ROLE OF GLI3 DURING INTRAMEMBRANOUS CALVARIAL BONE DEVELOPMENT

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LIST OF ORIGINAL PUBLICATIONS

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I Rice DP, Connor EC, Veltmaat JM, Lana-Elola E, **Veistinen L**, Tanimoto Y, Bellusci S, Rice R (2010). *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. *Human Molecular Genetics*. 19:3457-3467.

II **Veistinen L**, Takatalo M, Tanimoto Y, Kesper DA, Vortkamp A, Rice DP (2012). Loss-of-function of *Gli3* in mice causes abnormal frontal bone morphology and premature synostosis of the interfrontal suture. *Frontiers in Physiology*. 3:121.

III **Veistinen L**, Åberg T, Rice DP (2009). Convergent signalling through Fgfr2 regulates divergent craniofacial morphogenesis. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*. 312B:351-360.

IV Tanimoto Y, **Veistinen L**, Alakurtti K, Takatalo M, Rice DP (2012). Prevention of premature fusion of calvarial suture in *GLI-Kruppel family member 3* (*Gli3*)-deficient mice by removing one allele of *Runt-related transcription factor 2* (*Runx2*). *The Journal of Biological Chemistry*. 287:21429-21438.

V **Veistinen L**, Mustonen T, Takatalo M, Tanimoto Y, Kesper DA, Vortkamp A, Rice DP. Gli3 acts as a gatekeeper in a Runx2-Ihh regulatory loop to control osteoprogenitor development in intramembranous bone condensations. Manuscript.

ABBREVIATIONS

A	Activator
AER	Apical ectodermal ridge
AP	Anterior-posterior
BAC	Bacterial artificial chromosome
bHLH	basic Helix-Loop-Helix
Bmp	Bone morphogenetic protein
Boc	Brother of Cdo
BSA	Bovine serum albumin
Cbfβ	Core binding factor beta
Cdo	CAM-related/downregulated by oncogenes
Ck1α	Casein kinase 1 alpha
CNC	Cranial neural crest
CNS	Central nervous system
Col1a1	Alpha-1 type I collagen
Dhh	Desert hedgehog
Disp	Dispatched
Dll	Delta-like
Dlx	Vertebrate homologue of *Drosophila* distal-less (*Dll*) gene
DMEM	Dulbecco's minimal essential medium
E	Embryonic day
ECM	Extracellular matrix
En1	Engrailed 1
ENU	*N*-ethyl-*N*-nitrosourea
Eph	Ephrin receptor
EphA4	Ephrin type-A receptor 4
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
FL	Full-length
Fox	Forkhead box
Fuz	Fuzzy
Gas1	Growth arrest-specific 1
GCPS	Greig cephalopolysyndactyly syndrome
Gdf	Growth and differentiation factor
Gli	GLI-Kruppel family member
Gsk3β	Glycogen synthase kinase 3 beta
Hh	Hedgehog
Hip1	Hedgehog interacting protein 1
Ibsp	Integrin-binding sialoprotein
IFT	Intraflagellar transport
Ihh	Indian hedgehog
Jag1	Jagged 1
Kif	Kinesin family member
MSC	Mesenchymal stem cell

Msx	Vertebrate homologue of *Drosophila* muscle segment (*Msh*) gene
NBCCS	Nevoid basal cell carcinoma syndrome
NCAM	Neural cell adhesion molecule
Oc	Osteocalcin
OF	Osteogenic front
Op	Osteopontin
Osx	Osterix
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PHS	Pallister-Hall syndrome
PKA	Protein kinase A
PKC	Protein kinase C
PM	Paraxial mesoderm
Ptch	Patched
PTHrP	Parathyroid hormone related protein
R	Repressor
Rab23	Ras-associated protein 23
Runx2	Runt domain-containing transcription factor 2
Satb2	Special AT-rich sequence-binding protein 2
Shh	Sonic hedgehog
Ski	Skinny hedgehog
Smo	Smoothened
Spop	Speckle-type POZ protein
Sufu	Suppressor of fused
Tgfβ	Transforming growth factor beta
Tgfbr	Transforming growth factor beta receptor
Wnt	Vertebrate homologue of the *Drosophila* Wingless gene
Wt	Wild-type
ZPA	Zone of polarising activity

Gene names are stated in *italics* and protein names in roman. Mouse genes or proteins are in lower case letters and human in upper case letters.

ABSTRACT

The flat bones of the skull, the calvarial bones, develop by intramembranous ossification during which mesenchymal cells first condense and subsequently differentiate into osteoblasts. Sutures separate the calvarial bones and facilitate the synchronized growth of the underlying brain and the calvaria.

Hedgehog (Hh) signalling has an indisputable role in craniofacial development as well as during endochondral ossification. Yet, little is known about its function during intramembranous ossification of the calvarial bones. GLI-Kruppel family member 3 (Gli3) is a zinc-finger transcription factor that mediates Hh signalling. In the absence of Hh ligand Gli3 is proteolytically cleaved into a repressor that inhibits transcription of Hh target genes. Mutations in *GLI3* cause Greig cephalopolysyndactyly syndrome in humans, in which an infrequent, but significant feature is premature fusion of the metopic suture (interfrontal suture in mice). We have used *Gli3* loss-of-function mouse ($Gli3^{Xt\text{-}J/Xt\text{-}J}$) as a model to investigate the effects of aberrant Hh signalling during calvarial development.

In my thesis I describe how loss of *Gli3* causes craniosynostosis of the lambdoid as well as interfrontal sutures in mice. Elevated proliferation and ectopic differentiation of osteoprogenitors underlies this phenomenon. We were able to rescue craniosynostosis in these mice by two mechanisms. Firstly, by elevating fibroblast growth factor (Fgf) signalling in the suture prior to its fusion by imbedding Fgf2 soaked beads in tissue culture. This induced *Twist1* expression, which inhibits function of ectopically expressed *Runx2*. Secondly, craniosynostosis was prevented by genetically reducing *Runx2* activity by generating $Gli3^{Xt\text{-}J/Xt\text{-}J}$;$Runx2^{+/-}$ mice, which normalized elevated levels of Bmp signalling in the affected sutures. We also put forward a model of how Hh signalling helps to maintain the integrity of bone margins during calvarial development. The repressor isoform of Gli3 inhibits Runx2 activity in the early osteoprogenitor cells. Runx2, on the other hand, activates *Ihh* expression in the mature osteoblasts, which then induces osteogenesis by inhibiting the function of Gli3 repressor.

Our findings indicate that Gli3 and Hh signalling have an important role in mediating the location of osteoblast differentiation and the speed of bone formation in the developing calvaria. Uncovering the cellular and molecular mechanisms that underlie normal calvarial development, as well as pathological processes, is a vital step in developing treatment strategies for patients with craniosynostosis.

1. INTRODUCTION

The calvarial bones, the flat bones of the skull, provide vital protection to the brain. They differ from the axial and appendicular skeleton in many ways. Calvarial bones develop mainly by intramembranous ossification and sutures that separate these bones are fibrous joints, which are a unique feature of the skull. Calvarial development occurs mainly during embryogenesis and is carefully synchronized with the development of the neighbouring tissues. Sutures are important sites of calvarial bone growth. In a condition called craniosynostosis sutures fuse prematurely and bone growth terminates at the site of the fusion. Craniosynostosis has fundamental consequences from early stages of a child's life resulting in uncoordinated compensatory craniofacial development including deformity of the calvaria, orbits and the face. Without prompt treatment, obliterated suture also causes neurological symptoms. To date treatment options are limited, involving major surgery, and often, repeated operations during growth. Craniosynostosis occurs in 1/2500 live-births, but aetiology is revealed in only fraction of the cases. It is thus essential to reveal the basic mechanisms of normal development as well as of pathological situations in order to develop more elaborate treatment options.

Hedgehog (Hh) signalling pathway has a vital role in development as well as tumorigenesis. Mounting evidence suggests that Hh signalling pathway has a role in calvarial development, and ultimately in maintaining suture patency, as mutations in several Hh pathway members cause craniosynostosis in humans and in mice. Only preliminary data, however, exists on how Hh signalling pathway functions during calvarial morphogenesis.

The ultimate aim of this thesis was to unravel the role of Hh signalling during calvarial development. We used mouse as a model to study the function of the Hh mediator, GLI-Kruppel family member 3 (Gli3), and the Hh ligand, Indian hedgehog (Ihh), in calvarial development. We also investigated interaction of Gli3 and Runt domain-containing transcription factor 2 (Runx2) and we studied interaction of Gli3 with other signalling pathways: fibroblast growth factor (Fgf) signalling and bone morphogenetic protein (Bmp) signalling.

The following literature review entails the current understanding of embryonic calvarial development focussing on the intramembranous bones, following an overview of the key signalling pathways and transcription factors known to regulate calvarial development. The Hh signalling pathway and Fgf signalling pathway are reviewed in more detail. The role of Hh signalling during limb development is also covered as compared to the intramembranous ossification the role of Hh signalling during endochondral ossification is well known. As this thesis is based solely on findings obtained from mouse experiments all data presented will handle mice unless stated otherwise.

2. REVIEW OF THE LITERATURE

2.1 Calvarial development

The mammalian skull is made up of the neurocranium, which encapsulates and protects the brain, and the viscerocranium, which forms the face, enabling feeding and breathing functions. The neurocranium is composed of the superficial skull vault (calvaria) and the cranial base below the brain, which permits the passage of nerves and blood vessels. Calvaria is constructed from several flat bones, which are separated by the sutures. Bone growth occurs at these sutures, in the margins of the bones, facilitating synchronised growth with the underlying brain.

2.1.1 Anatomy and origin

Mammalian calvaria is mainly made up of five bones (Figure 1A). The paired frontal bones are anteriorly and are apically separated by the interfrontal suture (called metopic suture in humans). The coronal suture separates frontal bones from the parietal bones laterally. The sagittal suture lies between the pair of parietal bones apically. The single interparietal bone is situated posteriorly to the parietal bones, separated bilaterally by the lambdoid sutures. The intramembranous interparietal bone later fuses with the supraoccipital bone, which is formed by endochondral ossification, to form the posterior wall of the calvaria. In humans, the squamous part of the occipital bone represents the intramembranous interparietal bone, which fuses early with the supraoccipital bone forming the occipital bone. The squamous part of the temporal bone (squamosal) and the greater wing of the sphenoid bone (alisphenoid) also contribute to the lateral walls of the skull vault.

The majority of the calvarial bones are formed by intramembranous ossification between the dermal mesenchyme and the meninges, which separate the calvaria from the brain. The outermost meningeal layer is the dura mater made of dense fibrous tissue. External periosteal layer of the dura mater, the pericranium, is the endosteum covering the internal surface of the calvaria. It is continuous with the fibrous tissue in the calvarial sutures.

Calvarial bones develop from mesenchymal cells, which are derived from two distinct cell populations: the cranial neural crest (CNC) and the cephalic paraxial mesoderm (PM) (Figure 1B) (Couly et al., 1993; Jiang et al., 2002). Neural crest cells are a multipotent population of migratory cells, which form at the boundary between neural plate and non-neural ectoderm along the anterior-posterior (AP) length of the developing embryo during neurulation. PM is part of the mesoderm layer formed during gastrulation.

The frontal bones develop from the CNC, while the parietal bones are derived from the PM. The interparietal bone is a composite, its central portion being derived from the CNC and the lateral portions from the PM. The neural crest-mesoderm boundary lies between the frontal bone and the coronal suture, the coronal suture originating from the PM. The interfrontal and sagittal suture mesenchyme is derived from the CNC, while the lambdoid suture mesenchyme is derivative of the PM (Yoshida et al., 2008).

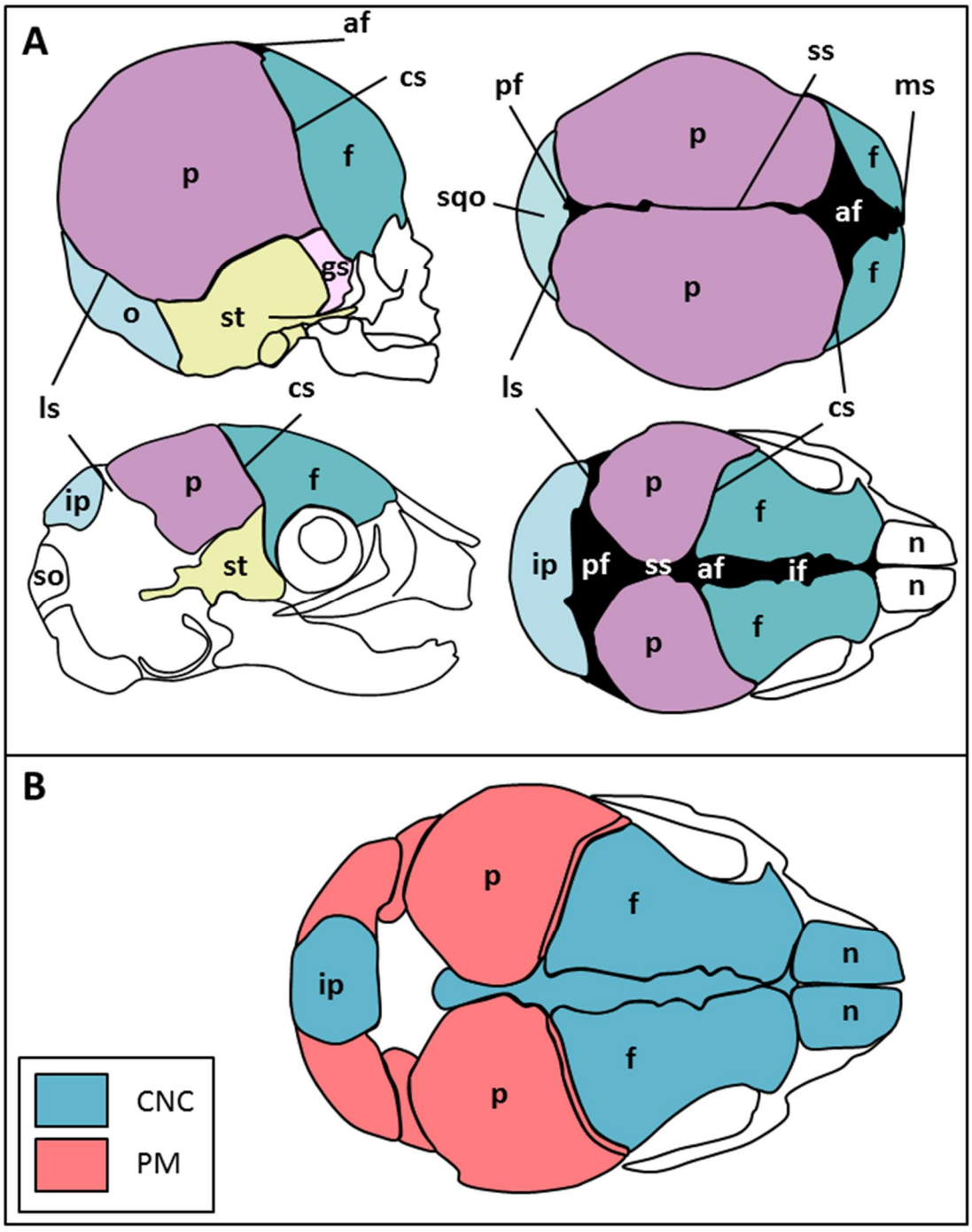

Figure 1. A) Schematic view of the anatomy of the human (top) and mouse (below) calvaria. af, anterior fontanelle; cs, coronal suture; f, frontal bone; gs, greater wing of sphenoid bone; if, interfrontal suture; ip, interparietal bone; ls, lambdoid suture; ms, metopic suture (interfrontal); n, nasal bone; o, occipital bone; p, parietal bone; pf, posterior fontanelle; so, supraoccipital bone; sqo, squamous part of occipital bone; ss, sagittal suture; st, squamous part of temporal bone.
B) Schematic view depicting the origin of calvarial bones in mice. CNC, cranial neural crest; PM, paraxial mesoderm.

2.1.2 Skeletal patterning of the calvaria

Calvarial bone development involves several phases: skeletal patterning and initial ossification of the bone anlages, followed by secondary growth of the bones at the bone margins as well as modelling and remodelling of the bones. During skeletal patterning, mesenchymal cells of CNC and PM origin first migrate to the frontonasal region by embryonic day (E) 9.5, where they form skeletogenic mesenchyme condensations. Most research on calvarial development has focused on the frontal and parietal bones. Their development is initiated at the supraorbital ridge where CNC and PM cells form rostral and caudal domains, respectively, between E10.5 and E11.5. From here, mesenchymal cells further migrate apically between the pre-existing dermal and meningeal mesenchymal layers and form skeletogenic mesenchyme condensations. Frontal bone precursors first move in caudal-to-rostral direction at E11.5 – E12.5 followed by apical migration at E13.5 (Yoshida et al., 2008).

Development of the interparietal bone remains ambiguous. In mammals, interparietal is formed from two pairs of ossification centres; the CNC derived medial pair and the PM derived lateral pair that fuse to form a single bone (Koyabu et al., 2012). CNC cells that contribute the interparietal bone migrate from the rostral hindbrain at E9.5 and localise to the surface ectoderm at E10.5. At E13.5 CNC cells are detected in the osteogenic mesenchyme, between the dermal and meningeal layers at the level of the cerebellum (Jiang et al., 2002; Yoshida et al., 2008).

CNC cells that form the calvarial structures arise at the cephalic region of the forebrain, the rostral midbrain and the hindbrain. They undergo epithelial-mesenchymal transition and subsequently migrate ventrally to colonize the frontonasal region. But what determines calvarial bone shape and location remains largely unknown. Hox homeodomain family of transcription factors that are vital regulators of skeletal patterning along the AP axis of the embryo are not expressed in the calvaria (Kmita and Duboule, 2003). On the contrary, ectopic expression of *Hoxa2* in the calvaria of avian embryo has been shown to inhibit calvarial bone development (Creuzet et al., 2002). There is evidence that some neural crest cells are precommitted to a specific lineage before the onset of migration, but majority differentiate as a result of the signals that they encounter during migration (Krispin et al., 2010; McKinney et al., 2013). Although during skeletogenesis of many craniofacial bones epithelial-mesenchymal interaction initiates condensation formation (Tyler and Hall, 1977; Hall, 1981; Tyler, 1983), there is no evidence to date of this phenomenon occurring in the calvaria. Signals from the dura mater are, however, known to influence calvarial bone development (Greenwald et al., 2000a; Greenwald et al., 2000b).

