



Published in final edited form as:

Genesis. 2019 January ; 57(1): e23248. doi:10.1002/dvg.23248.

A tale of two cities: the genetic mechanisms governing calvarial bone development

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Abstract

The skull bones must grow in a coordinated, three-dimensional manner to coalesce and form the head and face. Mammalian skull bones have a dual embryonic origin from cranial neural crest cells (CNCC) and paraxial mesoderm (PM) and ossify through intramembranous ossification. The calvarial bones, the bones of the cranium which cover the brain, are derived from the supraorbital arch (SOA) region mesenchyme. The SOA is the site of frontal and parietal bone morphogenesis and primary center of ossification. The objective of this review is to frame our current *in vivo* understanding of the morphogenesis of the calvarial bones and the gene networks regulating calvarial bone initiation in the SOA mesenchyme.

Keywords

skull bone; gene regulation; supraorbital arch mesenchyme; bone initiation

Introduction:

Skull bone development involves the formation of complex, three-dimensional structures in a spatially and temporally sensitive manner. The formation of the skull requires the convergence of multiple bones of complex shapes and sizes into a single unit. Cell fate selection, differentiation, and patterning of each bone occurs within close proximity to one another. In addition, two different stem cell progenitor populations contribute to the skull bones. Thus, spatial and temporal genetic regulation is required to coordinate early events in skull bone morphogenesis to ensure normal skull bone formation. Here, we will primarily highlight the morphogenesis of the mammalian calvaria and the genetic and epigenetic mechanisms required for calvarial bone initiation *in vivo*.

Characteristics of skull bone development:

In the context of bone formation, three traits characterize the development of mammalian skull bones. First, the skull bones are derived from two distinct cranial mesenchymal stem

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cell (CM) populations; the cranial neural crest cells (CNCC) and the paraxial head mesoderm (PM) (Fan et al., 2016; Hanken and Thorogood, 1993; Jiang et al., 2002; Yoshida et al., 2008). The CNCC is derived from the embryonic ectoderm layer and gives rise to the anterior skull bones. The PM, in contrast, is derived from the embryonic mesoderm germ layer and gives rise to the more posterior skull bones (Jiang et al., 2002; Yoshida et al., 2008) (Figure 1). Both the CNCC- and the PM-derived bones differ in their signaling requirements and timing of development, but ultimately coalesce and contribute to the cohesive structure of the skull (see below). The generation of tissue-origin restricted *in vivo* animal models are beginning to tease apart the developmental differences between the two stem cell populations (Fan et al., 2016). Second, the skull bones ossify through intramembranous ossification, in which the post-migratory CNCC- and PM-derived mesenchyme give rise to bone by direct ossification. Unlike endochondral ossification, which requires a cartilage template, the skull bones form from condensed mesenchyme that differentiate to mature osteoblasts and form mineralized bone (Dunlop and Hall, 1995; Hall and Miyake, 1992). Thus *SRY-box 9 (Sox9)*, the key determinant of cartilage formation which is required in endochondral ossification, is dispensable for skull bone formation (Bi et al., 1999; Mori-Akiyama et al., 2003). Third, the skull bones must differentiate and ossify in a unique environment within close proximity to other CNCC- and PM- derived tissues such as the dermis, meninges, sutures, and muscle (Goodnough et al., 2014; Kasner et al., 2013; Michailovici et al., 2015; Tran et al., 2010). The simultaneous differentiation of tissues within close proximity to one another poses developmental and genetic constraints for establishing the calvarial bone progenitors. Thus, many of the complex genetic questions pertaining to mammalian skull bone formation, such as tissue-tissue interaction and intramembranous ossification, are difficult to address using *in vitro* models. Conditional mouse genetics has allowed us to define some of the core set of transcription factors and signaling pathways required during skull bone initiation, and reveal spatial and temporal genetic mechanisms governing the establishment of the skull bone primordia. Disruptions in these genetic pathways can lead to defects in cell fate selection, ossification, and congenital skull bone defects (Bhatt et al., 2013; Fan et al., 2016; Morriss-Kay and Wilkie, 2005; Rice, 2005; Twigg and Wilkie, 2015).

The dual origin of the skull bones begins with the morphogenesis of the primordia, and we focus on the supraorbital arch (SOA) region which serves as the “organizer” for the calvarial bones (upper part of the skull). Most of the published studies have focused on understanding how the mesenchyme in the SOA (SOA-mesenchyme) is patterned and the signals required for specification of the mammalian frontal and parietal bone progenitors and the other lineages in the SOA (see below).

Embryonic development of the calvaria: morphogenesis.

The origin and site of specification of the mammalian calvarial bone precursors were identified by lineage analysis using lipophilic dye labeling and genetic lineage tracing (Jiang et al., 2002; Morriss-Kay and Wilkie, 2005; Roybal et al., 2010; Tran et al., 2010; Yoshida et al., 2008). Between E8.0-9.5, the CNCC-derived mesenchyme migrate from the mid-hindbrain region beneath the surface ectoderm to the anterior SOA region and give rise to the frontal bone precursors (Morriss-Kay and Wilkie, 2005; Yoshida et al., 2008). The PM

from the same region migrates to the posterior SOA to give rise to the parietal bone precursors (Figure 2a,b). The SOA-mesenchyme represents the site of specification and the location of mineralization initiation for the frontal and parietal bones of the calvaria. By E10.5, patterning of the SOA-mesenchyme is established with the calvarial bone precursors located between the meningeal mesenchyme medially and the dermal progenitors laterally under the surface ectoderm (Figure 2b). Between E10.5-12.5, the calvarial bone progenitors in the SOA are specified and express bone lineage-specific markers to form the frontal bone and parietal bone primordia (Figure 2c) (Deckelbaum et al., 2012; Han et al., 2007; Musy et al., 2018; Tran et al., 2010; Yoshida et al., 2008). The mesenchyme apical to the SOA is termed “early migrating mesenchyme” (EMM) and does not contribute to the calvarial bone primordia and does not ossify (Cesario et al., 2018; Roybal et al., 2010).

