs within <1 kilobase from a promoter sequence (H3k4me3 site), I will identify a set of putative direct Twist1 target genes for further analysis. Additionally, as another way to generate a list of candidate direct Twist1 targets, I will use other publically available, predictive computational approaches, such as TRED (Transcriptional Regulatory Element Database) and EEL (enhancer element locator), to compare Twist1 DNA-binding sites to putative regulatory elements (Hallikas et al., 2006; Zhao et al., 2005).

iii. Identify transcription factors that are direct targets of Twist1. I will functionally annotate the Twist1 direct targets using GREAT (Genomic Regions Enrichment of Annotations Tool), which queries several curated sets of mouse and human ontologies (McLean et al., 2010). Since I am interested in predominantly the transcription factors that exert genetic control of cell fate, I will focus on
transcription factors that are most differentially expressed as direct targets of Twist1.

iv. Identify direct transcription factor targets that may bind cooperatively with Twist1

I will further narrow my list of candidate transcription factors to Twist1 direct targets for which there is evidence of cooperative function, a hallmark of transcriptional regulation of cell fate decisions (Holmberg and Perlmann, 2012). I will therefore select direct Twist1 target transcription factors (Twist1-induced TFs) that also demonstrate putative DNA-binding sites near observed Twist1 binding sites at target genes. To identify transcription factor-binding sites near Twist1 binding sites, I will use available software cataloguing numerous experimentally-defined consensus DNA-binding sequences for an array of transcription factors, including programs such as GREAT, JASPAR, or EEL (McLean et al., 2010; Palin et al., 2006; Sandelin et al., 2004). I will identify which Twist1-induced TFs also have the highest number of putative binding sites near Twist1 binding sites (Figure 4.3). While I will not demonstrate definitive binding of Twist1-induced TFs near Twist1 at this time, evidence of putative transcription factor binding near Twist1 will allow me to focus on the most important target genes.

v. Functional analysis of target genes. To screen an array of transcription factors for importance during cranial bone progenitor specification, I will use a well-defined, high-throughput, in vitro primary osteoblast differentiation assay that recapitulates in vivo osteoblast specification, differentiation, and mineralization. E16.5 calvariae of wild-type mice will be dissected and introduced into single cell
suspension using a combination of collagenase P and 0.25% trypsin. The single cell suspension will be plated for cell culture in an osteogenic differentiation medium containing β-glycerophosphate and ascorbic acid. I will use pools of lentiviral, shRNA (an RNAi gene silencing strategy) for each candidate transcription factor (identified per criteria in Figure 4.3) to obtain functional genetic knock-down, which I will confirm by western blot or qPCR. The presence of osteoblast cell fate specification markers such as AP and Osx will be tested by real-time qPCR at day 3, 5, and 7 following lentiviral infection. Mineralization will be tested by alizarin red at 15 and 21 days.

3. Interpretation

I expect to identify at least 1-3 transcription factors that are required for osteoblast progenitor specification in vitro, as measured by an absence of Osx, and for mineralization, as measured by an absence of Alizarin Red staining. I would consider these as core transcription factors required for cranial bone progenitor specification, upon which to focus my efforts in generating new mouse knockouts.

D. Future Direction #3. Identify a set of transcription factors sufficient for direct induction of bone-forming cell fate from differentiated cells

1. Rationale

Ultimately, one clinical goal of studying the developmental pathways that control cranial bone progenitor specification is to recapitulate the process to generate de novo osteoblast progenitors that can expand and differentiate into functional bone-forming osteoblasts to replace lost or missing bone in patients. The ability of transcription factors to recapitulate the embryonic state was perhaps best
demonstrated when a defined set of transcription factors, over-expressed in human or mouse skin fibroblasts, could genetically and functionally coerce the cell back to one of the earliest developmental states, an embryonic stem cell (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Similarly, a set of transcription factors with essential roles in neuronal development were able to directly force embryonic fibroblasts to switch fates to neurons, bypassing the embryonic stem cell state completely (Vierbuchen et al., 2008). These data suggest that the right combination of transcription factors essential for osteoblast formation might force an unrelated, differentiated cell type to assume the identity and function of an osteoblast. My work has shown roles for $\beta$-catenin and Twist1 in osteoblast progenitor cell fate, and a number of additional factors essential have been described (cf Chapter I). Therefore, I propose to define a set of transcription factors that is sufficient to directly induce human adipocytes, a plentiful cell type, into a clinically functional osteoblast.

2. Experimental Strategy:

First, I will obtain human adipocytes or mouse/human adult dermal fibroblasts as starting material from collaborators. Next, I will clone or purchase individual lentiviral vectors, each using a constitutive promoter to drive expression of a transcription factor required for osteoblast formation. I will begin with vectors encoding the following factors: Twist1, $\beta$-catenin, Tcf4, Lef1, Runx2, Osterix, Msx2, Dlx5, and Alx4. Next, I will infect adipocytes, and human/mouse dermal fibroblasts, with a viral cocktail expressing a combination of all of the osteoblast factors, or an empty viral vector as a control. After culturing the
osteoblast-factor infected cells and control cells, I will test for direct conversion of factor-infected cells to osteoblast fate. I will assay for gene expression of an early marker of osteoblast fate, for instance qPCR for *Alkaline phosphatase* mRNA. Next I will test for genetic markers of osteoblast differentiation, such as *Osteocalcin* or *Osteopontin* mRNA by qPCR. Finally, I will test if the induced-osteoblasts form bone-matrix by alizarin red staining.

3. Interpretations:

If it is possible to convert differentiated mesenchymal cells such as fibroblasts or adipocytes directly into osteoblast progenitors, I expect that over-expression of the maximum combined number of transcription factors will induce osteoblast fate in fibroblasts/adipocytes. If the maximum number works, I will begin removing factors from the “osteoblast-factor” viral cocktail in an effort to define the minimum number of factors essential for conversion of differentiated mesenchymal cells to osteoblasts. There are broad clinical implications if direct induction of osteoblast fate is possible. First, it would offer the promise of engineering clinically viable osteoblasts from an abundant cell type for replacing acquired/congenital bone defects. Second, we could produce osteoblasts from skin biopsies of patients with genetic bone and mineral diseases and craniofacial defects. This in turn would allow us to better study the molecular basis of genetic skeletal defects, and would also provide a high-throughput system for screening potential therapies defective osteoblast development, differentiation, or homeostasis.
In summary, my proposed future directions will further elucidate the in vivo function of Twist1 from two perspectives: its functional role for *in vivo* specification of cranial bone progenitors, and its role in gene regulation at the genomic level. This approach to understanding Twist1 mechanism of action may offer the hope of identifying new factors underlying congenital cranial bone defects. Finally, if I elucidate a core set of transcription factors sufficient for direct specification of an osteoblast cell from an abundant cell type, my findings would be of great clinical utility in the diagnosis and treatment of an array of genetic skeletal defects.
Bibliography


Deficiency of PORCN, a regulator of Wnt signaling, is associated with focal dermal hypoplasia. *Nat Genet* 39, 833-5.  


essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development 130, 3063-74.