Factors that influence specifically calvarial bone condensation formation are yet unidentified to a large extent. Most data concerning condensations are obtained from the limb or craniofacial skeleton. Location-specific signals initiate condensation formation. Although the source and nature of these signals is largely unknown transforming growth factor β (Tgfβ) is able to initiate condensation formation by activating the glycoprotein fibronectin, which in turn regulates neural cell adhesion molecule (NCAM). NCAM is also a glycoprotein expressed on the surface of mesenchymal cells and it is important in maintaining the condensation state. Tgfβ also stimulates *N-cadherin* expression in mesenchymal cells that recruits cells to the condensations (Chimal-Monroy and Díaz de León, 1999). Condensation grows through cell proliferation. Proliferation is actively downregulated by cell surface proteoglycan receptor, Syndecan-3 that inhibits NCAM by binding to fibronectin and permitting differentiation of the mesenchymal cells. *NCAM* expression is yet again associated with osteoblast differentiation, while its expression must be downregulated during chondrogenesis (reviewed in Hall and Miyake, 2000).

2.1.3 Osteoblast differentiation and bone formation

During the second phase of calvarial bone development the mesenchymal cells of the condensations differentiate into osteoblasts. Differentiation begins at the site of the initial condensation in the supraorbital region, where preosteoblasts are first detected at E12.5 (Rice et al., 2003). From here, the differentiation wave proceeds apically, while growth of the bone anlage continues by proliferation of preosteoblasts. Mineralisation of the bone does not begin until the upgrowth of the bone anlage is complete, at E14.5. It is also initiated at the supraorbital region and proceeds in radial pattern (Ishii et al., 2003; Yoshida et al., 2008).

Once a miniature form of the bone is formed, the growth of the calvarial bones toward each other continues at the sutural margins of the bone rudiments, in the osteogenic fronts (OF) (Figure 2). This marks the third phase of calvarial bone development that also continues postnatally. The undifferentiated cells of the sutural mesenchyme were originally thought to account for the growth of the bones by getting incorporated into the OF after attaining osteoblastic fate. However, evidently only a fraction of cells of the sutural mesenchyme differentiate into osteoblasts (Lana-Elola et al., 2007). Mesenchymal osteogenic precursor cells that form the OFs are also derived from the supraorbital region of the bone rudiment from where they migrate towards the suture mesenchyme until an appropriate stimulus inhibits their progression (Yoshida et al., 2008; Ting et al., 2009; Roybal et al., 2010). Proliferation of these cells in the OF does also account for bone growth (Lana-Elola et al., 2007). It, however, is not clear whether OF is the leading edge of the migrating and proliferating cells of the bone rudiment, or if OF is established by a secondary wave of osteoblast induction and proliferation once the osteogenic mesenchyme has migrated apically (Holmes et al., 2009).

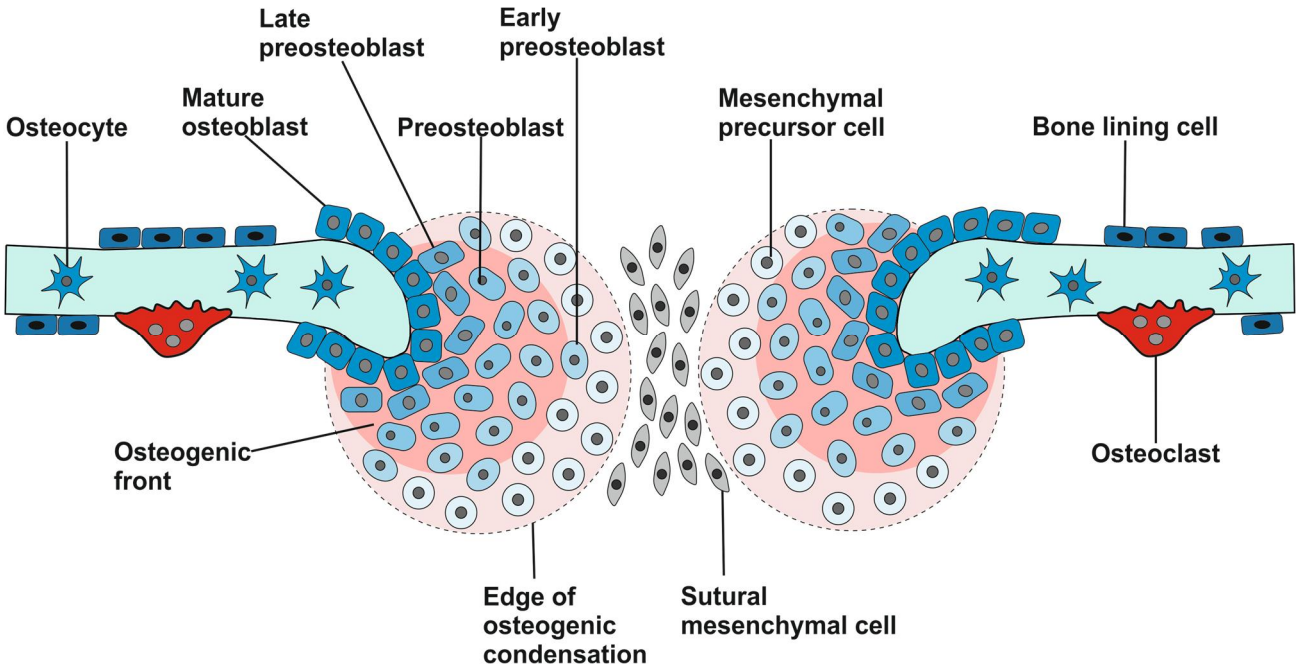

Figure 2. Schematic view of the osteogenic fronts (OF) and the suture. OFs are at the sutural edge of the developing calvarial bones, where active growth of the bone occurs. Mesenchymal stem cells migrate to the leading edge of the osteogenic condensation and begin to differentiate towards mature osteoblasts in the OFs. Mature osteoblasts reside on the surface of the bone. Part of the mature osteoblasts differentiates further into osteocytes or bone lining cells. Osteoclasts participate in modelling and remodelling of the bones. Sutural mesenchymal cells lie between two adjacent calvarial bone margins.

Osteoblasts and chondroblasts are both derived from common mesenchymal precursor cells, which first differentiate into skeletal precursors, characterised by *Sox9* expression (Figure 3) (Akiyama et al., 2005). The chosen differentiation lineage these skeletal precursors follow depends on the given transcriptional signals these precursors receive. Osteoblast differentiation can be divided into three stages: mesenchymal precursor cells, preosteoblasts and osteoblasts. Preosteoblasts are a heterogeneous group of proliferating cells. All preosteoblasts express *Runx2* and at more advanced stage they also express *osterix (Osx*; also known as *Sp7)*. *Osteopontin* (*Op*) is also expressed by preosteoblasts. Mature osteoblasts do not proliferate and they express high levels of *osteocalcin* (*Oc*). Osteoblasts secrete extracellular matrix (ECM), rich in type I collagen, called osteoid that mineralises through accumulation of hydroxyapatite, which is calcium phosphate. Part of the osteoblasts become trapped within the bone matrix and differentiate into osteocytes, which account for about 95% of cells in the mature bone tissue. Osteocytes regulate bone remodelling in response to both mechanical and hormonal signals. Rest of the osteoblasts either undergo apoptosis or become inactive bone-lining cells (reviewed by Bonewald, 2011).

In addition to osteoblast-mediated bone formation, osteoclasts are critical in remodelling the three-dimensional microarchitecture of the calvarial bones (Takahashi et al., 2002). Osteoclasts are haematopoietically derived cells that resorb bone.

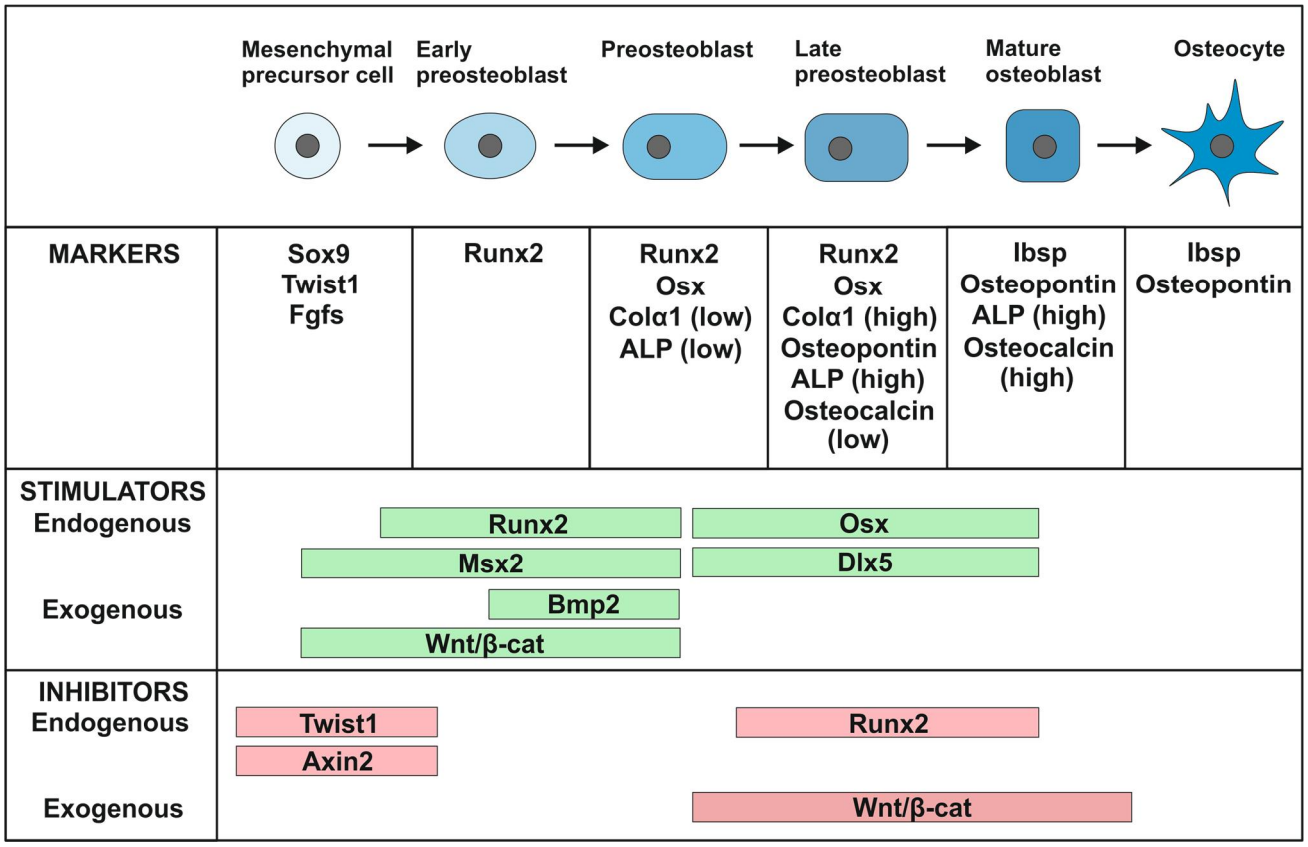

Figure 3. Schematic diagram of osteoblast differentiation. Osteoblasts differentiate from multipotent mesenchymal precursor cells. Mesenchymal cells first differentiate into preosteoblasts, which are a heterogeneous group of proliferating cells before reaching the mature osteoblast stage. Part of the osteoblasts differentiates further into osteocytes. Different factors either stimulate or inhibit osteoblast differentiation at different stages (refer to the text for details).

2.1.3.1 Role of Runx2 in osteoblast differentiation

Runx2 belongs to the Runx-family, consisting of Runx1 – 3, which are master regulators of different cell lineages. These transcription factors form heterodimers with transcriptional co-activator core binding factor β (Cbfβ) and bind to a consensus DNA sequence. Runx2 is a master osteogenic transcription factor as it is the most upstream molecule known in osteoblast differentiation and *Runx2* null allele mice show complete lack of osteoblasts (Komori et al., 1997; Otto et al., 1997). Haploinsuficiency of *Runx2* in mice and humans results in delayed closure of the sutures and fontanelles, which is characteristic of cleidocranial dysplasia in humans (Otto et al., 1997; Mundlos et al., 1997). On the contrary, when *Runx2* expression is activated early in the mesenchyme cells at E9.5, not only do calvarial osteoblasts differentiate prematurely, but sutures also fail to form resulting in multiple craniosynostoses (Maeno et al., 2011).

Runx2 is proposed to be the key coordinator of cell lineage specification, proliferation and growth. This is achieved in part by regulation of ribosomal RNA genes and ribosomal biogenesis (Young et al., 2007). Interestingly, Runx2 concomitantly inhibits maturation of osteoblasts, keeping preosteoblasts in a proliferative state and maintaining a supply of preosteoblasts. Unless *Runx2* expression is downregulated before the final stage of osteoblast differentiation, osteopenia results (Liu et al., 2001).

Runx2 is able to induce the expression of major bone matrix protein genes; *alpha-1 type I collagen* (*Col1a1*), *Op, integrin-binding sialoprotein* (*Ibsp*) and *Oc* in preosteoblasts, but Runx2 is not evidently essential for their expression (Komori, 2005). Runx2 regulates Osx, another transcription factor that is required to commit osteoblastic precursors to become osteoblasts and which later regulates terminal osteoblast differentiation (Nakashima et al., 2002). Osteoblasts also fail to differentiate in *Osx* deficient mice. Osx is downstream of Runx2 and *Osx* fails to be expressed in *Runx2* null allele mice, but it is also regulated by Runx2-independent mechanism. Bmp2 is able to stimulate *Osx* expression by activating Dlx5 in the absence of Runx2 in cell culture (Lee et al., 2003b).

Runx2 has two major isoforms, which are activated by individual promoters: Runx2-I (product of the proximal promoter (P2)) and Runx2-II (product of the distal promoter (P1)) (Xiao et al., 1998). The amino acid sequence of Runx2-I is almost identical to that of Runx2-II. The two isoforms share a common 509-amino-acid sequence, but a 5-amino-acid N-terminal sequence of Runx2-I differs from the 19-amino-acid N-terminal sequence of Runx2-II (Stock and Otto, 2005). The effect of this distinct N-terminal amino acid composition on their function in bone development is not well understood. *Runx2-I* is proposed to be involved in the initial commitment step as it is expressed in the sutural mesenchyme and in the preosteoblasts of the OF. *Runx2-II*, on the other hand, is expressed by more mature preosteoblasts that already express *Op* and *Oc*, and by mature osteoblasts. It is, thus, thought to act during later stages of osteoblast differentiation (Park et al., 2001; Choi et al., 2002).

2.1.4 Suture development

Sutures are fibrous joints consisting of mesenchymal cells and fibroblasts between two calvarial bone ends (Figure 2) (Opperman, 2000). Sutures are growth centres and thus need to remain patent. They also facilitate childbirth by allowing reshaping of the head. In mice, the posterior part of the interfrontal suture is the only suture that fuses this occurring during the first month of post-natal life. Other calvarial sutures remain patent (Bradley et al., 1996). In humans, the metopic suture also fuses first. It begins closing during the second year and is fused by 7 years of age. Most human calvarial sutures fuse at the third or fourth decade of life.

Sutures not only differ in fusion timing. They can be broadly divided into two categories depending on the conformation and location of the suture. The sagittal, interfrontal and lambdoid sutures belong to the first group, in which the sutural margins of the adjacent bones face each other. Bones are initially widely separate and the mesenchymal gap between them gradually narrow forming the suture (Morris-Kay and Wilkie, 2005). These sutures overlie interlobar spaces of the brain. The interfrontal and sagittal sutures overlie the midline between the cerebral hemispheres and the olfactory lobes, while the lambdoid suture is superior to the area between the cerebral hemispheres and the cerebellum.

The coronal suture represents the second group. The suture morphology differs in that the parietal bone margin overlaps the frontal bone margin. This overlap is present from the initial stage of mesenchymal condensation formation at E9.5 in mice. This neural crest–mesoderm boundary is initially situated over the telencephalon–diencephalon border. However, when cerebral hemispheres grow they extend caudally beneath the suture so that in the final anatomical relationship the suture lies over the cerebral hemisphere (Jiang et al., 2002). The coronal suture is thus not situated over an anatomical landmark of the brain.

Unanswered questions concerning the suture formation still remain. Is the suture formation prepatterned or do signals arising from neighbouring tissues inhibit the suture mesenchyme from ossifying? What governs the location of the sutures? It was previously thought that the suture formation was initiated when two edges of the growing bones approached each other and that sutural mesenchyme had osteoblastic fate. Novel evidence, however, indicates that suture morphogenesis may be traced to a considerably earlier stage of calvarial development (E10.5–12.5 in mice), preceding the expansion of adjacent skeletal rudiments. *Msx1* and *Msx2* are required to be expressed in the CNC from E10.5 onwards to inhibit ossification in a normally non-osteogenic, CNC-derived cell layer and thus prevent heterotopic ossification in the interfrontal suture (Roybal et al., 2010). By fate-mapping studies, Deckelbaum et al. (2012) also showed that mesenchymal cells of the PM origin migrate between E11 and E13.5 from the supraorbital region to form the coronal suture. The terminal fate of this sutural mesenchyme during physiological or pathological suture fusion remains unknown.

It is also noteworthy that sutures are not just passive, mechanical barriers between the bones (Lenton et al., 2005). Coronal suture has recently been proposed to act as a growth centre that actively regulates proliferation and differentiation rate of osteoprogenitors in the OFs (Deckelbaum et al., 2012).

New data indicate that postnatally sutures become an indispensable source of mesenchymal stem cells (MSCs), which give rise to the periosteum, dura as well as osteoblasts in the calvaria and are vital for tissue turnover and injury repair. Destruction of these MSCs postnatally leads to synostosis of all of the calvarial sutures (Zhao et al., 2015).