Until recently, the identity and source of the “osteo-inductive” signals for mammalian calvarial bone were not clear *in vivo*. However, functional mouse genetic experiments have offered some clues. Conditional mutants of well-known signaling pathways of endochondral bone development, such as BMP, FGF, and Wnts, demonstrate that they are required for development and morphogenesis of the skull bones, but they primarily function downstream of the inductive patterning event (reviewed in Fan et al., 2016; Bhatt et al., 1993). However, the Wnt signaling pathway and its effectors, such as TWIST1, are required for calvarial bone fate; thereby qualifying Wnts as a candidate osteo-inductive signal (Day et al., 2005; Goodnough et al., 2012, 2014, 2016; Hill et al., 2005; Tran et al., 2010).

As the bone initiation program (see below) progresses between E9.5 and E13.5, both the CNCC- and PM- derived cranial bone progenitors in the SOA must condense, proliferate, and expand in the baso-apical direction (Figure 2c). Between E10.5-12.5 transcription factors, such as the forkhead domain-containing *Fox* family and basic-helix-loop-helix transcription factor *Twist1*, are also expressed throughout the SOA-mesenchyme and are required for mesenchyme condensation and initiation of calvarial bone primordia (Bildsoe et al., 2009; Goodnough et al., 2012; Kume et al., 1998; Sun et al., 2013; Vivatbuttsiri et al., 2008). Temporally-inducible knockouts of *Twist1* with *UbcCre-ERT²* demonstrate the importance of condensation of the SOA-mesenchyme in the morphogenesis of calvarial bones (Fan et al., 2016). Inducible genetic lineage tracing of the SOA-mesenchyme between E10.0-11.5, resulted in the identification of lineage descendants of the SOA-mesenchyme in the apex of the frontal and parietal bones (Deckelbaum et al., 2012; Tran et al., 2010). These experiments are supported by more recent findings that non SOA-mesenchyme cannot be recruited to the calvarial bone progenitor pool (Cesario et al., 2018). After the onset of mineralization in the SOA at E14.0-14.5, frontal and parietal bones continue to grow apically and eventually meet to form the coronal, frontal, and sagittal sutures (Deckelbaum et al., 2012; Goodnough et al., 2012; Kaufman, 1992; Tran et al., 2010; Yoshida et al., 2008). These results together suggest that calvarial bones grow in the baso-apical direction intrinsically and the mechanisms underlying the cellular and tissue-level movements remain to be discovered (Figure 2c).

Transcriptional profiling experiments have identified several factors that are differentially expressed between the CNCC- and the PM-derived mesenchyme in the E9.5 head, highlighting regional signaling differences between the two stem cell populations (Fan et al.,

2016). In the next sections, we will focus on the *in vivo* genetic studies identifying a core set of transcription factors and signaling pathways involved in cranial bone initiation, and how the different genetic landscapes of the CNCC- and PM-derived mesenchyme impact skull bone formation.

The skull bone initiation program: transcription factor cascade for establishing bone progenitors

In mice, the first steps in establishing the skeletal progenitors that will differentiate and ossify into the skull bones occur between E10.5 to E13.5 with the emergence of mineralized ossified bone primordia after E14.0 (Han et al., 2007). The current *in vivo* data examining the initial establishment of the bone stem cells can be broken down into three tiers; the transcription factors required for the establishment of bone, signaling pathways regulating these factors, and the epigenetic mechanisms contributing to the spatial and temporal coordination of these processes. The transcription factor cascade required for the establishment of skull bone progenitors, which we will refer to as the “bone initiation program”, involves three primary factors; *Msh Homeobox 1 (Msx1)* and *2 (Msx2)*, *Runt Related Transcription Factor 2 (Runx2)*, and *Osterix (Osx/Sp7)* (Baek et al., 2014; Han et al., 2007; Ishii et al., 2003; Nakashima et al., 2002; Nishio et al., 2006).

The activation of the bone initiation program can be monitored with the CNCC- and PM-derived bone precursors expressing *Msx1* and *Msx2*. In the SOA-mesenchyme *Msx1* and *2* are expressed broadly at E10.5 and become progressively restricted to calvarial bone progenitors at E12.5 (Holland, 1991). *In vivo*, *Msx1* or *Msx2* null mutants, or compound *Msx1^{-/-}* and *Msx2^{+/-}* mutants, lead to defects in skull bone patterning and development (Han et al., 2007; Roybal et al., 2010; Satokata and Maas, 1994; Satokata et al., 2000). However, these mutants do not result in a complete loss of skull bones, demonstrating some level of functional redundancy between the two *Msx* genes. Compound *Msx1^{-/-}; Msx2^{-/-}* mutants and the inducible deletion of *Msx1* and *Msx2* in all cells using *CaggCreER* at E9.5 results in severe calvarial bone agenesis (Han et al., 2007; Roybal et al., 2010). In contrast, inducible deletion of *Msx1* and *Msx2* at E13.5 using *CaggCreER* results in little effect on the calvarial bones (Roybal et al., 2010). These functional mouse genetics studies highlight *Msx1* and *Msx2* as the first factors of the bone initiation program and demonstrate their temporal requirement for early events in bone formation.

The next key step in the program is the expression of *Runx2*, a transcription factor and a key determinant of bone fate selection required to establish the bone progenitors (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Deletion of *Runx2* in mice results in a complete loss of bone throughout the entire body (Komori et al., 1997). *Msx1* and *Msx2* expression is required for the expression of *Runx2* mRNA, as *Msx1^{-/-}; Msx2^{-/-}* compound mutants lack *Runx2* expression in the SOA-mesenchyme (Baek et al., 2014; Han et al., 2007; Nakashima et al., 2002; Nishio et al., 2006). Similar to long bones, RUNX2 protein expression decreases as the calvarial bone matures, further demonstrating its role primarily in early bone development (Kacena and Ciovacco, 2010).

Downstream of RUNX2 is *Osx*, a zinc finger-containing transcription factor required for intramembranous ossification (Baek et al., 2014; Nishio et al., 2006). Unlike the *Runx2* null mice, genetic deletion of *Osx* leads to a complete loss of ossified intramembranous bone specifically in the head and face. (Fan et al., 2016; Nakashima et al., 2002). *In vitro*, RUNX2 can bind to the *Osx* promoter in the 10T1/2 mesenchyme progenitor cell line; however, their interaction *in vivo* has not been demonstrated (Nishio et al., 2006). Indeed, *Runx2* null mice do not express *Osx*, but further experiments are required to determine if RUNX2 can trans-activate *Osx* in the skull bones.

Both the CNCC- and PM-derived bones use the bone initiation program, with some spatio-temporal differences. In mice, the bone initiation program is activated in the CNCC-derived frontal bone primordia roughly 24 hours prior to the PM-derived parietal bone primordia as indicated by the expression of *Msx1* and *Msx2*. At E10.5, the *Msx1* and *Msx2* expression can be observed in the frontal bone primordia with ossified bone detectable at E14.0. In contrast, the PM begins to express *Msx2* roughly at E11.5 with ossified bone being detectable by E14.5 (Deckelbaum et al., 2012; Han et al., 2007). These results show the regional differences between the CNCC- and the PM-derived calvarial bones and also suggests a difference in developmental timing of early events in bone initiation. In addition, conditional mutants in the CNCC using *Wnt1Cre* have revealed a level of communication between multiple tissues. Conditional deletion of genes such as *Twist1* or *Transforming Growth Factor Receptor β II (TGF β RII)*, or the FGF8 regulator, *Specificity Protein 8 (Sp8)*, in the CNCC using *Wnt1Cre* results in arrest of both frontal and parietal bone differentiation (Bildsoe et al., 2009; Ito et al., 2003; Kasberg et al., 2013; Sasaki et al., 2006; Yang et al., 2010). Conversely, expression of constitutively-active *Fibroblast Growth Factor Receptor 2 (Fgfr2)* in the PM does not affect the growth of the parietal bone, but leads to a truncation of the frontal bone and craniosynostosis by an unknown mechanism (Holmes and Basilico, 2012). These phenotypes demonstrate the potential cross-talk between the adjacent tissues during skull bone formation. As more cell-type restricted genetic tools become available, new insights will clarify the interdependence of the neighboring lineages in calvarial bone development.

Signaling factors regulating the calvarial bone initiation program

Many signaling pathways are required during various stages of calvarial bone development and have been reviewed in greater detail previously (Gou et al., 2015; Ishii et al., 2015; Karsenty, 2008; Liu and Lee, 2013; Szabo-Rogers et al., 2010). In the context of the bone initiation program, Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), and Wnt/ β -catenin signaling have been associated with the regulation of its factors (Cadigan and Nusse, 1997; Fan et al., 2016; Mark et al., 2009; Ornitz and Itoh, 2015; Rhinn and Dolle, 2012; Wang et al., 2014). BMPs are part of the Transforming Growth Factor Beta (TGF- β) super family and transduce intracellular signaling through phosphorylation of SMAD1/5/8. BMP 2/4 ligands and p-Smad1/5/8 are expressed in the SOA-mesenchyme at E12.5 (Roybal et al., 2010; Sun et al., 2013). *Msx1* and *Msx2* expression can be partially regulated by BMP. In multiple organisms such as zebrafish, *Xenopus*, and mice, BMP-signaling positively regulates *Msx* gene expression *in vivo*. (Bonilla-Claudio et al., 2012; Knecht and Bronner-Fraser, 2002; Suzuki et al., 1997; Tribulo et al., 2003). However, loss of

individual BMP ligands is not sufficient to result in a complete loss of *Msx1* or *2* gene expression, demonstrating functional redundancy between multiple BMP ligands (Bonilla-Claudio et al., 2012).

Another signaling pathway shown to regulate *Msx* gene expression is FGF signaling. FGF signaling pathway has 22 ligands and four FGF tyrosine kinase receptors (FGFR) (Ornitz and Itoh, 2015). FGFR2 and the pathway transducer, ERK1/2, are present in SOA mesenchyme, and FGF 8/10 ligands are expressed in the overlying surface ectoderm (Fan et al., 2016; Goodnough et al., 2014; Kim et al., 1998; Rice et al., 2000). Removal of FGFR2 in the SOA-mesenchyme results in decreased proliferation, leading to dome shaped, but ossified, skull (Yu et al., 2003). Similarly, overexpression of the FGF signaling antagonist *Sprouty1*, or deletion of *Sp8* in the CNCC using *Wnt1Cre* leads to diminished frontal bone (Kasberg et al., 2013; Yang et al., 2010). The presence of ossified bone in these mutants indicates the bone initiation program is not entirely disrupted. However, FGF signaling pathway mutants have loss of *Msx1* and *Msx2* mRNA expression in branchial arch facial mesenchyme, and FGF2 coated beads can induce *Runx2* expression in calvarial explants (Abu-Issa et al., 2002; Choi et al., 2005; Griffin et al., 2013; Kettunen and Thesleff, 1998; Kim et al., 2003; Omoteyama and Takagi, 2009). These results suggest that the calvarial defects observed in FGF mutants may be due to disruptions in *Msx1*, *Msx2*, and/or *Runx2* expression, but not a complete loss. Relative to BMP signaling, most FGF signaling pathway mutants have less pronounced effects on calvarial bone development, but FGF signaling is often perturbed in calvarial bone agenesis mutants and human craniosynostosis syndromes suggesting it is an integral pathway to calvarial bone development (Abu-Issa et al., 2002; Fan et al., 2016; Griffin et al., 2013; Nie et al., 2006; Rice et al., 2000; Su et al., 2014).