2.1.5 Abnormalities in calvarial development

Abnormalities affecting calvarial bone development, discussed below, include premature suture fusion, i.e. craniosynostosis, which is characterised by heterotopic ossification and, on the other hand, delay in suture closure involving reduced ossification.

2.1.5.1 Craniosynostosis

Craniosynostosis is a pathological, heterogeneous condition where one or more of the calvarial sutures fuse prematurely. Fusion may be partial or complete, but never the less, it terminates growth at the site of the fusion leading to asymmetrical growth of the skull as other sutures compensate,

while trying to accommodate the expansion of the underlying brain. Neurological symptoms do, however, develop unless treatment is initiated. Currently, treatment inevitably involves surgical emancipation of the fused suture. The affected suture also tends to re-ossify as it is impossible to eliminate the aberrant process that originally led to synostosis.

The incidence of craniosynostosis is estimated to be 1 in 2000 to 3000 live births, including the syndromic cases, as well as the more common non-syndromic cases (Wilkie and Morriss-Kay, 2001; Lenton et al., 2005). The sagittal suture is most frequently involved (40–55%) followed by the coronal suture (20–25%) and the metopic suture (5–15%). The lambdoid synostosis is rare (0–5%), even the multiple suture synostosis is more common (5–15%) (Cohen and MacLean, 2000; Rice, 2008a).

The aetiology of the non-sydromic craniosynostosis remains poorly understood, although they account for about 70% of the cases. Mutations in *EphrinA4* (*EFNA4*), *Fibroblast growth factor receptors 1, -2 and -3* (*FGFR1, -2, -3)* and *TWIST1* have been associated with non-syndromic craniosynostosis (Merrill et al., 2006; Lattanzi et al., 2012). Craniosynostosis is a clinical feature of at least 150 syndromes. Mutations in *FGFR1, -2, -3, TWIST1, EphrinB1* (*EFNB1), Msh homeobox 2* (*MSX2)* and *RAS-associated protein 23* (*RAB23*) unequivocally cause craniosynostosis (Passos-Bueno et al., 2008). *Fibrillin1* (*FBN1*) (Sood et al., 1996), *Transforming growth factor β receptor type I and II* (*TGFBR1, -2*) (Loeys et al., 2005), *Cytochrome p450 reductase* (*POR*) (Flück et al., 2004) and *GLI3* (McDonald-McGinn et al., 2010; Hurst et al., 2011) have also been occasionally associated with premature suture fusion, but penetrance has been low. Mutations in these 12 genes account for approximately 30% of the syndromic cases (Cohen and MacLean, 2000; Rice, 2008a; Passos-Bueno et al., 2008). At least another 10% of the syndromic cases are explained by the chromosomal alterations (duplications, deletions, copy number variants etc.) (Passos-Bueno et al., 2008). Genetic analysis of syndromic craniosynostosis has revealed many of the important pathways participating in suture development and closure (Passos-Bueno et al., 2008; Ting et al., 2009).

FGFR-mutations cause most of the syndromic craniosynostoses. They are inherited autosomally dominantly and confer gain-of-function to the mutated receptor. Several de novo *FGFR*-mutations originate exclusively paternally and incidence increases with father's age (Moloney et al., 1996; Glaser et al., 2000). FGFR-related craniosynostoses are discussed in more detail in the chapter 2.3, regarding the Fgf signalling pathway (refer to page 33). Mutations in *TWIST1* and *MSX2* are also autosomally dominantly inherited, while *EFNB1*-mutations, causing craniofrontonasal syndrome, are linked to the X-chromosome and paradoxally affecting mostly females (~95%). Carpenter syndrome, caused by mutations in *RAB23,* is the only autosomally recessive condition.

Non-syndromic craniosynostosis may also be caused by extrinsic factors such as metabolic causes. Vitamin D deficiency and hyperthyroidism, for example, have both been associated with premature suture fusion (McCarthy and Reid, 1980; Hirano et al., 1995). Also some brain malformations, such as, microcephaly and encephalocele, predispose to craniosynostosis.

It is important to distinguish deformational plagiocephaly from craniosynostosis. Deformational plagiocephaly refers to asymmetrical head shape caused by repeated pressure to the same area of the head, although sutures remain patent. Intrauterine pressure, muscular torticollis, prematurity and back sleeping have all been associated with abnormal head shape. Experimental studies have revealed that calvarial mesenchymal cells do respond to mechanical strain. Mesenchymal cells express markers of proliferation and differentiation in response to tensile or cyclic loading, while compressive loading favors osteogenesis (Kopher and Mao, 2003; Collins et al., 2005; Vij and Mao, 2006).

2.1.5.2 Delay in suture closure

Another anomaly affecting the calvaria is delayed suture closure, which is an opposite phenomenon to premature suture fusion. Delayed suture closure is a feature of cleidocranial dysplasia, caused by heterozygous loss-of-function mutation in *RUNX2* (Otto et al., 1997; Mundlos et al., 1997). In cleidocranial dysplasia, intramembranous ossification of calvarial as well as clavicle bones is defected. All the calvarial bones are smaller in size and the fontanelles are larger.

Related condition is enlarged parietal foramina, which is an inherited condition caused by mutation in either *MSX2* or *ALX4* (Wilkie et al., 2000; Mavrogiannis et al., 2001). It is characterised by symmetrical and circular enlarged openings in the two parietal bones. The size of the foramen varies, ranging from a few millimetres to several centimetres wide. The condition is due to impaired parietal bone ossification. Parietal foramina are normally seen in parietal bones during the foetal period, but they usually close by the fifth month of pregnancy.

2.1.6 Molecular regulation of calvarial development

There are several signalling molecule families that direct the development of different organs and which are conserved among different species. A signalling family consists of extracellular ligands, cell membrane receptors, intracellular signalling factors, transcription factors, co-factors and antagonists. These molecule families are named after soluble growth factors that control the activities of cells through intercellular communication even over long distances. The growth factor signal binds to its receptor on the cell surface in the recipient cell, which activates an intracellular transduction cascade leading to target gene transcription in the nucleus.

The role of the important growth factor families that regulate calvarial development is described below, apart from Hh and Fgf signalling pathways, which have been dedicated their own chapters owing to their important role in this thesis. Following the growth factor families, important transcription factors in calvarial development are introduced.

2.1.6.1 TGFβ Superfamily signalling pathways

The Tgfβ superfamily includes Tgfβs, Bmps, and Growth and differentiation factors (Gdfs) that all mediate calvarial development at multiple stages. These Tgfβ superfamily ligands form dimers that bind to heterodimeric receptor complexes that consist of type I and type II receptor subunits with serine/threonine kinase domains. Ligand binding activates type II receptor, which in turn activates the type I receptor by phosphorylation, initiating a Smad-dependent signalling cascade that induces or represses transcriptional activity. These Smad proteins are intracellular mediators of the Tgfβ superfamily signalling, which are also phosphorylated upon activation (Whitman 1998; Sakou et al., 1999; Massagué and Chen, 2000). Smads 1, 5 and 8 mediate Bmp signalling, while Smads 2 and 3 mediate Tgfβ signalling (Massagué and Chen, 2000; Ross and Hill, 2008). Smads 6 and 7 are inhibitory Smads, with Smad6 regulating Bmp signalling and Smad7 Tgfβ signalling (Massagué and Chen, 2000).

Tgfβ and Bmps are both involved in the earliest step of ossification, in initiating the skeletogenic condensations. Bmps are associated with growth of the condensations i.e. recruitment of cells, while noggin, an antagonist of Bmps, has an important role in restricting condensation size and duration. In the absence of noggin condensations become hyperplastic. Since neither the initial condensation phase, nor the cell proliferation is affected, it has been proposed that the expansion of the condensation is due to increased recruitment of cells (Hall and Miyake, 2000). Tgfβ signalling also plays a vital role in mesenchymal osteoprogenitor maintenance and proliferation during bone development (Derynck and Akhurst, 2007). In the frontal bone anlage it has been shown to control

proliferation by activating Fgf signalling (Sasaki et al., 2006). Conditional inactivation of the receptor *Tgfbr2* in the calvarial mesenchyme in mice leads to complete absence of interparietal and parietal bones as well as hypoplastic frontal bones (Spagnoli et al., 2007).

Tgfβ superfamily also participates in determining the osteogenic fate of the condensations. Both Tgfβ1 and Bmp2 stimulate *Runx2* expression and function (Ryoo et al., 2006). Bmp2 specifically regulates *Runx2-II* and *Osx* expression by activating Dlx5 (Lee et al., 2003a; Lee et al., 2003b). It is important to note that neither *Dlx5* nor *Osx* are expressed in the skeletal primordium of *Runx2*$^{-/-}$ mice indicating that Bmp signalling is involved in regulating a later stage of osteoblast differentiation (Ryoo et al., 2006). Bmp and Tgfβ signalling also promote osteoblast differentiation by stimulating the expression of *Msx1, Msx2* and *Dlx5* (Bei and Maas, 1998; Sasaki et al., 2006). Overactivation of Bmp signalling, however, directs cells to differentiate toward the chondrogenic lineage, by inducing *Sox9* expression, while inhibiting *Runx2* and *Op* expression (Abzhanov et al., 2007).

Bmp2, *Bmp4* and *Bmp7* are all expressed in the frontal bone rudiment (Kim et al., 1998; Rice et al., 1999). Conditional deletion of *Bmp2*, *Bmp4* and *Bmp7* from the CNC leads to development of very truncated frontal bones (Bonilla-Claudio et al., 2012). Bonilla-Claudio et al. (2012) showed that *special AT-rich sequence-binding protein 2* (*Satb2*) is a direct target of Smad1/5. Satb2 enhances osteoblast lineage development by regulating *Runx2* and *activating transcription factor 4* expression. *Bmp2* is also expressed in the OFs of the calvarial bones where it controls differentiation and proliferation of osteogenic mesenchymal cells. It has been proposed that differentiation of the proliferative preosteoblasts in the OF into more mature osteoblasts in the mineralizing bone involves a reduction in phosphorylated Smad1/5/8, which indicates decreased Bmp signalling (Ting et al., 2009). On the other hand, conditional inactivation of *Tgfbr2* from the CNC cells has indicated that Tgfβ signalling is required for the terminal osteoblast differentiation in the frontal bones (Sasaki et al., 2006).

All members of the Tgfβ superfamily have been implicated in calvarial suture closure. Bmp2, Bmp4 and Bmp7 have been proposed to promote suture fusion by activating their downstream targets Dlx5 and Msx2 (Holleville et al., 2003; Rice et al., 2003). Recent evidence has shown that enhanced Bmp signalling through the Bmp type IA receptor in the CNC causes craniosynostosis of the interfrontal suture in mice (Komatsu et al., 2013).

The antagonist *Noggin* is expressed in the sutural mesenchyme of patent sutures enforcing suture patency by blocking the positive-feed-back loop of Runx2-induced Bmp2 stimulating *Runx2* expression. Noggin is downregulated by Fgf signalling during suture fusion (Warren et al., 2003). Noggin can also bind to Gdf6 and inhibit its signalling ability (Chang and Hemmati-Brivanlou, 1999). Gdf6 participates in coronal suture development, as in *Gdf6* null allele mice, coronal suture is not established, the primordia of frontal and parietal bones being fused already before the onset of ossification (Clendenning and Mortlock, 2012; Settle et al., 2003). The function of *Gdf6* is not yet fully revealed, but it is known that it is expressed in the frontal bone primordia.

Gain-of-function mutations in the *TGFβ* receptors *TGFBR1* and *TGFBR2* in humans cause Loeys-Dietz syndrome, an autosomal dominant aortic aneurysm syndrome, of which many patients have craniosynostosis (Roth et al., 1997; Loeys et al., 2005). These mutations evidently increase cellular response to TGFβ. Increased Tgfβ signalling has also been associated with posterior interfrontal suture fusion in rodents and downregulation of Tgfβ signalling is required for suture patency (Opperman et al., 1993; Opperman et al., 1995). Smad7 maintains suture patency by downregulating Tgfβ signalling by inhibiting Smad2/3 activation (Zhou et al., 2014). Tgfβ signalling has also been suggested to regulate suture closure through the Erk-MAPK pathway. Opperman et al. were able to hinder *Tgfβ2*-induced suture closure by an Erk kinase inhibitor, which blocked Erk1/2 function (Opperman et al., 2006).

2.1.6.2 Canonical Wnt signalling pathway

Three wingless-type MMTV integration site (Wnt) signalling pathways have been characterised: the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. The canonical Wnt pathway utilises β-catenin through which it regulates gene transcription, while the non-canonical planar cell polarity pathway, which controls the cytoskeleton and the non-canonical Wnt/calcium pathway that regulates calcium inside the cell, are both β-catenin independent pathways (reviewed by Nusse, 2012).

The canonical Wnt signalling pathway is activated by the binding of the Wnt-protein ligand to a transmembrane receptor called Frizzled to activate intracellular cascade that is transduced by unphosphorylated β-catenin. β-catenin is stabilised and enters the nucleus where it binds to Tcf/Lef transcription factors to activate the expression of Wnt-target genes. In the absence of Wnt-ligands, β-catenin is phosphorylated and degraded by a destruction complex, which includes axin, adenomatosis polyposis coli, protein phosphatase 2A, glycogen synthase kinase 3 (Gsk3), and casein kinase 1α (Ck1α). Activated Frizzled receptor interacts with another transmembrane protein, LRP, which in turn binds to axin and thus restricts the function of the destruction complex (Logan and Nusse, 2004; Moon et al., 2004; MacDonald et al., 2009).

Canonical Wnt signalling has an important role in calvaria development from initial stage onwards as conditional deletion of *β-catenin* from neural crest cells results in a complete loss of calvarial bones and dramatic brain malformation indicating that β-catenin has a role in neural crest cell survival and/or differentiation (Brault et al., 2001).

In mesenchymal condensations Wnt/β-catenin signalling is required to determine osteoblastic fate. When β-catenin is absent from the condensations cells that normally differentiate into dermis and calvarial bones acquire cartilaginous fate (Day et al., 2005; Tran et al., 2010). Normally β-catenin represses chondrogenesis by activating *Twist1* expression in the calvarial mesenchymal condensations, which in turn inhibits *Sox9* expression (Goodnough et al., 2012). Although there is evidence that Wnt/β-catenin signalling is able to stimulate *Runx2* expression, it is not obligatory for *Runx2* expression. When *β-catenin* is conditionally deleted in the head from E11.5-12.5, *Runx2* is expressed, although mesenchymal cells fail to differentiate further into functional osteoblasts (Gaur et al., 2005; Tran et al., 2010). β-catenin also promotes proliferation of osteoprogenitors by activating cyclin D (Mirando et al., 2010).

On the other hand, high levels of Wnt/β-catenin signalling evidently inhibit ossification of embryonic calvarial mesenchyme. During posterior interfrontal suture closure, which occurs by endochondral ossification, there is a sharp decrease in canonical Wnt signalling and this decrease is not detected in the patent sagittal suture (Quarto et al., 2010). Endochondral ossification is proposed to be initiated by decrease in *Twist1* expression due to downregulation of Wnt signalling leading to chondrogenesis (Behr et al., 2010).

Axin2 is a transcriptional target of Wnt signalling and also functions as an inhibitor of Wnt signalling, among others. The role of Axin2 in calvarial development is controversial. It is expressed in CNC cells, in the OFs and in the sutural mesenchyme. It has been shown to repress osteoprogenitor proliferation and osteoblast differentiation (Yu et al., 2005). Deletion of *Axin2* in mice causes premature fusion of the interfrontal suture at an early postnatal stage, evidently due to elevated Bmp signalling, which in turn controls β-catenin activity (Yu et al., 2005; Liu et al., 2007). To support this, feedback regulation has also been shown to exist between Runx2 and Axin2 as in mesenchymal progenitor cells Axin2 inhibits Wnt/β-catenin signalling, and thus inhibits Wnt/β-catenin mediated *Runx2* activation. Runx2, on the other hand, represses *Axin2* transcription during early stages of osteoprogenitor cell commitment (McGee-Lawrence et al., 2013). There is, however, novel and contradicting evidence to show that *Axin2* deletion has an opposite effect on frontal bone

development leading to a delay in ossification and absence of posterior interfrontal suture fusion (Behr et al., 2013).

Wnt5a is a Wnt-ligand that is able to inhibit as well as activate Wnt/β-catenin signalling depending on the location and timing. *Wnt5a* overexpression in mice causes induction of Wnt/β-catenin signalling in the meninges, which has been proposed to cause reduced calvarial ossification at E18.5 (van Amerongen et al., 2012).

In more mature bone, canonical Wnt signalling has been shown to affect bone homeostasis and influence bone mass by activating osteoblasts and inhibiting osteoclasts (Glass et al., 2005). Disruption of β-catenin signalling in osteoblasts causes osteopenia and increased numbers of osteoclasts in postnatal mice (Holmen et al., 2005).

2.1.6.3 Notch signalling pathway

The mammalian notch signalling pathway consists of four single-pass transmembrane receptors (Notch1-4) and five canonical ligands: Jagged 1 (Jag1), Jag2, Delta-like 1 (Dll1), Dll3 and Dll4. Notch ligands are also transmembrane proteins and so the receptor activation requires direct cell-to-cell contact. Ligand binding activates the receptor and an intracellular part of the receptor is cleaved off and translocated to the nucleus, where it binds to the DNA-binding protein CBF1/Suppressor of Hairless/LAG-1 and activates the transcription of Notch target genes (reviewed in Andersson et al., 2011).

Notch signalling has a vital role during development in boundary formation and cell fate determination. Increased activation of Notch pathway in the neural crest cells in mice (Wnt1Cre;Rosa(Notch)) causes abnormal and deficient neural crest cell migration leading to severe craniofacial malformations including exencephaly (Mead and Yutzey, 2012).

Notch signalling also contributes to coronal suture formation as disruption of notch ligand, *Jag1*, in mice and in humans (Alagille syndrome) alike, causes craniosynostosis of the coronal suture (Yen et al., 2010; Kamath et al., 2002). Deletion of *Jag1* from the PM-cells of the coronal suture, where it is normally expressed, alters the identity of the sutural mesenchymal cells from E12.5 onwards and disrupts the boundary between osteogenic and non-osteogenic cells. *Notch2* expression and Notch signalling activity were concomitantly increased in the sutural mesenchyme. Yen et al. revealed that Jag1 and Notch2 are both downstream effectors of Twist1 in the developing coronal suture (Yen et al., 2010).