The Wnt/ β -catenin signaling pathway has 19 ligands and binds to Frizzled and LRP5/6 receptors. The intracellular signaling pathway is transduced by β -catenin which binds to TCF/LEF transcription factors to regulate gene transcription in diverse contexts during embryonic development (Brugmann et al., 2007; Cadigan and Nusse, 1997; Day et al., 2005; Gaur et al., 2005; Niemann et al., 2004). Of the three signaling pathways discussed in this review, conditional mutants of the Wnt signaling pathway result in the most dramatic skull bone defects with complete agenesis of the CNCC- and PM-derived skull bones (Day et al., 2005; Goodnough et al., 2012, 2014; Hill et al., 2005). During early skull bone development, Wnt/ β -catenin signaling is required for the proliferation and survival of craniofacial structures. Deletion of Wnt/ β -catenin signaling in the CNCC using *Wnt1Cre* results in a complete agenesis of the head and face (Brault et al., 2001). Conditional deletion of β -catenin in the SOA mesenchyme by E12.5 using multiple *Cre* lines leads to a disruption of the *Runx2* expression pattern, and subsequent loss of *Osx* expression in frontal and parietal bone primordia with little impact on *Msx1* and *2* expression (Brault et al., 2001; Fan et al., 2016; Goodnough et al., 2012; Hill et al., 2005). Conditional deletion of individual Wnt ligands results in mild effects on bone differentiation, suggesting functional redundancy (Bennett et al., 2005; Später et al., 2006). Deletion of *Wntless*, which is required for secretion of all Wnt ligands, in the surface ectoderm using *CreEct* leads to loss of canonical Wnt/ β -catenin signaling in the SOA-mesenchyme and agenesis of the skull bones by E12.5 (Bänziger et al., 2006; Goodnough et al., 2014). Unlike other signaling pathway mutants, β -catenin deletion in the SOA and facial mesenchyme leads to ectopic expression of a key

chondrogenic determinant, *Sox9*, and conversion of calvarial bones and non-osteogenic mesenchyme into cartilage. It is currently unclear as to how β -catenin promotes the expression of the bone initiation program and represses the cartilage fate.

One candidate mechanism is that β -catenin signaling function is mediated by TWIST1. (Goodnough et al., 2012, 2014; Hill et al., 2005). The deletion of *Twist1* leads to smaller calvarial bones, increased fontanelle size, and ectopic chondrogenesis in the posterior calvarial bones, similar to β -catenin mutants (Bildsoe et al., 2009; Goodnough et al., 2012, 2016; Krawchuk et al., 2010; Loebel et al., 2012). A second candidate mechanism is that β -catenin deletion leads to stabilization of SOX9 protein, and higher SOX9 levels negatively regulate *Runx2* transcription and bone formation *in vivo* (Eames et al., 2004; Zhou et al., 2006) Additional possibilities are shown in Figure 3 (Akiyama et al., 2004; Goodnough et al., 2012; Hoffmeyer et al., 2017; Liu and Lefebvre, 2015; Mead et al., 2013; Reinhold et al., 2006; Weston et al., 2002; Yasuhara et al., 2010).

Based on these *in vivo* data, it appears that BMP and FGF signaling establish the *Msx1* and *2* positive bone precursors, and Wnt/ β -catenin signaling is required for the cell fate selection and commitment to bone. Similar to the spatio-temporal differences in expression observed in the factors of the bone initiation program, the signaling pathways regulating them also exhibit spatio-temporal differences between the CNCC- and the PM-derived mesenchyme. Compared to the PM, the CNCC has increased expression of FGF and Wnt ligands (Fan et al., 2016). In contrast, many BMP signaling components are preferentially expressed in the PM (Fan et al., 2016). However, compound loss of BMP2/4/7 ligands in the CNCC using *Wnt1Cre* leads to a near complete loss of CNCC-derived bones with little impact on the PM-derived bones (Bonilla-Claudio et al., 2012), showing there is a cell/tissue autonomous role for BMP signaling in CNCC-derived frontal bone despite lower expression compared to the PM. Additional differences in the transcriptional profile of the CNCC and the PM have been identified and reviewed previously (Fan et al., 2016). To date, the majority of the studies examining the bone initiation program are focused on either null mutations or CNCC-specific conditional mutants. A more systematic effort examining the bone initiation program and its regulatory factors in the PM-derived mesenchyme are required to obtain novel mechanistic insights into the regulation of skull bone development.

Epigenetic regulation of the bone initiation program:

Epigenetic modifications allow for the modular regulation of transcription in a reversible and heritable manner (Bracken et al., 2006; Messerschmidt et al., 2014; Rothbart and Strahl, 2014; Schübeler, 2015). During development, the two primary forms of epigenetic regulation are DNA methylation and histone modifications. DNA methylation involves the addition of a methyl group to cytosine residues in cytosine-guanine dense regions (CpG islands) and is linked to transcriptional repression. In mammals, three methyltransferases have been identified; DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a and 3b (DNMT3a, DNMT3b), with DNMT3a and 3b functioning as the primary DNA methyltransferases during development (Goll and Bestor, 2005; Gopalakrishnan et al., 2008; Okano et al., 1999). Histone modifications are post-translational modifications of histones that affect chromatin structure. In contrast to DNA methylation, which is thought to be

established early in development, histone modifications are thought to enable transient transcriptional regulation throughout development (Lynch et al., 2012; Schübeler, 2015; Tanay et al., 2007). The two most studied histone modifications are methylation and acetylation which can be associated with either transcriptional activation or repression (Greer and Shi, 2012; Madrigal and Krajewski, 2015; Roth et al., 2001).

Much of the current understanding regarding epigenetic modifications is based on *in vitro* data. In various cell types *in vitro*, epigenetic modifications have been shown to regulate transcription or occupy loci of the bone initiation program factors such as *Runx2*, *Osx*, and regulatory factors such as the BMP, Wnt, and FGF signaling pathways (Bracken et al., 2006; Lee et al., 2006; Rosenbloom et al., 2013; Sinha et al., 2010; Yang et al., 2013a; Zhang et al., 2015). However, *in vitro*, the epigenetic profile of a single cell type can vary based on the specific culture conditions (McEwen et al., 2013). As a result, the extent by which *in vitro* data accurately represents *in vivo* conditions is limited. In contrast to *in vitro*, the role of epigenetic regulation on skull bone development *in vivo* appears to be relatively limited. Disruptions in many epigenetic modifier pathways result in limited phenotypes (Table 1) (Haberland et al., 2009; Jacques-Fricke et al., 2012; Ueda et al., 2006; Yang et al., 2013b; Zhang et al., 2015). Conditional mouse mutants have identified the Polycomb Repressive Complex 2 (PRC2), a methyltransferase, as the primary regulator of skull bone formation as a loss of PRC2 function results in a loss of multiple bones (Dudakovic et al., 2015; Schwarz et al., 2014).

H3K27me3 is the primary epigenetic modifier during skull bone formation *in vivo*

PRC2 is a histone modifier that catalyzes the tri-methylation of the 27th lysine on the third histone (H3K27me3) and is typically associated with transcriptional repression. In mammals, PRC2 is a multi-protein complex primarily composed of *Enhancer of Zeste 2* (*EZH2*), *Suppressor of Zeste 12* (*SUZ12*), and *Embryonic Ectoderm Development* (*EED*). *EZH2* is the methyltransferase catalytic component of PRC2 and is required for the H3K27me3 modification (Figure 4) (Margueron and Reinberg, 2011). In mammals, a homologue of *EZH2*, *EZH1*, has also been shown to be associated with the PRC2 complex and possess methyltransferase activity (Shen et al., 2008). However, *EZH1* null mice exhibit no phenotype and are viable, indicating that *EZH2* is the primary methyltransferase during development (Ezhkova et al., 2011). Thus, most *in vivo* studies investigating PRC2 have focused on *Ezh2* mutants.

In comparison to other epigenetic mechanisms, *Ezh2* mutants can result in the most severe skull phenotypes. In mice, conditional deletion of *Ezh2* can result in a complete loss of multiple skull bones. However, the severity of the phenotype is highly dependent on the specific timing of the *Cre* line. For example, loss of *Ezh2* in the CNCC at E8.5 using *Wnt1Cre* leads to a decrease in *Runx2* and *Osx* expression in the first branchial arch and a complete loss of the CNCC derived skull bones (Schwarz et al., 2014). In contrast, loss of *Ezh2* primarily in the PM at E9.5 using *Prx1Cre* leaves the skull bones intact, but results in fusion of multiple sutures at 3 weeks of age (Dudakovic et al., 2015). Furthermore, deletion

of *Ezh2* in both the CNCC and the PM at E9.5 or E10.5 using *Dermo1Cre* or *En1Cre* results in no skull bone phenotype (Ferguson et al., 2017; Snitow et al., 2016).

The mechanism by which PRC2 regulates the skull bone in the CNCC has not been fully elucidated. Deletion of *Ezh2* using *Wnt1Cre* results in the down regulation of both *Runx2* and *Osx* in the first branchial arch (Schwarz et al., 2014). Because H3K27me3 is typically associated with transcriptional repression, an anti-osteogenic intermediate factor may be involved. One potential group of factors proposed to inhibit the skull bone in the *Ezh2* mutants are the *Hox* genes. In both the CNCC- and the PM-specific *Ezh2* mutants, multiple *Hox* genes are upregulated (Dudakovic et al., 2015; Schwarz et al., 2014). *Hox* genes have been associated with repression of *Runx2* in skull bone *in vivo* (Carroll and Capecchi, 2015; Santagati et al., 2005). In addition, the SOA-mesenchyme lacks expression of all *Hox* genes, resulting in a potential sensitivity to ectopic *Hox* gene expression. Functional studies demonstrating that the ectopic *Hox* gene expression in the *Ezh2* mutants leads to the disruption in the bone initiation program and craniofacial defects are still required.

The specific cause of the dramatic phenotypic differences between each *Ezh2* mutant is not fully clear. One possibility is that *Ezh2* is required preceding the induction of the bone initiation program. The phenotypic differences observed between *Ezh2* conditional mutants are due to the temporal differences between the *Cre* drivers. *Wnt1Cre* mutants are a result of deletion of *Ezh2* by E8.5, and *Dermo1Cre* mutants are a result of deletion of *Ezh2* at E9.5. Using *Wnt1Cre*, the *Ezh2* target genes may be sufficiently dysregulated prior to the onset of the bone initiation program around E10.5 in the CNCC-derived mesenchyme. In contrast, *Ezh2* activity is not sufficiently lost by E10.5 in *Dermo1Cre* mutants resulting in normal activation of the bone initiation program in the CM (Ferguson et al., 2017). Temporally inducible genetic clonal analysis will be required to understand when calvarial bone precursors become lineage-restricted and if *Ezh2* has a role in this process. A second possibility is due to inherent differences between the CNCC- and the PM-derived mesenchyme. Loss of *Ezh2* in the CNCC with *Wnt1Cre* lead to a loss of skull bone, but loss of *Ezh2* in the PM with *Prx1Cre* does not lead to loss of skull bone. Multiple histone modifications have been shown to work in tandem to coordinate stem cell identity and position (Minoux et al., 2017). The variations in the other methylation modifications between the CNCC and the PM could account for the differences in phenotype.

The *Ezh2* mutants highlight a level of complexity by which epigenetics may function during development. PRC2 has been associated with the regulation of multiple signaling factors in a spatial and temporal manner. As a result, the specific mechanism by which *Ezh2* regulates skull bones has not been fully demonstrated. Genetic studies utilizing tissue-restricted and inducible conditional mutants will provide new insights into the complex dynamics of PRC2 and epigenetic modifications in skull bone development.

Conclusions:

The formation of the skull is a complex process requiring the coordination of multiple genetic mechanisms and tissues. The mechanisms governing the establishment of the stem cells, the bone initiation program, and the epigenetics coordinating these processes require

spatial and temporal regulation. As a result, skull bone development is susceptible to congenital and environmental perturbations (Trainor, 2007). A greater understanding of the cellular and genetic mechanisms controlling these processes will provide insights into the pathogenesis of birth defects, tissue engineering, and wound/fracture healing.