Notch signalling also affects calvarial osteogenic cells by keeping them in the proliferating preosteoblast state, restricting further differentiation prior to Osx activation (Hilton et al., 2008). Decreased proliferation has been proposed to cause widened interfrontal suture and reduced frontal bone formation at E18.5 in mice that lack Notch signalling from the neural crest cells (Wnt1Cre;RBP-J(f/f)) (Mead and Yutzey et al., 2012).

2.1.6.4 Eph-ephrin signalling pathway

Ephrin receptors (ephs) are receptor tyrosine kinases, which are activated by ephrin ligands. Both Ephs and ephrin ligands are transmembrane proteins that require direct cell-cell communication for signal transduction. Ephs have a unique capacity of bidirectional signalling that affect both receptor- and ephrin-expressing cells (reviewed in Lisabeth et al., 2013). Eph-ephrin signalling is a critical regulator of embryonic development including axon guidance, formation of tissue boundaries, cell migration, and segmentation.

Anti-adhesive interaction, where eph repulses the ephrin ligand, is proposed to play a significant role in restricting cell mixing across boundaries (Poliakov et al., 2004). Indeed, deletion of *ephrin type-A receptor 4* (*EphA4*) in mice causes premature fusion of the coronal suture (Merrill et al., 2006; Ting et al., 2009). Likewise, mutations in ephrin ligands: *EFNA4* and *EFNB1*

(Craniofrontonasal syndrome), are known to cause craniosynostosis of the coronal suture in humans (Twigg et al., 2004; Wieland et al., 2004; Merrill et al., 2006).

Eph-ephrin signalling functions downstream of Twist1 and Msx2 to regulate the apical migration of osteoprogenitor cells from the frontal and parietal bone primordia in the supraorbital ridge to the leading margins of the developing bones. Concomitantly it inhibits these osteoprogenitor cells from entering the coronal suture (Merrill et al., 2006; Ting et al., 2009).

2.1.6.5 Twist1

Twist1, basic Helix-loop-Helix (bHLH) transcription factor, is a critical regulator of calvarial development. *Twist1*-null allele mice die by E11.5 exhibiting major craniofacial defects (Chen and Behringer, 1995). Twist1 is required for early migration and survival of cranial mesenchyme and for proper osteogenic differentiation of both PM and CNC cells, as well as for reciprocal tissue interaction. Conditional deletion of *Twist1* from CNC causes complete failure of the frontal and supraoccipital bones to develop, and only remnants of the parietal and interparietal bones are formed (Bildsoe et al., 2009). Likewise, conditional deletion of *Twist1* from PM leads to absence of parietal and interparietal bones, but also the size of frontal bones is severely reduced. Twist1 was also found to maintain cells derived from PM in a mesenchymal progenitor state and to inhibit transition of these cells to an epithelial architecture (Bildsoe et al., 2013).

Twist1 has a critical role in maintaining boundaries between osteogenic and non-osteogenic compartments in the calvaria (Ting et al., 2009). *Twist1*-heterozygous mice ($Twist1^{+/-}$) present a postnatally occurring synostosis of the coronal suture, as well as the lambdoid suture associated with enlarged interparietal bone (Bourgeois et al., 1998; Carver et al., 2002; Ting et al., 2009). *Twist1* is expressed from E9.5 onwards in the calvarial mesenchyme to control intramembranous bone progenitor specification. Twist1 inhibits Runx2 function by interacting with its binding domain. Osteoblast differentiation in the mesenchymal condensations is not initiated until E13 when *Twist1* expression is decreased. The calvarial defects in $Twist1^{+/-}$ mice may be in part explained by the genetic interaction of *Twist1* and *Runx2* as in $Twist1^{+/-}$;$Runx2^{+/-}$ mice the calvarial development is normalized (Bialek et al., 2004).

$Twist1^{+/-}$ mouse models the Saethre-Chotzen syndrome, which is caused by loss-of-function mutations in *TWIST1* and features a coronal synostosis (El Ghouzzi et al., 1997; Howard et al., 1997). Most research has thus focussed on the coronal suture, where *Twist1* is expressed in the sutural mesenchyme and in the OFs of the frontal and parietal bones (Rice et al., 2000; Johnson et al., 2000). The 'Twist box' that controls Runx2 function does not explain coronal craniosynostosis in Saethre–Chotzen syndrome patients as *TWIST1* missense mutations cluster in the bHLH region of the protein only (Morris-Kay and Wilkie, 2005). Twist1 has been shown to be vital in maintaining the neural-crest mesoderm boundary during coronal suture development. In $Twist1^{+/-}$ mice, neural crest cells cross the boundary into the mesoderm domain of the coronal suture (Merrill et al., 2006). Twist1 controls the guidance of migratory osteogenic mesenchymal cells to the leading margin of the frontal and parietal bones and excludes osteogenic cells from the coronal suture by activating Notch signalling (Jag1) and Eph-ephrin signalling (EphA4) (Merrill et al., 2006; Ting et al., 2009; Yen et al., 2010).

Twist1 also regulates suture organisation and osteoblast differentiation in the OFs by controlling Fgf signalling. It does this by forming functional homodimers as well as heterodimers with bHLH E-proteins, which are ubiquitously present. These dimers have distinct activities and regulate the expression of different sets of genes. Homodimers are present in the OFs, where they upregulate *Fgfr2* expression, while heterodimers exist in the sutural mesenchyme to downregulate *Fgfr2* expression (Connerney et al., 2006; Connerney et al., 2008).

2.1.6.6 Msx1 and Msx2

Homeobox genes *Msx1* and *Msx2* encode transcriptional regulators that participate in calvarial development from E9.5 onwards in mice. *Msx1* and *Msx2* are both expressed in the CNC-derived frontal bone anlage from E10.5, while only *Msx2* is detected in the developing parietal bone at E12.5, once the osteoblast differentiation is already initiated (Kim et al., 1998; Han et al., 2007).

Loss-of-function mutations in *MSX2* cause enlarged parietal foramina in humans (Wilkie et al., 2000). Likewise, *Msx2* null allele mice have a large ossification defect in the frontal bones (Satokata et al., 2000). In mice that lack both *Msx1* and *Msx2* all calvarial bones fail to form (Satokata et al., 2000; Han et al., 2007). *Msx*-genes act in parallel pathways with Twist1 to control the proliferation and differentiation of the CNC-derived mesenchyme that forms the frontal bones (Ishii et al., 2003; Han et al., 2007). The parietal bone defect is postulated to be a result of compromised CNC-derived meninges (Han et al., 2007).

Msx-genes control the initial step of osteoblast lineage specification in frontal bone primordium, but later on they inhibit the terminal differentiation keeping preosteoblasts in a proliferating state (Dodig et al., 1999; Hu et al., 2001). In the absence of both *Msx1* and *Msx2*, *Runx2* fails to be expressed in the frontal bone condensation (Han et al., 2007). Msx2, on the other hand, has been shown to suppress the promoter of *Runx2-II* (Lee et al., 2005; Kawane et al., 2014). There is also evidence that *Msx*-genes control *Dlx5* expression; Msx2 has been shown to inhibit *Dlx5* expression, while Msx1 is required for *Dlx5* expression in the developing frontal bone anlage (Lee et al., 2005; Chung et al., 2010). *Msx2* is also evidently a downstream target of Bmp2, while Msx1 is activated by both Bmp and Fgf signalling (Kim et al., 1998; Kim et al., 2004; Choi et al., 2005).

Msx-genes also contribute to suture development as overexpression of *Msx2* in mice causes craniosynostosis of the coronal and sagittal sutures, which mimics the human condition, Boston-type craniosynostosis, caused by activating mutation in *MSX2* (Liu et al., 1995). These mice exhibit an increased number of proliferating preosteoblasts in the OFs at the early postnatal stage (Liu et al., 1999). Msx2 also functions downstream of Twist1 to control Eph-ephrin signalling in coronal suture development to maintain the neural crest-mesoderm boundary between frontal and parietal bones (Merrill et al., 2006). Together they control the guidance of migratory osteogenic cells to the leading margin of the frontal and parietal bones.

Interestingly, Msx1 and Msx2 function early during calvaria development to supress osteogenic program in the interfrontal suture. When *Msx1* and *Msx2* are conditionally deleted only from the CNC from E9.5 or E10.5 onwards, frontal and parietal bones do develop, but frontal bone morphology is defected and heterotopic ossification is present in the interfrontal suture. Roybal et al. showed that *Msx*-genes are required to supress ossification in a normally non-osteogenic, CNC-derived cell layer within which the frontal bone anlages grow. This heterotopic ossification was associated with increased Bmp signalling (Roybal et al., 2010).

2.1.6.7 Dlx3, Dlx5 and Dlx6

Distal-less homeobox (*Dlx*) genes encode transcription factors that control development of the appendages of the main body axis. *Dlx*-genes function in specifying the differential fates of the CNC cells and govern epithelial-mesenchymal interactions (Morasso et al., 1995; Bendall and Abate-Shen, 2000). Consequently they are important regulators of the branchial arch patterning (Depew et al., 2005).

Out of the six *Dlx*-genes *Dlx3*, *5* and *6* are known to contribute to calvarial development. *Dlx5* is expressed in CNC-derived calvarial mesenchymal cells already before the onset of calvarial ossification (Holleville et al., 2003). Calvarial ossification is delayed in *Dlx5* null allele mice (Depew et al., 1999; Acampora et al., 1999). Dlx5 most likely has redundant functions with Dlx6 during

calvarial bone patterning as *Dlx5*$^{-/-}$;*Dlx6*$^{-/-}$ compound mutant mice lack all calvarial bones (Depew et al., 2002; Merlo et al., 2002; Robledo et al., 2002).

Dlx-genes are vital regulators of osteoblast differentiation. Dlx3 functions in early stages of osteoblast maturation, while Dlx5 is associated with more mature osteoblasts. *Dlx5* is expressed in cells that are undergoing differentiation in the OFs of calvarial bones, where it specifically activates the expression of *Runx2-II* by directly binding to the distal *Runx2* (P1) promoter (Lee et al., 2005; Kawane et al., 2014). Dlx3 and -5 are both targets of Bmp2 signalling (Holleville et al., 2003). Although Bmp2/Dlx5-pathway is not the initial inducer of *Runx2* expression, as there is normal *Runx2* expression in *Dlx5*$^{-/-}$ mutant mice, Dlx5 is capable of activating Runx2 target genes in the absence of *Runx2* (Choi et al., 2005; Hassan et al., 2006). Dlx5 also promotes maturation of osteoblasts by regulating *Oc* expression (Ryoo et al., 1997; Newberry et al., 1998; Depew et al., 1999).

2.1.6.8 En1

Homeodomain-containing transcription factor Engrailed 1 (En1) regulates several developmental processes, such as dorsoventral patterning of the limb, mid-hindbrain specification as well as skeletal development (Loomis et al., 1996; Wurst et al., 1994). En1 has a dual role during calvarial development. During early stage it regulates coronal suture formation by positioning and maintaining the PM/CNC boundary. *En1* expression is initiated in the calvarial osteogenic mesenchyme at E11.5, following the establishment of supraorbital regulatory centre across the PM/CNC lineage boundary. Evidence suggests that En1 restricts the invasion of CNC cells into the PM derived parietal bone and the coronal suture by regulating early *Msx2* and *Twist1* expression (Deckelbaum et al., 2012).

Later on, En1 promotes calvarial osteoblast differentiation and proliferation by activating Fgfr2 signalling in the bone anlage. In the suture progenitors it, in turn, prevents ossification by inhibiting Fgfr2 signalling (Deckelbaum et al., 2005; Deckelbaum et al., 2012).

2.1.6.9 Foxc1

Foxc1 is a forkhead box transcription factor that controls the differentiation of osteogenic precursor cells and is required for the apical growth phase during calvarial bone development. In *Foxc1*$^{-/-}$ mutant mice only rudimentary calvarial bones form at the sites of initial mesenchymal cell condensations. Foxc1 has been shown to regulate Bmp-mediated induction of *Alx4* and *Msx2* expression. Reduction of *Alx4* and *Msx2* expression in *Foxc1*$^{-/-}$ mutant mice leads to decreased osteoprogenitor cell proliferation (Rice R. et al., 2003). Sun et al. have recently shown that Foxc1 restricts differentiation of osteogenic precursors to the frontal bone primordium, where Bmp signalling is active. Foxc1 limits *Msx2* expression to this osteogenic zone by setting a transcriptional threshold for the Bmp-dependent *Msx2* activation (Sun et al., 2013). There is also evidence that *Msx2* is a direct target of Foxc1 during early stages of osteoblast differentiation (Mirzayans et al., 2012). *Foxc1* expression, on the other hand, is regulated by Fgf2, which indicates that Foxc1 has a role in integrating Bmp and Fgf signalling pathways during calvarial development (Rice et al., 2005).

2.1.7 Regulation of calvarial development by the brain, the meninges and the dura mater

Despite the rapid evolution of the vertebrate head, the brain and the calvaria have retained a tight fit (Nieman et al., 2012). The brain and skull morphogenesis are inevitably linked through coordinated integration of signalling pathways, but the processes remain unknown to a large extent. Signalling that regulates certain tissue can evidently have direct influence also on other tissues. Sympathetic

nervous system, for example, has been shown to inhibit proliferation of preosteoblasts (Karsenty and Wagner, 2002).

Mounting evidence now supports the original functional matrix theory by Moss (Moss, 1962), which states that the growing brain produces mechanical pressure on the developing calvarial bones, which causes morphological remodelling of the bones to reduce the strain by mirroring the shape of the brain (Henderson et al., 2005). Mechanical forces deform the cell membrane and cytoskeleton, which have an effect on cell signalling, cell differentiation, and cell proliferation (Mao and Nah, 2004). ECM and the cytoskeleton also communicate (Ingber, 2003; Temiyasathit and Jacobs, 2010). Application of strain to preosteoblasts has been shown to activate Wnt signalling pathway by causing accumulation of β-catenin in the cell cytoplasm and nucleus (Case et al., 2008).

The meninges connect the brain and the calvarial surfaces, and it is capable of transmitting signals, but the nature and mechanisms of communication that underlie their interaction remain elusive. The meninges may serve as a scaffold on which the calvarial bone condensations take shape as brain and bone follow their own developmental programs (Richtsmeier and Flaherty, 2013). Initiation of apical growth of the frontal and parietal bones and differentiation of meningeal precursors begin at the same time and progress jointly toward the apex of the head. In *Foxc1* null allele mice failure of the apical growth of the bone primordia is associated with disrupted meningeal development, which begins correctly, but mature meninges fail to form. This indicates that the early development of meninges and the calvarial bones are closely linked (Vivatbutsiri et al., 2008).

The composition of the dura mater varies regionally and it has age specific functions (Warren et al., 2003; Loeys et al., 2005). Especially immature dura mater secretes many osteogenic growth factors, cytokines and ECM molecules, which are vital for ossification of calvarial bones (Greenwald et al., 2000a; Greenwald et al., 2000b). Inductive stimulus from the dura mater is also required during suture formation before the suture is able to maintain patency independent of the surrounding tissues (Opperman, 2000). The dura mater has also been shown to influence fusion of the posterior frontal suture in rats (Opperman et al., 1993; Roth et al., 1996; Levine et al., 1998).

Tgfβ signalling originating from the dura mater has been shown to have many roles during calvarial development. Tgfβ signalling regulates suture morphogenesis by controlling cell numbers within the sutural mesenchyme and OFs. Disruption of Tgfβ signalling from the CNC severely impairs cell proliferation in the dura mater, resulting in calvaria agenesis. Ossification of the parietal bone also requires interaction with the CNC derived meninges (Jiang et al., 2002). CNC derived dura mater may induct the frontal and parietal bone formation (Ito et al., 2003).

The role of the dura mater has also been investigated in Apert syndrome mouse models with mixed findings. Dural cells transfected with the *Fgfr2*^{P253R} allele were shown to promote ossification of the co-cultured osteoblasts (Ang et al., 2010). On the other hand, occurrence of Apert mutation (S252W) solely in the dura mater is unable to induce osteogenic fusion of the coronal suture in mice (Holmes and Basilico, 2012).

The mutations that lead to craniosynostosis have also been shown to affect interactions between genes and regulatory networks that communicate during head development. Same genes participate in brain and calvaria development. In the *FGFR*-related craniosynostosis syndromes, for example, evidently both the brain and the calvaria are primarily affected. Expression of *Fgfr1* and *-2* has been shown to determine brain size (Stevens et al., 2010).

2.2 Hedgehog signalling

The Hh pathway has a crucial role in development, as well as in tumorigenesis. Three Hh ligands have been identified in mammals; Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehog, which differ

primarily in tissue distribution (Echelard et al., 1993). *Shh* is expressed, for example, in the nervous system, in the limb bud and in many epithelial tissues, and has a vital role in early embryogenesis and patterning. Ihh is indispensable for endochondral ossification, while *Dhh* is expressed in the peripheral nervous system and reproductive organs.