To this end, *in vivo* studies are required to provide a greater picture into the dynamics governing skull bone formation. With the loss of spatial and temporal information *in vitro*, advancements in the available *in vivo* tools are providing a greater understanding of these developmental processes. New *Cre*-recombinase drivers specific to either the CNCC or the PM will help tease apart the genetics and cross-talk between the two tissues enabling skull bone formation. New *in vivo* cell reporters and live imaging technology will provide advancements in the understanding of the formation of the SOA and the mechanism underlying the apical expansion of skull bones. Sequencing technology such as Hi-ChIP, Assay for Transposase Accessible Chromatin (ATAC), and single cell RNA sequencing, which all facilitate the generation of large data sets from relatively few cells will enable the identification of new sub population and relevant target genes *in vivo* (Mumbach et al., 2016). As the genetic mechanisms governing the formation of the skull bones are uncovered, a greater picture of the complex processes of skull bone formation will begin to become clear.

Acknowledgments:

Thanks to previous and current members of the Atit laboratory for excellent contributions and discussion, in particular L.Henry Goodnough, Gregg DiNuoscio, and Thu Tran. Figures were modified from: Ferguson, J.W. (2018) *The Spatial and Temporal Role of Ezh2 in Skull Bone Formation* (doctoral dissertation). Retrieved from OhioLINK (Accession Number: case1530898825341447)

Funding: This work was supported by the following grants: NIH National Institute of Dental and Craniofacial Research R01 DE-01870 (R.P.A.), National Institutes of Health (NIH) T32 AR-007505 (J.F.)

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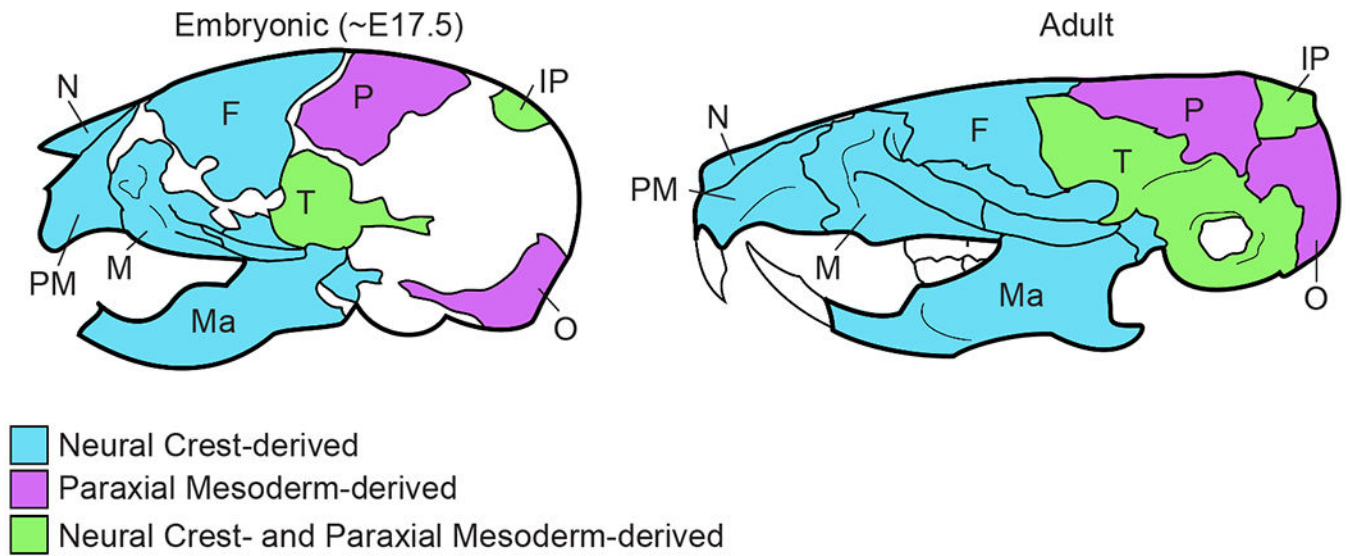


Figure 1: The bones of the mammalian skull are derived from two different stem cell populations.

The anterior bones are derived from the CNCC and the posterior bones are derived from the PM. The temporal and parietal bones have contributions from both stem cell populations. N = nasal bone; M = maxilla; PM = premaxilla, Ma = mandible; F = frontal bone; P = parietal bone; IP = interparietal bone; T = temporal bone; O = occipital bone