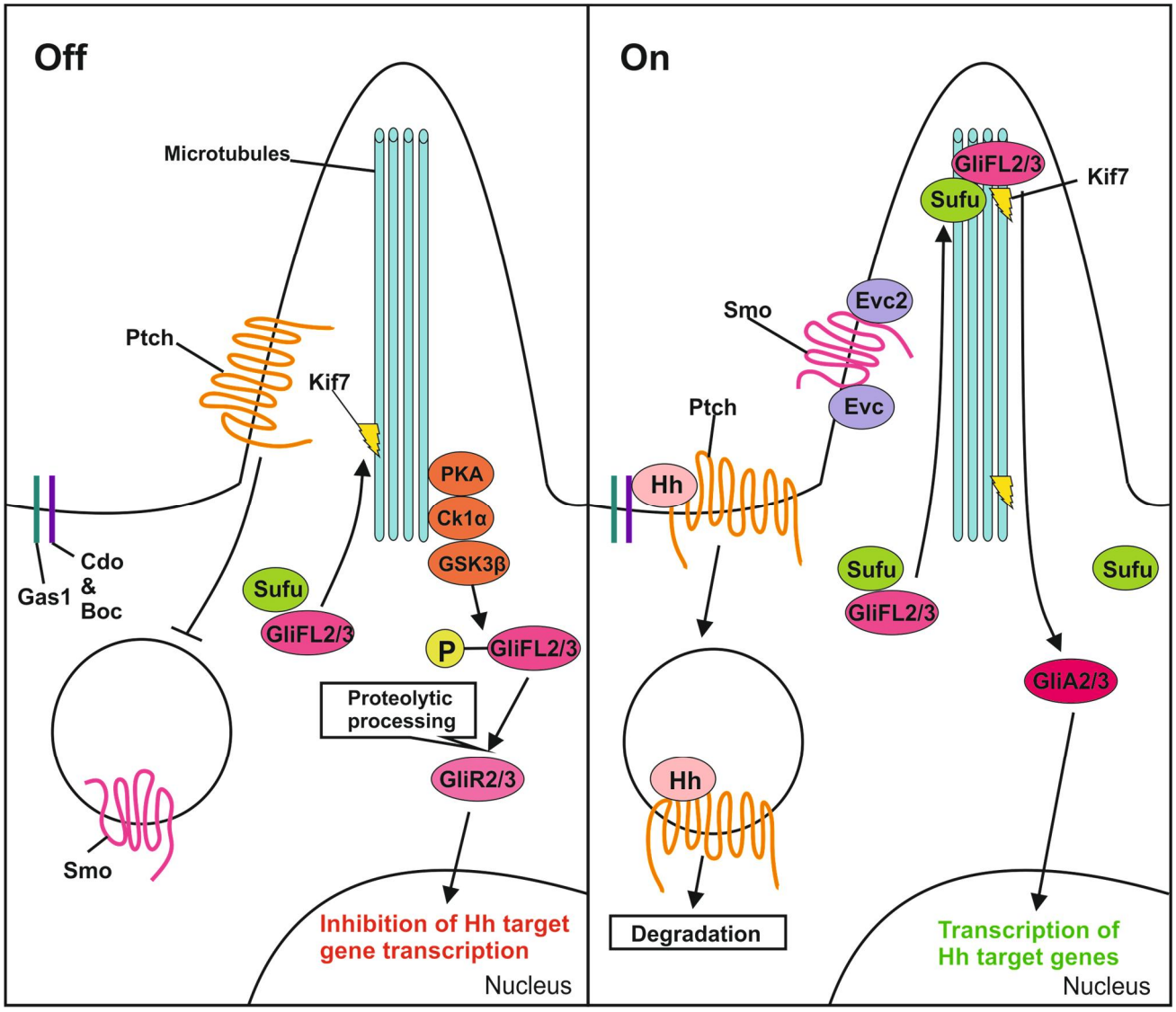

Figure 4. Schematic view of the vertebrate Hedgehog (Hh) pathway depicting the Hh-receiving cell when Hh ligand is absent (Off) and present (On).
Off: In the absence of Hh ligand Ptch resides in the primary cilium and inhibits Smo from entering the ciliary membrane from the cytosol. Sufu retains the full-length (FL) forms of Glis in the cytosol. Kif7 is required to recruit PKA, GSK3β and CK1α, which then phosphorylate Gli2FL and Gli3FL. These FL-Glis are further processed proteolytically into repressor (R) forms, which enter the nucleus and actively inhibit Hh target gene transcription.
On: When Hh ligand binds to the receptor Ptch and to the co-receptors, Gas1, Cdo and Boc, Smo enters the ciliary membrane, forming a complex with Evc and Evc2. This complex relieves the inhibition of Gli proteins and Kif7 then transports Gli-Sufu complex to the tip of the cilium. GliFL proteins are processed into activators (GliA2/3), which enter the nucleus to activate transcription of Hh target genes. Modified from Briscoe and Thérond, 2013.

2.2.1 Hh signalling pathway

Hh ligands are secreted proteins. Following translation, Hh precursor peptides go through two independent lipid modifications, which are essential for normal Hh signalling. A cholesterol residue is first added to the C-terminus by cholesterol-dependent autocatalytic cleavage in the endoplasmic reticulum (ER) lumen (Porter et al., 1996; Chen et al., 2011). The subsequently cleaved C-terminal fragment is degraded in the ER. Then Hh acyltransferase, called Skinny hedgehog (Ski), catalyses the attachment of palmitate to the N-terminal end of Hh ligand (Chamoun et al., 2001). This hydrophobic Hh ligand is membrane-associated, but the exact mechanism of Hh release is unknown. Multispanning membrane protein Dispatched (Disp) and secreted protein Scube are known to be essential for secretion and long-range Hh signalling (Burke et al., 1999; Ma et al., 2002; Tukachinsky et al., 2012). They recognise the cholesterol-moiety and affect the solubility of Hh ligand. Cholesterol and palmitate moieties are also required to form active multimeric Hh complexes enabling long-range spread (Zeng et al., 2001).

Hh is able to signal both short- and long-range. Recent evidence suggests that long-range activation of Hh signalling is mediated through direct receptor-ligand interaction between cell membranes that utilize specialized class of actin-based filopodia called cytonemes, which are long cytoplasmic extensions (Sanders et al., 2013). Hh ligand, together with its co-receptor CAM-related/downregulated by oncogenes (Cdo), is transported in exovesicles via these cytonemes (Gradilla et al., 2014). The spread of Hh proteins is also regulated by proteins responsible for Hh reception, which are situated on the membranes of the receiving cells. The receptor Patched 1 (Ptch) and the vertebrate-specific Hh-interacting protein 1 (Hip1) both limit diffusion of Hh ligands (Chuang et al., 2003; Chen and Struhl, 1996).

Primary cilia, microtubule-based, non-motile structures that protrude from the surface of most cells of the body, are essential for Hh signal transduction in vertebrates (Huangfu et al., 2003) (Figure 4). Intraflagellar transport (IFT) proteins are required for primary cilia production and maintenance, and facilitate transport of proteins into and out of the cilia (Pedersen and Rosenbaum, 2008). At the base of the ciliary shaft is the basal body from which the microtubules originate and in this transition zone cytoplasmic proteins are being loaded to the IFT system. Also what proteins enter the ciliary membrane is tightly controlled. Novel evidence indicates that primary cilia serve as signalling centres and other signalling pathways, including platelet-derived growth factor-, Notch- and Wnt signalling, are also associated with primary cilia (Goetz and Anderson, 2010).

The Hh receptor Ptch is a transmembrane protein that has a dual role in Hh signalling serving as an inhibitor of Hh signalling in the absence of the ligand, as well as a receptor for the Hh ligands (Rohatgi et al., 2007). *Ptch1* is also a direct transcriptional target of Hh signalling. In the absence of the Hh ligand it resides in the primary cilium where it inhibits another transmembrane protein Smoothened (Smo) from entering the cilium (Taipale et al., 2002). Smo's structure resembles a G-protein-coupled receptor and it is a signal transducer of Hh signalling (Corbit et al., 2005).

Ptch requires Hh co-receptors: Cdo, Brother of Cdo (Boc), and Growth arrest-specific 1 (Gas1) for Hh ligand binding (Beachy et al., 2010). These co-receptors are situated on the cell membrane, where they form multimolecular complexes with Ptch and facilitate high-affinity Hh binding. These co-receptors, however, have an opposing role in pathway regulation compared to Ptch; the co-receptors promote Hh signalling while Ptch inhibits it.

2.2.1.1 Transcription factors Gli1, 2 and 3 mediate Hh signalling

GLI-Kruppel family members 1, 2 and 3 (Gli1, 2, 3) are zinc finger-containing transcription factors that mediate transcriptional output of Hh signalling (Buttitta et al., 2003; Motoyama et al., 2003). Gli proteins function as repressors (R) as well as activators. Gli1 contains only an activator domain in the C-terminal and functions solely as an amplifier of activation (Park et al., 2000). Gli2 and Gli3, on the

other hand, contain, in addition, N-terminal R-domains and can be cleaved into R-forms by removal of their carboxy-terminal trans-activation domains (Dai et al., 1999; Sasaki et al., 1999). *Gli1* and, to lesser extent, *Gli2* are direct targets of Hh signalling, while high concentration of Hh ligand downregulates transcription of *Gli3* (Marigo et al., 1996). Primary cilia are evidently mandatory for Gli-protein metabolism and transcriptional activity (Kim et al., 2009; Wen et al., 2010).

In the absence of the Hh ligand the full-length (FL) activator forms of Gli2 and Gli3 are kept in the cytosol by Suppressor of Fused (Sufu), which further promotes phosphorylation of the C-terminal residues of the FL Gli's by activating protein kinase A (PKA) (Figure 4). Sufu thus acts as a negative regulator of Hh signalling. GliFL is further phosphorylated by Gsk3β and Ck1α. The kinesin family member 7 (Kif7) is required to recruit PKA, Gsk3β and Ck1α (Jiang and Hui, 2008). E3 ubiquitin ligase, βTrCP, then degrades C-terminal peptides and the modified proteins are then partially proteolytically processed by the proteasome to generate the R-forms of Gli2 (Gli2R) and Gli3 (Gli3R). The processing of Gli3 is significantly more efficient compared to Gli2, and so the Gli3R, in particular, enters the nucleus and actively inhibits the translation of Hh target genes. Gli2FL is likely being completely degraded by the proteasome (Pan et al., 2006).

Binding of the Hh ligand causes Ptch to relieve its inhibition on Smo, which is then phosphorylated, allowing its' translocation into the ciliary membrane (Figure 4) (Corbit et al., 2005; Rohatgi et al., 2007; Goetz and Anderson, 2010). There Smo forms a complex with Evc2 and Evc in the EvC zone of the cilium (Dorn et al., 2012; Yang et al., 2012). This active Hh signalling complex relieves the inhibition of the Gli-proteins by PKA and Gli-Sufu complex is able to translocate to the tip of the cilium (Dorn et al., 2012). Kif7 is thought to act as an anterograde motor in the cilium that facilitates Gli-protein transport to the tip of the cilia. Kif7 is needed to form the Gli transcriptional activators in the presence of Hh ligand (He et al., 2014). Evc2 further regulates the dissociation of Gli and Sufu in the cilia. Gli-FLs then enter the nucleus where they are modified to transcriptional activators of the Hh target genes (Huangfu and Anderson, 2006; Varjosalo and Taipale, 2007). Gli2FL is more potent transcriptional activator compared to Gli3FL (Matise et al., 1998).

Dissociation from Sufu also results in an increased rate of Gli-protein degradation. In the nucleus, GliFL binds to the MATH-BTB-domain-containing E3-ubiquitin ligase called Speckle-type PDZ protein (Spop) that mediates its degradation. The balance between Gli-Sufu and Gli-Spop formation is indicated to be important in regulation of the amount and activity of Gli-proteins (Chen et al., 2009).

2.2.1.2 Syndromes caused by mutations in GLI3

Mutations in *GLI3* are responsible for five different autosomal dominant syndromes in humans depending on the location of the defect. GCPS is caused by N-terminal, loss-of-function mutations leading to haploinsufficiency of *GLI3*, which manifests as reduced amount of both GLI3R and GLI3FL, respectively (Kalff-Suske et al., 1999; Vortkamp et al., 1991). Features of GCPS include preaxial polydactyly in feet and postaxial polydactyly of hands, as well as variable cutaneous syndactyly and craniofacial abnormalities, such as hypertelorism, broad nasal bridge, macrocephaly with frontal bossing, and rarely craniosynostosis (Johnston et al., 2005). Central nervous system (CNS) anomalies, agenesis of the corpus callosum, and cognitive impairment have also occasionally been reported.

The extra-toes mouse ($Gli3^{Xt\text{-}J/Xt\text{-}J}$) was originally introduced by D.R. Johnson already in 1967 (Johnson, 1967). It was later shown to represent a *Gli3* null allele, containing an intragenic deletion of *Gli3*, and to model GCPS (Vortkamp et al., 1992; Hui and Joyner, 1993). As the name implies, $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice show fore and hind limb polydactyly, as well as syndactyly. They die at birth having respiratory difficulties, kidney anomalies, severely abnormal brain morphology, rudimentary eyes, neural tube closure defects, and high incidence of exencephaly.

Pallister-Hall syndrome (PHS) is caused by mutations that lead to C-terminally truncated GLI3 (Kang et al., 1997). Repressor function of Gli3 is thus increased in relation to the activator function (Krauss et al., 2009). Common PHS features are central polydactyly, syndactyly, imperforated anus, gastrointestinal, epiglottis and larynx defects, abnormal kidney development, and absence of adrenal glands. Böse et al. have also generated a mouse model of PHS, which displays all of these features (Böse et al., 2002).

GLI3 is also the causative gene of Preaxial polydactyly type IV, as well as Postaxial polydactyly type A and B, which only present as digit abnormalities. Fifth syndrome, diagnosed in two patients with *GLI3* mutations, is Acrocallosal syndrome characterised by postaxial polydactyly, macrocephaly, agenesis of the corpus callosum, and severe developmental delay (Elson et al., 2002; Speksnijder et al., 2013).

2.2.2 Role of Hh signalling during calvarial development

Hh signalling has a significant role in development of the head. It is known to control the width of the face and the skull (Bergmann et al., 2010). Loss-of-function mutations in *SHH* cause holoprosencephaly in humans and mice, which is a cephalic disorder, where the forebrain does not divide to form bilateral cerebral hemispheres, and it is also associated with midline facial dysmorphism (Chiang et al., 1996). Humans and mice with inactivating mutations of the Hh signalling repressor, *GLI3*, on the other hand, exhibit wider faces. It has been proposed that excessive Hh signalling leads directly to increased proliferation of CNC cells, which contributes to the increase in facial width (Bergmann et al., 2010; Tabler et al., 2013).

2.2.2.1 Loss of Shh signalling causes holoprosencephaly

Shh is vital for the patterning of the face and Hh signalling plays a crucial role in CNS development. Decrease in Shh signalling has been associated with holoprosencephaly, cleft palate, and disrupted tooth development. *Shh* null allele mice have severe deficiencies in head structures due to early defects in the axial mesoderm (Chiang et al., 1996). This has prevented the analysis of possible later roles for Shh in the regulation of calvarial morphogenesis. Inactivation of *Shh* in the CNC derived cells leads to severe defect in the frontal bone development, and Shh was found to be essential for developmental steps involving postmigratory CNC cells (Jeong et al., 2004). Intriguing novel data also shows that significant population of the PM, from which calvarial mesenchyme is derived from, are Hh-responsive *Gli1*-expressing cells. These cells express *Gli1* transiently at E7.5 to E8.5. The frontal bones develop from the CNC, but they have also been shown to receive contribution from the Hh-responsive mesodermal lineage (Deckelbaum et al., 2012).

Deletion of *Disp1* in mice leads to similar phenotype as lack of *Shh*. In *Disp1* null allele mice Hh ligand accumulates in the Hh-secreting cell causing a weak activation of Hh target genes, but mice die already at E9.5 (Caspary et al., 2002; Kawakami et al., 2002). Tian et al. (2004) generated a hypomorphic *Disp1* mouse, which lacked parietal bone completely and also other skull bones derived from cephalic PM were truncated or misshapen. These findings suggest that Shh also contributes to patterning of the cephalic mesoderm.

Loss of Hh co-receptor *Gas1* also causes mild holoprosencephaly in mice. Calvarial bones of *Gas1*$^{-/-}$ mice were normal and sutures patent at birth. Interestingly, genetic reduction of *Shh* from *Gas1*$^{-/-}$ mice (*Gas1*$^{-/-}$;*Shh*$^{+/-}$), not only caused more severe holoprosencephaly, as anticipated, but also craniosynostosis of the coronal suture was detected (Seppälä et al., 2007). Cause of this premature suture fusion was not investigated further, but the finding is intriguing considering the facts that

neither *Gas1*$^{-/-}$ nor *Shh*$^{+/-}$ mice bare coronal synostosis, and that *Shh* null allele mice die before the onset of calvarial development.

2.2.2.2 Role of Ihh in calvarial development

The role of Ihh during endochondral ossification is well established, but the data on its functions during intramembranous ossification have been controversial. The original phenotype characterisation of *Ihh* null allele mouse reported the surprising finding that although osteoblasts failed to differentiate in endochondral bones, normal, albeit truncated, calvarial bones developed (St-Jacques et al., 1999). *Ihh* is expressed in the OFs and it is known that loss of *Ihh* delays calvarial ossification resulting in widened sutures, but the mechanism is debated on (Jacob et al., 2007). Abzhanov et al. postulated that Ihh signals from more mature osteoblasts to preosteoblasts to repress osteogenic lineage differentiation. In the absence of *Ihh* the preosteoblasts would differentiate faster and the proliferation of preosteoblasts would decrease leading to smaller bones (Abzhanov et al., 2007). On the other hand, there is increasing amount of evidence to suggest that the role of Ihh in the calvaria is pro-osteogenic. Ihh has been shown to promote osteoblast differentiation of isolated calvarial mesenchymal cells by activating Gli2, which physically interacted with Runx2 leading to activation of *Runx2* expression and function (Shimoyama et al., 2007). In the same study Gli3 was shown to inhibit Ihh-dependent osteoblastogenesis, but interaction between Runx2 and Gli3 was not detected. Ohba et al., however, revealed that Gli3R competes for the same binding site with Runx2 and so inhibits expression of Runx2 target genes and thus ossification (Ohba et al., 2008). Loss of *Ihh* has been shown to result in reduction of osteogenic marker expression in the calvaria. *Bmp2* and *-4* expression was also downregulated, which indicates that Bmp2/4 lie downstream of Ihh in the developing calvaria (Lenton et al., 2011). In zebrafish Ihh regulates outgrowth and shaping of a craniofacial intramembranous bone called opercle by controlling location specific proliferation (Huycke et al., 2012).

2.2.2.3 Increased Hh signalling causes craniosynostosis

Mounting evidence links elevated Hh signalling with craniosynostosis in humans. Dominant mutations in *GLI3* cause Greig cephalopolysyndactyly syndrome (GCPS) characterised by frontal bossing of the skull, hypertelorism, and in some cases, premature metopic suture fusion (Hurst et al., 2011; McDonald-McGinn et al., 2010). Microduplication at the *IHH* locus is also associated with craniosynostosis of the sagittal suture and cloverleaf skull. The critical duplicated region serves as a long-range enhancer of *IHH*, specifically regulating *IHH* expression, causing increased Hh signalling (Klopocki et al., 2011). RAB23 is a membrane-associated protein that regulates intracellular trafficking. Rab23 has negative impact on Hh signalling, as it has been shown to promote Gli3R production (Eggenschwiler et al., 2006). Recessive mutations in *RAB23* cause Carpenter syndrome in humans and some patients present premature synostosis of the metopic and sagittal sutures. In severe cases cloverleaf skull has also been reported, where the lambdoid sutures are also fused (Jenkins et al., 2007). *Rab23* null allele mice are exencephalic and they die between E12.5-13.5 (Eggenschwiler et al., 2001).

Nevoid basal cell carcinoma syndrome (NBCCS), also known as Basal Cell Nevus Syndrome or Gorlin Syndrome, is caused by heterozygous mutation of *PTCH1*, which leads to increased activation of Hh pathway. Craniofacial features include macrocephaly, frontal and parietal bossing, hypertelorism, and intracranial ectopic calcification. Microdeletion causing NBCCS has also lead to metopic craniosynostosis, but the causative gene could be other than *PTCH1* (Muller et al., 2011). Mouse models of NBCCS have not been very informative on the role of Ptch1 in calvaria development, as *Ptch1* null allele mice die at E9 with failure of the neural tube closer (Goodrich et al., 1997; Hahn et al., 1998). Transgenic mice that overexpress *Shh* in the basal epithelium from early

stages of embryogenesis, on the other hand, have almost complete absence of skull vault. Frontal bones are very truncated due to neural crest cell sensitivity to abnormal Hh signalling levels. Complete failure of mesodermal derived parietal and interparietal bone development may be secondary to increased signalling in the dorsal neural tube and consequent brain overgrowth (Cobourne et al., 2009). Novel ENU-induced recessive mouse model, however, represents a hypomorphic allele of *Ptch1* and these mice have a dome shaped calvaria, widened interfrontal suture with heterotopic ossification and craniosynostosis of the lambdoid suture (Feng et al., 2013).

Ciliopathies entail pathological conditions caused by defects in primary ciliogenesis, which lead to abnormal processing of Gli proteins. One syndrome caused by mutations in genes encoding IFT proteins in humans is Sensenbrenner syndrome, and subpopulation of the patients have premature synostosis of the sagittal suture (Sensenbrenner et al., 1975; Arts et al., 2011). Several mouse models of ciliopathies have been generated with variable calvarial phenotypes. Partial disruption of *Ift144* in mice enhances Hh signalling and causes exencephaly. Frontal bones fail to develop, and parietal, as well as interparietal bones are very truncated (Ashe et al., 2012). Deletion of IFT gene called *Kif3a* from neural crest cells causes truncation of primary cilia leading to gain of Hh signalling. The head of these mice are much wider in the frontal bone area; the frontal bones are truncated and the interfrontal suture is widened. In the anterior part of the interfrontal suture ectopic midline bones are detected (Bergmann et al., 2010).

Intriguing new evidence indicates that postnatally the role of Hh signalling in controlling suture patency is somewhat the opposite compared to the embryonic stage. Zhao et al. (2015) have recently shown that postnatally the calvarial sutures become a source of MSCs that express *Gli1*. Ihh, secreted from the OFs, is an important regulator of the differentiation of these *Gli1*-positive cells. Postnatal ablation of *Gli1* in mice from one month of age causes a severe reduction of MSCs that eventually leads to premature fusion of all of the calvarial sutures in two months. They also showed that in a mouse model of Saethre-Chotzen syndrome ($Twist^{+/-}$ mice), in which the coronal suture fuses postnatally, the *Gli1*-positive cells were reduced in all of the calvarial sutures. These results implicate that postnatally occurring craniosynostosis may be caused by reduction of these MSCs, which are regulated by Hh signalling (Zhao et al., 2015).

2.2.3 Role of Hh signalling during endochondral ossification

In this thesis we have studied the role of Ihh and Gli3 during osteoblast differentiation in intramembranous bones. As the role of Hh signalling during osteoblast differentiation in the endochondral bone is well established, an overview is given here.

Most of the bones of the skeleton, including the long bones of the limbs, develop by endochondral ossification. During limb development, mesenchymal cells originating from the lateral plate mesoderm first condense and differentiate into chondrocytes, which form a cartilage anlage. These chondrocytes proliferate and secret the ECM. Fibroblast-like cells surrounding the anlage form the perichondrium. Initially all the chondrocytes in the anlage proliferate elongating the anlage. Then cells residing in the middle exit the cell cycle and undergo cellular hypertrophy forming the hypertrophic zone. After E14.5 three morphologically distinct groups of chondrocytes form the embryonic growth plate. Distally are the round, low-proliferating, periarticular chondrocytes, which differentiate into flat columnar chondrocytes that proliferate actively, while hypertrophic, non-proliferating chondrocytes reside centrally (Figure 5). Osteoblasts first appear in the perichondrium adjacent to the hypertrophic zone, where they form the bone collar. Vasculature from the surrounding tissue then invades and triggers the removal of hypertrophic cartilage. Vasculature also brings osteoblast precursors that initiate ossification and marrow formation form the primary

ossification centres inside the bone, later forming a network of irregular spicules called the primary spongiosa (Erlebacher et al., 1995).

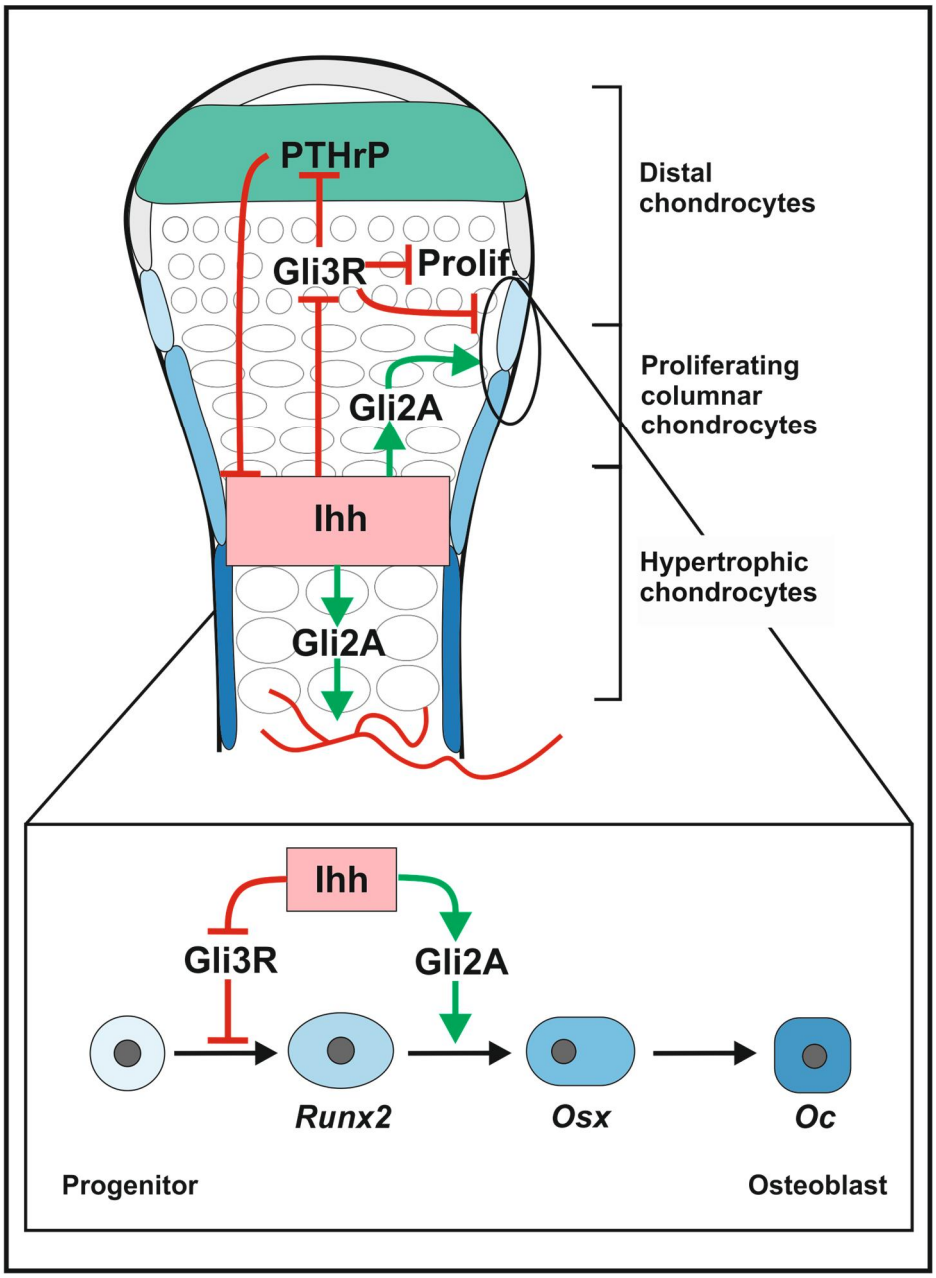

Figure 5. Schematic diagram of the role of Hedgehog (Hh) pathway during endochondral ossification of the limb. During endochondral ossification *Ihh* is expressed by hypertrophic chondrocytes. It activates chondrocyte proliferation by stimulating the differentiation of distal chondrocytes into columnar chondrocytes by downregulating Gli3R, which prevents distal to columnar chondrocyte differentiation and proliferation. Ihh also signals to immature chondrocytes to inhibit the onset of chondrocyte hypertrophy by activating PTHrP in the distal periarticular chondrocytes again by inhibiting Gli3R formation. Gli3R is an inhibitor of PTHrP expression, while PTHrP, in turn, inhibits the proliferating columnar chondrocytes from differentiating further. Ihh later induces ossification in the overlying perichondrium. Ihh accomplishes this by repressing Gli3R formation, which inhibits *Runx2* expression, and by activating Gli2A, which facilitates further osteoblast differentiation. By activating Gli2A, Ihh also mediates vascularization of the hypertrophic cartilage. Modified from Joeng and Long, 2009.

Ihh is a key regulator of endochondral ossification mediating the linkage between chondrocyte maturation and osteoblast differentiation (Figure 5). *Ihh* null allele mice exhibit reduced chondrocyte proliferation, malposition of mature chondrocytes, and failure of osteoblast differentiation in the endochondral bones (St-Jacques et al., 1999). Runx2 induces *Ihh* expression in the prehypertrophic chondrocytes and the expression is sustained in hypertrophic chondrocytes (Yoshida et al., 2004). Firstly, Ihh activates chondrocyte proliferation by stimulating the differentiation of periarticular chondrocytes into columnar chondrocytes (Kobayashi et al., 2005). Secondly, Ihh signals to immature chondrocytes to inhibit the onset of chondrocyte hypertrophy by activating another secreted growth factor, Parathyroid hormone related protein (PTHrP), in the distal periarticular chondrocytes. PTHrP, in turn, inhibits the proliferating columnar chondrocytes from differentiating further. Ihh achieves these two steps by downregulating Gli3R, which prevents distal to columnar chondrocyte differentiation and is also a strong inhibitor of *PTHrP* expression. *Gli3* is expressed by the distal and columnar chondrocytes. Genetic removal of *Gli3* from $Ihh^{-/-}$ mutant mice rescues the chondrocyte proliferation and hypertrophy defects (Hilton et al., 2005; Koziel et al., 2005).

Thirdly, Ihh later induces ossification in the overlying perichondrium (Kronenberg, 2003). Both Gli3R and activator isoform of Gli2 (Gli2A) mediate this process. Activation of Gli2A in *Ihh* null allele mice is sufficient to rescue vascularization of the hypertrophic cartilage (Joeng and Long, 2009). Osteoblast differentiation in the perichondrium, on the other hand, requires both; repression of Gli3R formation by Ihh, as Gli3R inhibits *Runx2* expression, as well as activation of Gli2A, which facilitates further osteoblast differentiation (Joeng and Long, 2009; Kesper et al., 2010).

It is noteworthy that although *Runx2* fails to be expressed in the perichondrium in the absence of *Ihh*, forced expression of *Runx2* alone does not rescue the osteoblast differentiation in $Ihh^{-/-}$ mutant mice (Tu et al., 2011). This indicates that Runx2 is not the only effector of Ihh. In effect, Ihh has also been shown to induce canonical Wnt signalling in perichondrial cells (Hu et al., 2005). Even though Gli2 and Gli3 evidently mediate all processes governed by Ihh during endochondral ossification, Gli-proteins are able to compensate for each other. Disruption of any one *Gli*-gene in mice has only minor consequences on endochondral ossification (Mo et al., 1997; Miao et al., 2004; Koziel et al., 2005).

2.3 Fibroblast growth factor signalling

In vertebrates at least 18 identified extracellular Fgf ligands and four Fgfrs have been implicated in numerous developmental processes from early embryonic stages onwards (reviewed by Ornitz and Itoh, 2015). Binding of Fgf ligand together with heparin sulphate activates these tyrosine kinase receptors through homodimerization (Figure 6). This results in phosphorylation of cytoplasmic tyrosine residues, which further activates three different cytoplasmic signal transduction pathways.

Ras/ERK pathway controls proliferation and differentiation, the Akt pathway is associated with cell survival, while the protein kinase C (PKC) pathway regulates cell morphology and migration. *Fgfr*s undergo alternative splicing in their extracellular domain to generate a wide variety of receptors with different affinities for their ligands (Zhang et al., 2006). The genes encoding Fgfr1, -2 and -3 use either exon IIIb or exon IIIc (Miki et al., 1992; Johnson and Williams, 1993). Cross talk between mesenchymal and epithelial cells is facilitated by the differential expression of ‘b’ and ‘c’ isoforms in epithelia versus mesenchyme (Ornitz et al., 1996). For example, *Fgfr2IIIb*, predominantly expressed in the epithelial cells, binds Fgfs 3, 7, 10 and 22, which are secreted by mesenchymal cells.

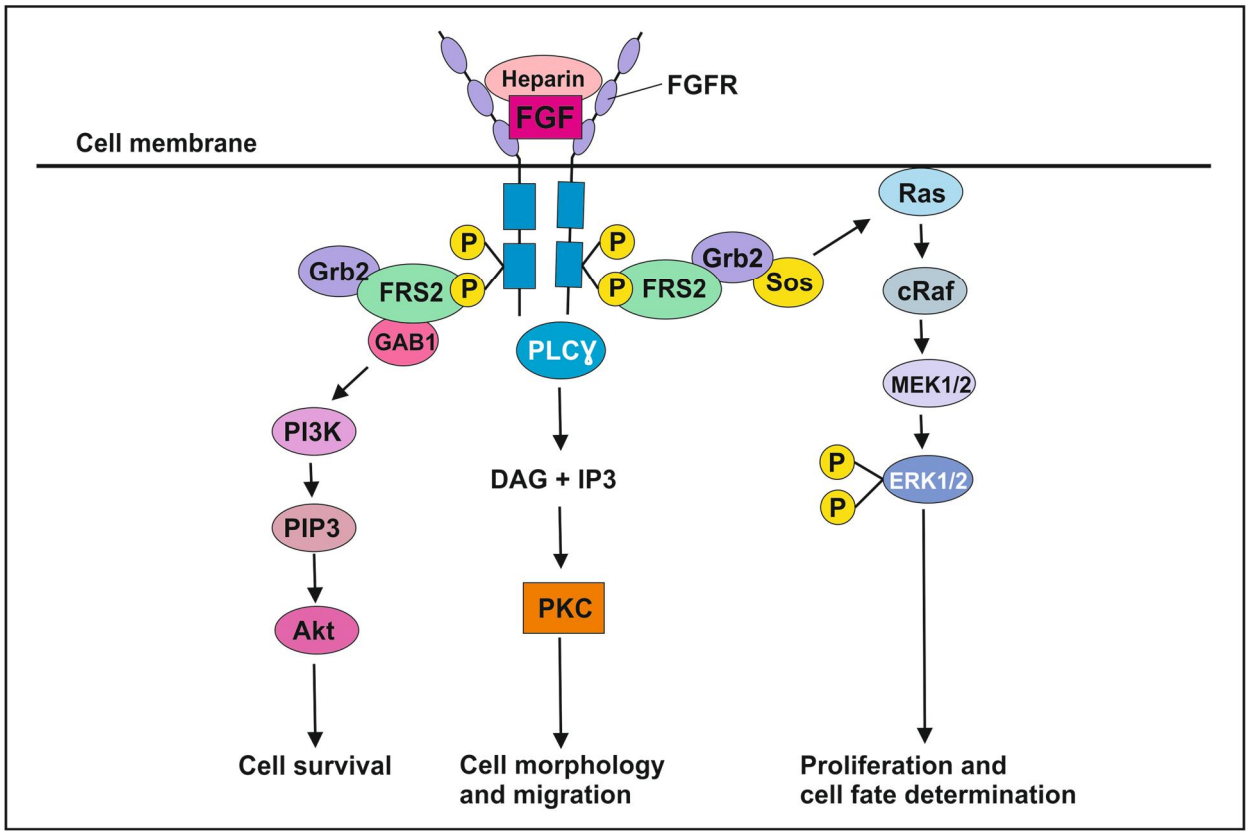

Figure 6. Schematic view of the Fgf pathway. Fgfr molecule is composed of an extracellular region harbouring two or three immunoglobulin-like domains, a transmembrane element and an intracytoplasmic tyrosine kinase domain, which become phosphorylated upon activation. Binding of Fgf ligand together with heparin sulphate dimerizes the Fgfr molecule and triggers Fgfr signalling, which is transduced cytoplasmically by PI3 kinase, PLC-pathways and Map kinases, which in turn regulate cell survival, cell morphology and migration, and proliferation and cell fate determination, respectively. Modified from Wagner and Siddiqui, 2007.