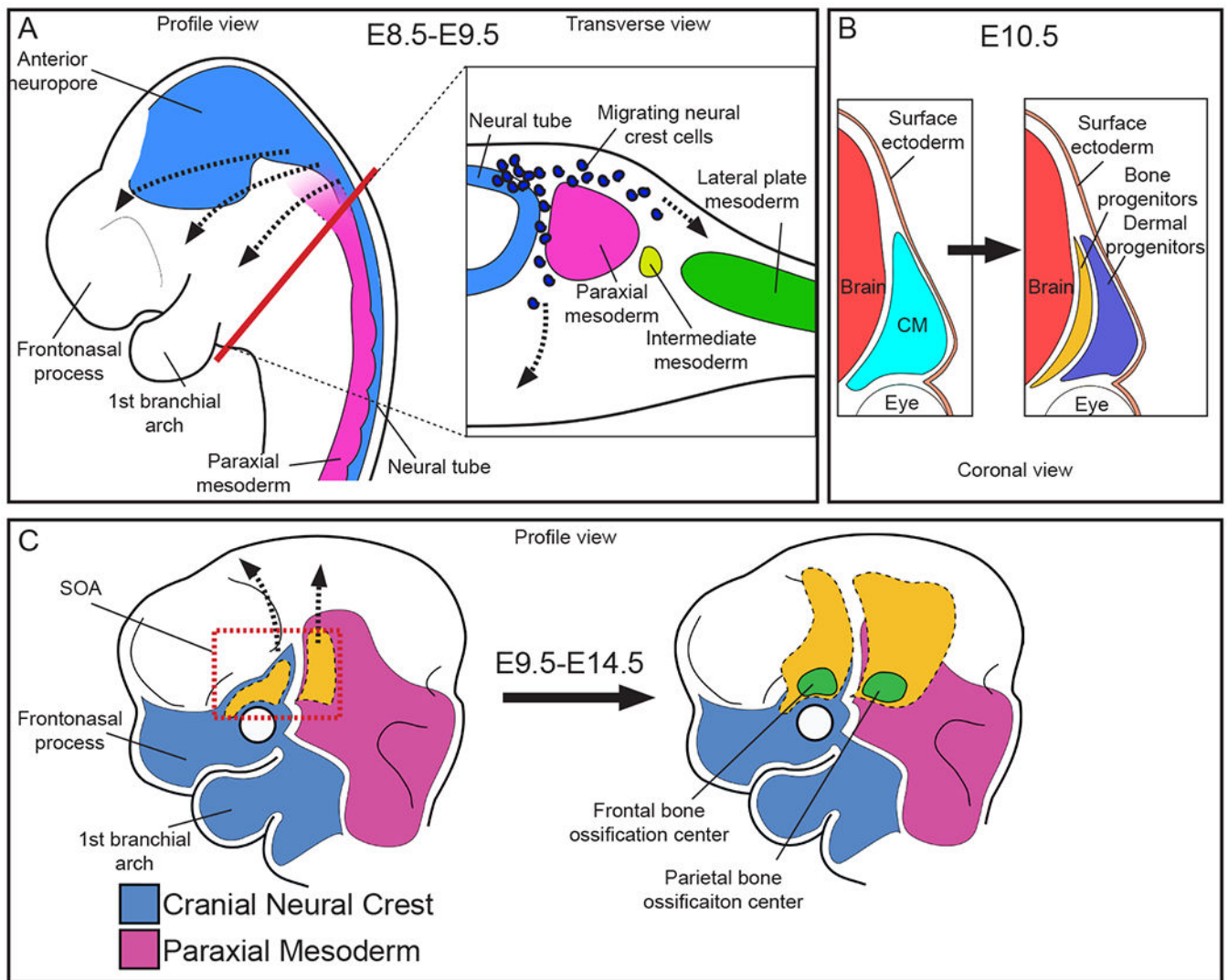


Figure 2: Origins and morphogenesis of the mammalian calvarial bones.

(A) The CNCC migrate from the neural tube to populate the anterior region of the head and face. The paraxial mesoderm flanks the neural tube and populates the posterior region of the head and face. In the head and face, both the CNCC and the PM are considered CM. (B) The CM in the SOA (SOA-mesenchyme) is located between the surface ectoderm and the brain, and gives rise to both the bone and dermal progenitors. (C) As the bone and dermal progenitors develop and differentiate, they grow apically over the brain.