Fgfr2IIIc, on the other hand, is expressed by the mesenchymal cells and binds Fgfs 2, 4, 6, 9 and 18 that are synthesized by the epithelial cells. In general, Fgfrb isoforms are more selective compared to Fgfrc isoforms.

2.3.1 Role of Fgf signalling in calvarial development

The significance of Fgf signalling during calvarial development is undebated as elevated Fgf signalling causes craniosysnostosis. *FGFR*-related craniosynostosis syndromes are caused by dominantly acting mutations in *FGFR1*, *2* and *3* and affect specific regions of the proteins. *FGFR3* harbors the mutation underlying Muenke syndrome, the most common syndromic form of craniosynostosis, and a rare variant of Crouzon syndrome associated with skin manifestations. Thanatophoric dysplasia type II, also caused by mutations in *FGFR3*, is characterised by cloverleaf skull, which involves a trilobar skull deformity usually caused by synostosis of coronal, lambdoid, metopic, and sagittal sutures, as well as dwarfism (Langer et al., 1987). *FGFR1* mutations are associated with Pfeiffer syndrome. Mutations in different regions of *FGFR2*-gene cause Pfeiffer syndrome, Apert syndrome, Crouzon syndrome, Beare-Stevenson syndrome, *FGFR2*-related isolated coronal synostosis and Jackson-Weiss syndrome, respectively. Muenke syndrome and *FGFR2*-related isolated coronal synostosis are characterised only by uni- or bicoronal craniosynostosis; the

remainder are characterised by bicoronal craniosynostosis or cloverleaf skull, distinctive facial features, and variable hand and foot findings, as well as CNS abnormalities (reviewed in Rice, 2008b).

The reciprocal epithelial–mesenchymal system of Fgf–Fgfr signalling described above, does not apply in the calvaria, as it is derived solely from mesenchyme. *Fgfrc* isoforms expressed in the calvaria are activated by a large number of Fgf ligands and all *Fgf* ligands, except *Fgf3, -4, -5, -6* and *-8* are expressed in mouse calvaria (Iseki et al., 1997; Hajihosseini and Heath, 2002). *Fgf2* and *Fgf9* are expressed in the sutural mesenchyme and in the OFs, while *Fgf18* and *Fgf20* are expressed solely in the OFs (Rice et al., 2000; Hajihosseini and Heath, 2002). Fgf2 is also secreted by the dura mater, which influences calvarial ossification (Warren et al., 2003). The receptors are expressed in the OFs. *Fgfr2* is expressed in the proliferating osteoprogenitors, while post-proliferative osteoblasts express *Fgfr1* (Iseki et al., 1999; Johnson et al., 2000). *Fgfr3* is expressed at low levels in the OF, overlapping with the expression domains of *Fgfr1* and *Fgfr2* (Johnson et al., 2000).

Fgf pathway has an early role in neural crest cell migration. It is also important in condensation formation and maintenance. Fgf ligands promote ossification in several ways. Fgf2 regulates cell fate decisions of mesenchymal stem cells between adipocyte and osteoblast differentiation (Xiao et al., 2010). Indeed, many Fgf ligands (Fgf2, -4, -8, -18) have been shown to stimulate *Runx2* expression through Fgfr activation (Zhou et al., 2000; Kim et al., 2003). Fgf2 and -18 also activate Runx2 protein through protein kinase C pathway (Kim et al., 2003). Fgf2 can also interact directly with Runx2 to influence osteoblast proliferation via effects on the ECM (Teplyuk et al., 2009; Marie, 2012). Fgf2 and Fgf18 enhance Bmp activity through modulation of *Runx2* expression and by suppressing expression of the Bmp antagonist *Noggin* (Warren et al., 2003; Reinhold et al., 2004; Choi et al., 2005). Fgf2 also stimulates osteoblasts differentiation in part by activating Wnt/β-catenin signalling (Fei et al., 2011).

Fgf2 is also proposed to have an inhibitory effect on calvarial ossification as implanting Fgf2-soaked beads into calvarial explants have shown to induce *Twist1* expression (Rice et al., 2000). Relationship of Fgf signalling and Twist1 is complex as Twist1 also regulates the expression of *Fgfr2*. Twist1/E-protein heterodimers repress *Fgfr2* expression in the sutural mesenchyme, while Twist1 homodimers activate *Fgfr2* expression in the OFs (Connerney et al., 2006; Connerney et al., 2008).

Null allele *Fgfr1* or *Fgfr2* mice die before gastrulation (Arman et al., 1998; Yamagouchi et al., 1994). To investigate the function of these receptors during calvarial development *Fgfr*-genes have been conditionally disrupted. An *Fgfr1* hypomorph revealed that mainly *IIIc* isoform of this gene is essential already at the initial stage of calvarial development, during paraxial mesoderm induction and patterning (Partanen et al., 1998). Deletion of the *Fgfr2c* isoform in mice causes a delay of calvarial bone differentiation and mineralization at E14.5, but somewhat incoherently these mice later show a premature fusion of medial part of the coronal suture at postnatal day 14 (Eswarakumar et al., 2002). Conditional inactivation of *Fgfr2* in the osteoblast and chondrocyte lineages, on the other hand, did not lead to craniosynostosis (Yu et al., 2003). However, both of these studies came to the same conclusion as Iseki et al. (1999); *Fgfr2* is essential for osteoblast proliferation but not differentiation. Low level of Fgf signalling activates *Fgfr2* (Iseki et al., 1999).

Fgfr1 activation through increased level of Fgf signalling, on the other hand, is required for osteoblast differentiation. Mouse model of Pfeiffer syndrome, $Fgfr1^{250/+}$, supports these experimental findings by Iseki et al. (1999). Synostosis of the sagittal and coronal sutures in these mice is associated with accelerated osteoblast differentiation. Premature expression of *Runx2* suggests that Fgfr1 signalling is upstream of *Runx2* (Zhou et al., 2000). Hajihosseini et al. (2004) have demonstrated a dosage effect of *Fgfr1* expression on osteogenic differentiation in the calvarial sutures directly by introducing variable copy numbers of a hypermorphic *Fgfr1* mutation carried by a

bacterial artificial chromosome (BAC). They showed that increased severity of the sutural fusions correlated with increasing the BAC copy number. In conclusion, at the OFs is an Fgf gradient that at least in part controls the increasing maturation of osteoblasts from proliferating osteoprogenitors expressing *Fgfr2* to post-proliferative osteoblasts expressing *Fgfr1*. Mature osteoblasts express neither receptor (Iseki et al., 1999; Johnson et al., 2000).

The prevailing hypothesis is that increased FGF signalling leads to craniosynostosis by triggering a switch from *FGFR2* expression and proliferation to *FGFR1* expression and differentiation (Morriss-Kay and Wilkie, 2005). Evidence from several studies has elucidated the mechanisms by which the mutations in *FGFR*-genes increase FGF signalling. Pfeiffer syndrome patients who have a mutation in exon IIIc acceptor splice site, as well as Apert syndrome patient with Alu insertions both show ectopic expression of the *FGFR2IIIb* isoform in the calvaria (Oldridge et al., 1999). Furthermore, mutant FGFR2IIIc splice form receptors acquired from Apert syndrome patients show novel binding to the FGFR2IIIb-specific ligand FGF10 (Yu et al., 2000). Hajihosseini et al. (2001) generated a mouse with heterozygous abrogation of *Fgfr2* exon *IIIc* to model this splicing switch. Additional deletion of *Fgf10* from these mice was able to rescue the coronal synostosis, which highlights the surprising role Fgf10 has in the calvaria in pathological situations (Hajihosseini et al., 2009).

In most mouse models of Fgfr-related craniosynostosis the coronal suture fusion is reported to occur postnatally. Holmes et al. (2009) used $Fgfr2^{S252W/+}$ mice to study the effects of increased Fgf signalling on earlier embryonic calvarial development. Coronal suture changes were detected already at E13.5. The mutated osteoprogenitor cells were shown to both proliferate and differentiate faster in the OF and invade the area where normally the presence of undifferentiated mesenchyme marks the coronal suture (Holmes et al., 2009).

The coronal suture and the OF of the frontal bone mark the PM/CNC boundary, respectively. Ectopic cell mixing causing disruption of this lineage border between mesoderm and neural crest has been proposed to be one mechanism of coronal suture fusion. However, Fgf signalling does not seem to have a role in maintaining this boundary. Holmes and Basilico (2012) generated mice that expressed the gain-of-function $Fgfr2^{S252W}$ allele solely in the mesoderm or neural crest, respectively. Interestingly, coronal synostosis only occurred when $Fgfr2^{S252W}$ was expressed in the mesoderm. (Holmes and Basilico, 2012)

Function of Fgfr3 signalling in the cranial sutures is not known. *Fgfr3* null allele mice are viable. Although they show excessive growth of long bones, caused by increased proliferation of the growth plate chondrocytes, no calvarial abnormalities were detected (Colvin et al., 1996; Deng et al., 1996). A Muenke syndrome mouse model has a P244R mutation in *Fgfr3*. However, these mice very rarely bear craniosynostosis (Twigg et al., 2009).

3. AIMS OF THE STUDY

Hh signalling is known for its fundamental role in craniofacial development and in endochondral ossification, respectively. However, very little is known about the Hh pathway in the intramembranous ossification of the calvarial bones.

The aims of this thesis were to:

1. Study the role of Hh-Gli3 signalling during calvarial bone development.

2. Investigate the interaction of Fgf and Hh-Gli3 pathway in calvarial development.

3. Attempt to rescue craniosynostosis induced by loss of *Gli3* by downregulating *Runx2*.

4. MATERIALS AND METHODS

4.1 Mating and genotyping

All animal experiments were approved by the University of Helsinki, Helsinki University Hospital, and the Southern Finland Council animal welfare and ethics committees.

NMRI wild-type (Wt) mice (Jackson Laboratories, USA) were maintained in Helsinki University, Experimental animal unit.

Fgfr2b null allele mice were obtained from mating heterozygous *Fgfr2b* mice, maintained on a pure C57BL/6 background and supplied by Dr. Clive Dickson's laboratory, Cancer Research, UK (De Moerlooze et al., 2000). For genotyping see De Moerlooze et al. (2000).

Fgf10$^{-/-}$ and Wt littermates were also kindly provided by Dr. Clive Dickson's laboratory, Cancer Research, UK (Min et al., 1998).

Gli3$^{Xt\text{-}J}$ mice were obtained from The Jackson Laboratory (stock No. JR0026: The Jackson Laboratory, Bar Harbour, ME) and maintained on a pure C57BL/6 background. Genotyping has been previously described by Maynard et al. (2002).

Runx2$^{+/-}$ mice, maintained on mixed NMRI/C57BL/6 background, were provided by Prof. Irma Thesleff's laboratory (University of Helsinki, Finland). For maintenance and genotyping see Åberg et al. (2004). *Gli3;Runx2* compound mutant mice were generated by mating *Gli3*$^{+/Xt\text{-}J}$; *Runx2*$^{+/-}$ mice.

The age of the embryos was determined by the day of the appearance of the vaginal plug (E0) and by morphological criteria.

4.2 Tissue culture and bead implantation assays

E15.5 calvaria were dissected from embryos, and the brain and the skin were removed. The explants were placed on Nucleopore polycarbonate filters supported by grids and cultured in Dulbecco's minimal essential medium (DMEM) (Sigma) supplemented with 10% bovine calf serum (Sigma), glutamax and penicillin/streptomycin (Sigma).

For bead assays heparin-coated acrylic beads (Sigma) were incubated in 25 ng/µl recombinant human FGF2, FGF10 (R&D Systems) or bovine serum albumin (BSA) at 37 °C for 40 minutes before being placed on the explant. Bead assays were cultured from 24 hours up to 4 days.

4.3 Histological analyses

For all the histologic staining embryonic tissue were fixed either in 4% paraformaldehyde (PFA) or 10% neutral buffered formalin (pH 6.8) at 4 °C overnight, embedded in paraffin and sectioned at 7 µm intervals. Before staining according to specific protocol, slides were dewaxed with Xylene and rehydrated in ethanol series.

4.3.1 Haematoxylin and eosin staining

Slides were stained with Mayer's Haematoxylin (Merck) for 30 seconds, followed by running water for 15 minutes. Then slides were washed with 95% ethanol and stained for 90 seconds in 1% EosinY solution. Slides were then dehydrated and mounted.

4.3.2 Alizarin red
Slides were stained with 2% Alizarin red S solution (pH 4.2) for 2 minutes followed by dehydration and mounting.

4.3.3 Alcian blue
Sections were stained with 1% Alcian blue 8GX solution (pH 2.5) (Sigma) for 30 minutes at room temperature following counterstaining by nuclear fast red.

4.3.4 VonKossa
Slides were treated with 5% silver nitrate solution ($AgNO_3$) under 60W bulb until black stain appeared in the bone matrix (for approximately 1 hour), rinsed with distilled water followed by 5% sodium thiosulphate for 3 minutes and rinsed again. The slides were counterstained with nuclear fast red.

4.3.5 Toluidine blue
Slides were rinsed in distilled water followed by 10 minute staining with 1% Toluidine blue and rinsed again.

4.3.6 Alkaline phosphatase
Sections were washed first in phosphate buffered saline (PBS) and circled with Dako pen (Dako). Slides were then washed three times with NTMT and then stained with NBT/BCIP (BM Purple, Roche) in the dark until the blue colour developed. The colour reaction was terminated by washing slides in PBS.

4.4 Skeletal staining

4.4.1 Alcian blue alizarin red skeletal staining
Prior to fixation in 95% ethanol (overnight) the skin is removed from the embryos. For cartilage staining the embryos were incubated overnight in alcian blue staining solution (1 volume glacial acetic acid, 4 volumes 95% ethanol and 150 µg/ml of alcian blue 8GX (Sigma)). Samples were then washed in 95% ethanol for 1 hour and then cleared in 2% KOH for 1-4 hours depending on the age. Tissues were transferred into alizarin red staining solution (1% KOH, 75 µg/ml alizarin red-S) for 2-15 hours to stain the bones. Samples were then cleared in 20% glycerol, 1% KOH and then stored in 50% glycerol, 50% ethanol.

4.4.2 Alizarin red staining for calvarial explants
Explants were fixed overnight in 95% ethanol and then bone was stained with 1% KOH with 75 µg/ml alizarin red. Explants were then cleared in 1% KOH in 20% glycerol for up to 3 hours. The stained explants were stored in 50% glycerol 50% ethanol.

4.5 BrdU incorporation

Measurement of the incorporation of the modified nucleotide (BrdU) during DNA synthesis was used to assay cell proliferation. For BrdU incorporation, pregnant females were injected intraperitoneally with 1 ml/100 g body weight of undiluted BrdU solution (Zymed). After 2 hours the mothers were sacrificed and the embryos collected, fixed in 10% neutral buffered formalin and embedded into paraffin.

For organ culture explants, BrdU solution was added to the medium (1:200) for 3 hours before fixation and paraffin embedding.

The BrdU staining kit (Invitrogen) was used, where BrdU incorporated cells were detected using biotinylated monoclonal anti-BrdU and visualized with streptavidin-biotin staining system according to the manufactures instructions. Sections were counterstained with haematoxylin.
BrdU-positive cells were counted in an area defined by a grid.

4.6 *In situ* hybridisation

4.6.1 ^{35}S in situ hybridisation

Paraffin section (7 µm) were deparaffinised in xylene and rehydrated in ethanol series. Tissue sections were permeabilised with 7 µg/ml proteinase K and fixed with 4% PFA for 20 minutes. To prevent background and non-specific binding of probes to the slides, the slides were treated with acetic anhydride. Tissues were hybridised overnight at 52 °C with ^{35}S-UTP labelled riboprobes (Table 1). Hybridisation was followed by high stringency washes; 30 minute in Wash 1 (5xSSC, 10 nM DDT) at 50 °C and 1 hour in Wash 2 (50% deionised formamide, 2xSSC, 20 mM DTT) at 65 °C. Slides were then washed in NTE (500 nM NaCl, 10 mM Tris-HCl, 5 mM EDTA) at 37 °C and treated with ribonuclease A (20 ng/ml in NTE) to remove non-specifically bound and excess probe. Following 30 minutes in Wash 2, and then 2xSSC and 0.1xSSC 15 minutes each, the tissue was dehydrated in ethanol series. The slides were coated with autoradiography liquid emulsion NTB (Kodak) diluted 1:1 with H_2O and exposed in a dark box for 10-18 days at 4 °C. The slides were developed and fixed (Kodak) in a dark room and then counterstained with haematoxylin.

4.6.2 Whole mount in situ hybridisation

Calvaria were dissected from E15.5 aged embryos and fixed in 4% PFA overnight. Explants were bleached in 6% H_2O_2 for 1 hour and then treated with 10 µg/ml proteinase K (Sigma) in PBT for 10 minutes at 37 °C. Tissues were then washed in 2 mg/ml glycine in PBST followed by PBST and then fixed in 4% PFA for 20 minutes. Tissues were prehybridised in PBST and hybridisation buffer (1:1) and then in hybridisation buffer (50% deionised formamide, 5xSSC pH5, 1% SDS, 50 µl/ml yeast tRNA, 50 µg/ml heparin in sterile DEPC treated H_2O) for 2 hours. The aliquot of Digoxigenin-labelled probe was denatured at 80 °C for 5 minutes and chilled on ice before mixed with hybridisation buffer at a concentration of 1 µg/ml (Table 1). Tissues were hybridised at 64 °C overnight followed by stringency washes; first three times for 30 minutes at 70 °C in Wash 1 (50% deionised formamide, 5xSSC pH4.5, 1% SDS) and then three times for 30 minutes in Wash 2 (50% deionised formamide, 5xSSC pH4.5). Next, explants were washed three times in MABT for 5 minutes and pre-blocked with 2% BBR (Roche), 10% heat activated goat serum and 200 mM levamisole in MAB for 3 hours at room temperature. Then tissues were washed in 2% BBR, 1% goat serum, 200 mM levamisole and anti-Dig-antibody coupled to alkaline phosphatase (Roche) at a dilution of 1:2000 with MAB overnight at 4 °C. Tissues were washed in MABT for 3 x 5 minutes followed by 5 x 1 hour and then left in MABT at 4 °C overnight. The following day the explants were washed three times in NTMT (100 mM).