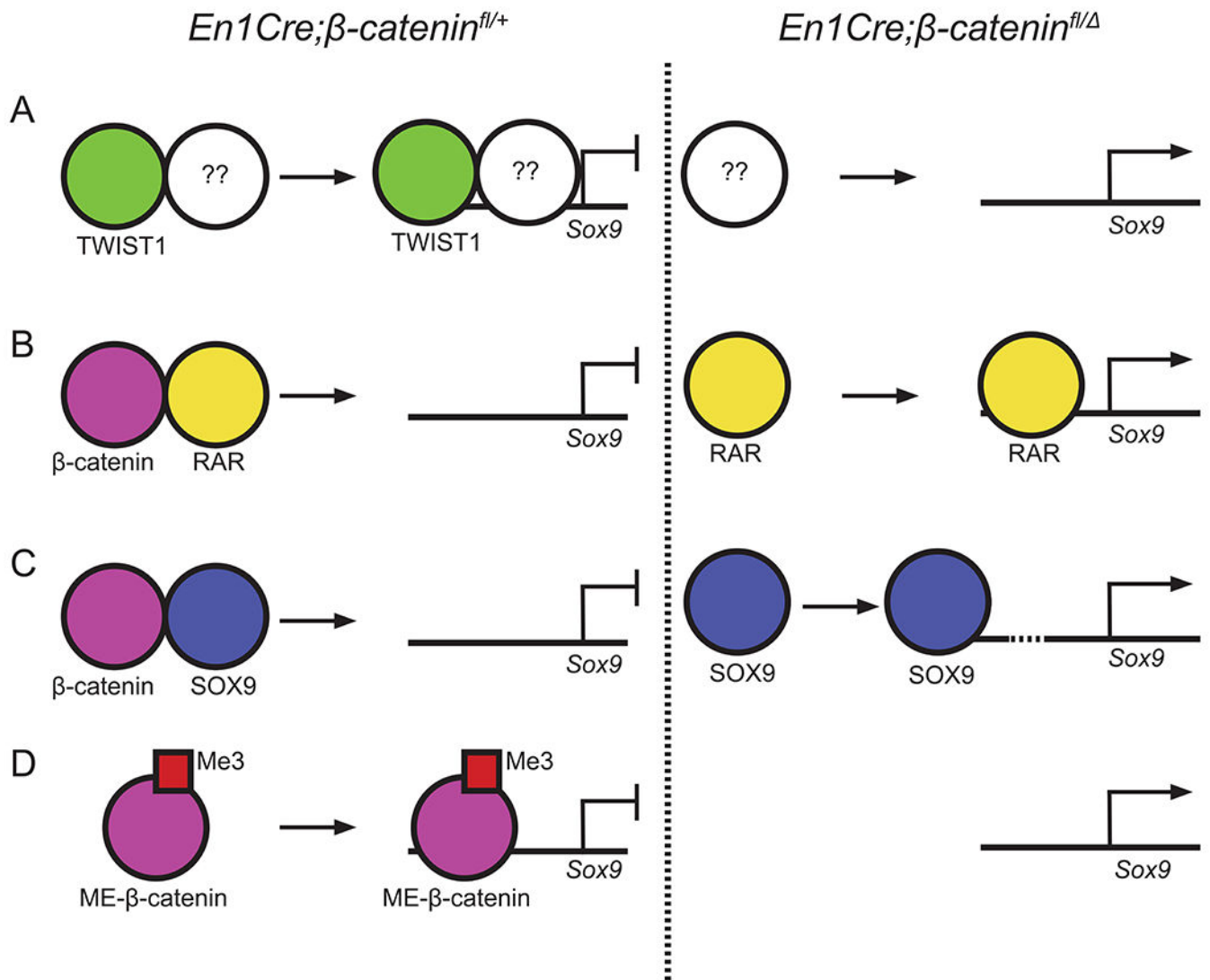


Figure 3: Proposed mechanism by which β -catenin suppresses chondrogenesis.

(A) TWIST1 heterodimerizes with an unidentified factor to repress *Sox9* transcription. In the absence of β -catenin, *Twist1* mRNA is absent leading to the derepression of *Sox9* (B) β -catenin dimerizes with retinoic acid receptors (RAR) to prevent the activation of *Sox9* transcription. (C) β -catenin dimerizes with SOX9 protein to prevent the auto-regulation of *Sox9* transcription. (D) A methylated form of β -catenin acts as a repressor and directly inhibits *Sox9* transcription.

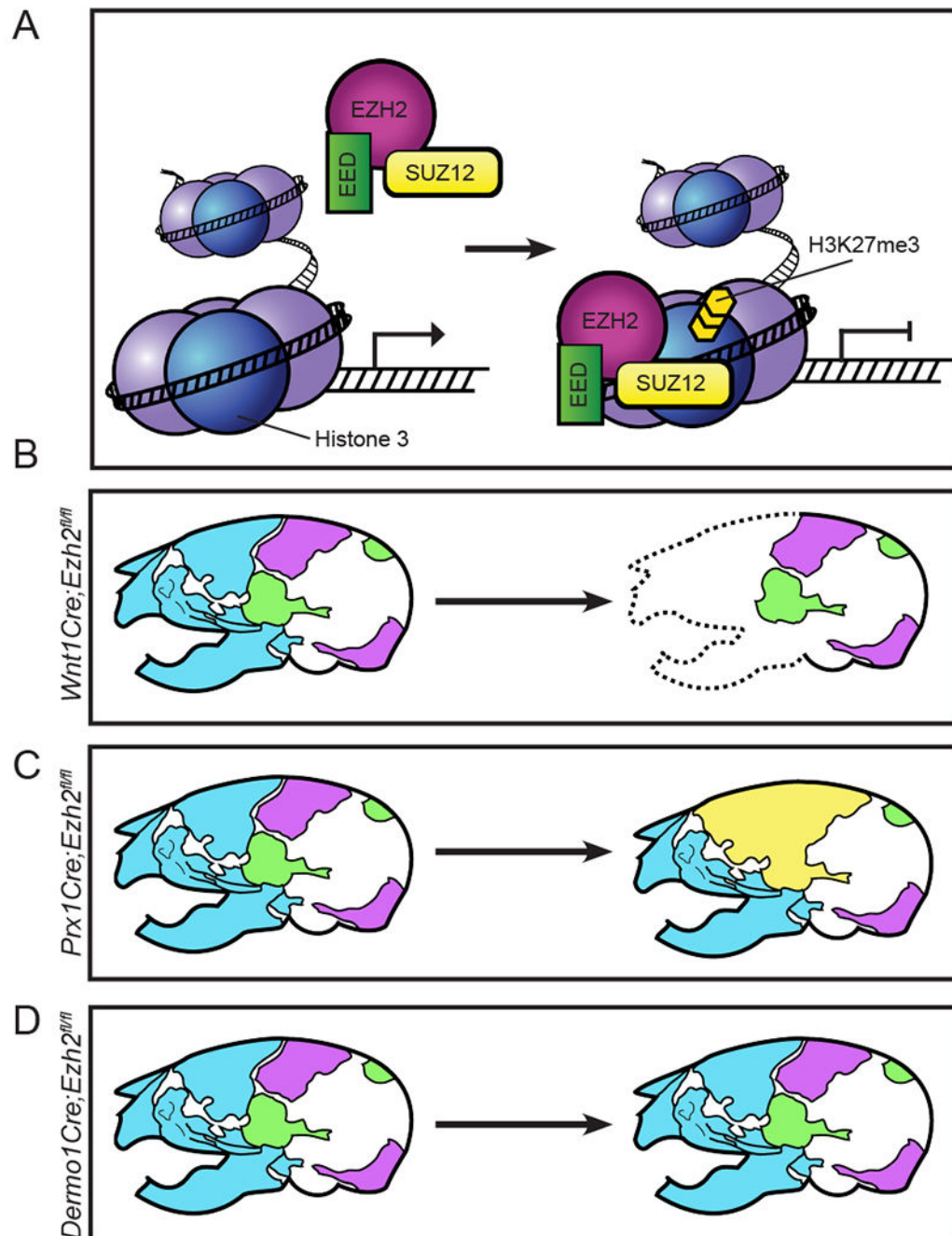


Figure 4: Spatial and temporal requirements for *Ezh2* in skull bone formation.

(A) EZH2 is the catalytic component for PRC2. PRC2 binds to DNA and tri-methylates the 27th lysine on the third histone (H3K27me3). H3K27me3 is typically associated with transcriptional repression. (B) Deletion of *Ezh2* in the CNCC around E8.5 leads to a complete loss of CNCC-derived skull bones (blue). (C) Deletion of *Ezh2* in the PM around E9.5 leads to a fusion of the frontal, parietal, and temporal bones (yellow). (D) Deletion of *Ezh2* in the CNCC and PM around E9.5 results in normal skull bone phenotype.