Table 1. Probes used in *in situ* hybridisations in Studies I-V.

Probe	***Reference***	***Used in***
Bmp2	Åberg et al., 1997	IV
Dhh	Rice et al., 2006	I
Dlx5	Liu et al., 1997	IV
Fgf3	Kettunen et al., 2000	III
Fgf7	Kettunen et al., 2000	III
Fgf10	Kettunen et al., 2000	III
Fgfr2IIIb	Kettunen et al., 1998	III
Fgfr2IIIc	Kettunen et al., 1998	III
Gli1	Rice et al., 2006	V
Gli2	Rice et al., 2006	IV, V
Gli3	Rice et al., 2006	I, IV, V
Gli3_whole mount	I	I
Ibsp	Rice et al., 1999	I, II
Ihh	Rice et al., 2006	I, IV, V
Msx2	Rice D et al., 2003	IV
Noggin	McMahon et al., 1998	IV
Oc	Rice D et al., 2003	IV, V
Osx	Rice D et al., 2003	V
Ptch1	Rice et al., 2004	I, IV, V
Runx2	Rice D et al., 2003	I, II, IV, V
Runx2-I	IV	IV
Shh	Rice et al., 2004	I
Twist1	Rice et al., 2000	I

4.7 Protein isolation and immunoblotting

Calvaria were dissected from E15.5 Wt embryos and tissue samples taken from OFs of the frontal bone and from interfrontal suture respectively. Tissue samples were pooled from three calvaria of the same litter. Brain tissue of same aged Wt and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ embryos were used as controls. Samples were lysed in radio-immunoprecipitation assay buffer (RIPA, Sigma-Aldrich) supplemented with Complete protease inhibitor mixture (Roche Diagnostics). Protein concentration was determined with Pierce BCA protein assay kit (Thermo) and 10 µg of each sample was probed for anti-GLI3 polyclonal antibody (AF3690, R&D Systems, Minneapolis, MN), anti-GLI1 monoclonal antibody (Cell Signalling) and anti-α-tubulin antibody (DM1A, Sigma-Aldrich). The signals were quantified by Odyssey detection system (LI-COR).

4.8 Immunohistochemistry

Whole heads of embryos aged E15.5 were dissected, fixed with 4% PFA at 4 °C overnight, and sectioned at 7 µm intervals. Tissue sections were deparaffinised and rehydrated. Sections were heated in the microwave in citric acid (pH 6.0). Sections were then washed in PBS and endogenous

peroxidase activity was blocked with H_2O_2 in methanol for 30 minutes at room temperature. Sections were then incubated with primary antibody: Polyclonal anti-phospho-Smad1/Smad5/Smad8 (Ser463/465) antibody (Millipore, Temecula, CA) overnight at 4 °C followed by the secondary antibody: anti-rabbit biotinylated. Immunoreactivity was visualized with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with haematoxylin.

4.9 Calvarial osteoblasts and siRNA treatment

E15 NMRI mouse calvaria were dissected and separated from the overlying skin and the underlying meninges. Calvarial cells were isolated by four sequential trypsin-treatments. After 15 minutes 0.25% trypsin incubation, the first patch of cells was discarded, and the cells from the following trypsin treatments were pooled and cultured in T75 cell culture bottles in DMEM (Lonza, supplemented with 100 M Na-pyruvate, 2 mg/l L-glutamine, 1% penicillin-streptomycine, 10% FBS). At passage 2 the cells were transfected with control siRNA (Ambion Silencer Select control #1 siRNA, 4390843) and anti-RUNX2 siRNAs (Ambion Runx2 Silencer Select Pre-designed siRNA, 4390771) using Lipofectamine RNAiMAX reagent (Life Technologies, 13778) according to the manufacturer's instructions. Cells were kept in the siRNA transfection complex for three days, after which the medium was changed to osteogenic medium (DMEM (Lonza) supplemented by 100 M Na-pyruvate (Lonza), 2 mg/l L-glutamine (Lonza), 10% FBS, 10 mM -glycerophosphate (Sigma) , 50 g/ml ascorbic acid (Sigma), and 100 ng/ml BMP2 (R&D Systems)) for 24 hours. For western blotting the cell samples were lysed in radio-immunoprecipitation assay buffer (RIPA, Sigma-Aldrich) with Complete protease inhibitor mixture added (Roche Diagnostics).

4.10 Statistical analyses

One-way ANOVA and independent samples *t*-test were used for the statistical analysis of normally distributed samples. When ANOVA was used for multiple comparisons, in order to determine which groups were different from which, Post Hoc tests were performed using the Bonferroni corrections in order to adjust from an inflated probability of a type I error. Mann-Whitney tests were chosen for non-normal samples. A *p*-value of less than 0.05 was considered statistically significant.

Table 2. Methods used in this study.

Method	***Used in***
BrdU incorporation	I, III, IV
Calvarial osteoblasts and siRNA treatment	V
Histological analyses	
Alcian blue	I
Alizarin red	I
Alkaline phosphatase	II
Haematoxylin Eosin staining	I, III
Toluidine blue	II
VonKossa	I
In situ hybridisation	
Preparation of probes	I, IV
Radioactive	I, II, III, IV, V
Whole mount	I, IV, V
Immunohistochemistry	IV
Mating and genotyping	I, II, III, IV, V
Protein isolation and immunoblotting	IV, V
Skeletal staining	I, II, IV, V
Statistical analyses	I, II, III, IV, V
Tissue culture and bead implantation assays	I

5. RESULTS AND DISCUSSION

5.1 Expression of Hh signalling pathway members in the Wt mouse calvaria (I, IV, V)

The fundamental role of Hh signalling during endochondral ossification prompted us to investigate its role during intramembranous ossification of calvarial bones. We studied the expression of Hh pathway members during the embryonic development of the mouse calvaria by *in situ* hybridisation (I, III, IV) (Figure 7). *Ihh* was the only Hh ligand detected, *Shh* and *Dhh* were not expressed (I). At E13.5 expression patterns were investigated at the frontal bone primordia. *Ihh* expression was seen as a narrow strip at the medial edge of the frontal bone primordia.

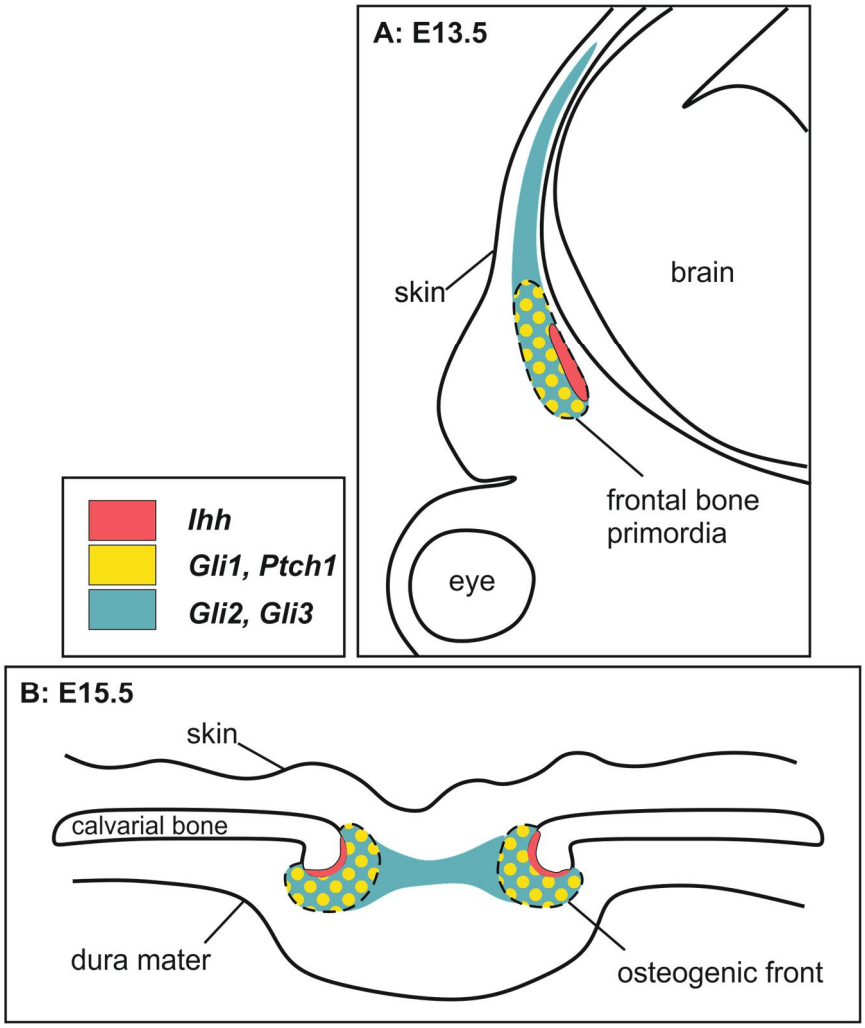

Figure 7. Schematic diagram of the expression pattern of Hedgehog (Hh) pathway members E13.5 in the frontal bone primordia at E13.5 (A) and in the sagittal suture at E15.5 (B).
A: At E13.5 *Ihh* is expressed in the border of the frontal bone primordia. *Gli1* and *Ptch1* are detected across the frontal bone primordia, while *Gli2* and *Gli3* are expressed in the frontal bone primordia as well as in the undifferentiated mesenchyme apically.
B: At E15.5 *Ihh* is detected again at the border of osteogenic front by restricted group of cells. *Gli1* and *Ptch1* are expressed across the whole osteogenic front. *Gli2* and *Gli3* are detected in the osteogenic front, as well as across the whole sutural mesenchyme.

The transmembrane receptor *Ptch1* and transcription factor *Gli1* were intensely expressed across the whole frontal bone primordia. At E15.5 *Ihh* was expressed at the OFs of the calvarial bones. Compared to *Ptch1* and *Gli1*, also expressed in the OF, the expression domain was again restricted to a small group of cells.

mRNA levels of Hh pathway members that we detected are concise with results previously described by Abzhanov et al. (2007). They showed that in dermal bones/mandible *Ihh* is expressed by more mature osteoblasts, while *Ptch1* and *Gli1* are detected in preosteoblasts.

We also investigated expression of the important mediators of Hh signalling: *Gli2* and *Gli3*. Both *Gli2* and *Gli3* had a diffuse expression pattern. At E13.5 they were detected across the frontal bone primordia, but expression was also seen more apically, in the undifferentiated mesenchymal cells. Two days later *Gli2* and *Gli3* were detected in the OFs as well as in the suture mesenchyme, where *Gli3*'s expression was more intense. Furthermore, expression of *Gli3* was strongest in the interfrontal suture and at the lateral edges of the interparietal bone (I).

In conclusion, *Ihh* was expressed by restricted group of more mature osteoblasts, but Ihh protein affects preosteoblasts in the frontal bone primordia at E13.5 and in the OF at E15.5 as *Ptch1* and *Gli1*, the direct transcriptional targets of Hh signalling, are expressed by these cells. The expression of *Gli2* and *Gli3*, on the other hand, extends to a broader area indicating that they function in cells that are devoid of the Hh signal, in less differentiated mesenchymal cells. It is known that *Gli2* and *Gli3* are not direct targets of Hh signalling, but what activates their transcription remains ambiguous. There is evidence that Wnt signalling is able to activate *Gli3* transcription in the spinal cord and in the retina (Alvarez-Medina et al., 2008; Yu et al., 2008; Borday et al., 2012).

5.2 Loss of *Gli3* causes craniosynostosis in mice (I, II, IV, V)

Elevated Hh signalling is associated with craniosynostosis in humans as loss-of-function mutations in Hh-repressor *RAB23* cause carpenter syndrome representing premature suture fusion as a feature (Jenkins et al., 2007). Recent evidence also indicates that GCPS caused by loss-of-function mutations in *GLI3* occasionally features premature fusion of the metopic suture (McDonald-McGinn et al., 2010; Hurst et al., 2011). To investigate effects of disturbed Hh signalling in the calvaria we used $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mouse as a model (I, II, IV, V). Interestingly, on the contrary to the human patients, bilateral craniosynostosis of the lambdoid suture was a 100% penetrant feature of $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice. Premature fusion of this suture was detected from E16.5 onwards (I, IV, V). The interfrontal suture was also abnormal. Paradoxally, the frontal bone margins remained wider apart, but ectopic ossification was detected in the interfrontal suture from E16.5 onwards. In some specimens this lead to premature fusion of the interfrontal suture by E18.5 (II). As $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice die at birth we could not verify if additional high percentage of the interfrontal sutures would fuse later. The coronal suture remained patent in $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice. The sagittal suture was wider compared to Wt samples.

The morphology of all the calvarial bones was abnormal in $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice. The size of the interparietal bone was larger compared to the Wt sample and lateral margins of the bone extended more ventrally. The architecture of the frontal bones differed significantly, overall bone area being greater compared to the Wt sample. The shape of the parietal bones was also abnormal, the size being fractionally smaller. In conclusion the morphology of the whole calvaria was altered in $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice. The integrity of the lambdoid and interfrontal sutures was lost and the shape as well as the size of all the bones was abnormal.

In many aspects $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice model GCPS (Vortkamp et al., 1992). In regard to the calvarial development, recent evidence indicates that craniosynostosis of the metopic suture is an occasional feature of GCPS (McDonald-McGinn et al., 2010; Hurst et al., 2011). Craniosynostosis of the lambdoid suture, which is a fully penetrant feature of $Gli3^{Xt\text{-}J/Xt\text{-}J}$ mice, is not seen in GCPS

patients. This might be explained by the very early fusion of the intramembranous squamous part of the occipital bone (interparietal) with the endochondral supraoccipital bone. In study II we show that *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice present heterotopic ossification also in the interfrontal suture that in severe cases leads to its premature fusion. As in humans, the phenotype in mice varies; at E18.5 only part of the interfrontal sutures were fused. The phenotype in mice varies depending on the amount of heterotopic ossification in the interfrontal suture, but in all of the studied *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice the frontal bone morphology was abnormal and heterotopic ossification was seen in all samples at E18.5. Premature metopic suture fusion in GCPS patients may have also been underdiagnosed as metopic suture fuses early in humans, beginning already at 1 year of age.

Heterotopic ossification detected in the interfrontal suture of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice resemble Wormian bones, which arise from abnormal ossification centres in the calvaria that develop in addition to those present normally. Recent evidence indicates that Wormian bones may be associated with craniosynostosis and it is thus possible that the heterotopic ossification seen in the interfrontal suture is secondary phenomenon caused by the lambdoid synostoses. Studies, however, suggest that that Wormian bones always develop in a specific site in relation to the synostosis. In case of unilateral lambdoid synostosis, for example, Wormian bones are detected on the contralateral side. In case of midline metopic synostosis, Wormian bones form along the midline of the calvaria (Sanchez-Lara et al., 2007).

5.2.1 Gli3 affects both proliferation and differentiation of osteoblasts (I, II, IV)

Osteoprogenitor proliferation affects the size of the calvarial bones. We found that loss of *Gli3* significantly increased proliferation of cells in the OFs as well as in the suture mesenchyme of the interfrontal and lambdoid sutures in mice at E15.5 (I, IV). In concordance, the size of the frontal as well as interparietal bones was also enlarged (V). Gli3 is thus needed to restrict proliferation in the calvaria and uncontrolled proliferation contributes to the abnormal calvarial bone size and shape.

Increased proliferation of CNC cells as well as PM cells already at the patterning stage may also contribute to the size of the bones in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice and could be further investigated. Excessive Hh signalling has been shown to lead directly to uncontrolled proliferation of neural crest cells causing hypertelorism; widely set eyes (Brugmann et al., 2010). Hypertelorism is a feature of GCPS as well as Gorlin syndrome, caused by mutations in *PTCH1* or *SMO* leading to ectopic Hh pathway activation (Aszterbaum et al., 1998; Xie et al., 1998). In fact, during mouse limb development, Gli3 has been shown to directly restrict the expression of regulators of the G_1–S cell-cycle transition and constrain S phase entry (Lopez-Rios et al., 2012).

We detected ectopic osteoblast differentiation in the sutural mesenchyme of interfrontal and lambdoid sutures at all stages of ossification studied. During frontal bone development ALP; an early marker of ossification was ectopically detected apically in relation to the frontal bone primordia in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice already at E13.5 (II). Later on at E15.5 *Runx2-II*, *Dlx5*, *Osx*, *Ibsp* and *Oc* were all expressed ectopically in the interfrontal suture (II, IV, V). In the lambdoid suture aberrant *Runx2-II* and *Dlx5* expression was also detected at E15.5, while *Twist1*, a repressor of Runx2, was downregulated (I, IV). Expression of *Runx2-I* was reduced especially in the interfrontal suture of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice at E15.5 indicating that the process of forming ectopic bones had already started at E15.5, one day before ectopic bones appeared (IV).

5.2.2 Abnormal cartilage formation in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice (I, IV)

We found abnormalities in cartilage formation to be associated with premature interfrontal and lambdoid suture fusion. Primary cartilage, ectocranial to the lambdoid suture, was thicker compared to the Wt specimens (I). Ectopic secondary cartilage was also detected in association with ectopic ossification of the interfrontal suture (II).

Transient cartilage formation has been associated previously with normal intramembranous ossification of calvarial bones, and chondrocyte markers Sox9 and type II collagen are normally detected in the calvaria (Markens, 1975; Åberg et al., 2005). Sahar et al. (2005) have shown that the posterior section of the interfrontal suture fuses postnatally by endochondral ossification. Posterior frontal suture fusion is not possible to study in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice as they die at birth. Cartilage formation has, however, also been identified in pathological situations. Heterotopic cartilage has been reported in sagittal suture of Apert syndrome mouse model (*Fgfr2*$^{+/S252W}$) prior to premature suture fusion (Wang et al., 2005). Altered mechanical forces in the dura mater have also been related to transient secondary cartilage rod formation (Solem et al., 2011). However, Gli3 may directly affect chondrocyte differentiation in the calvaria as during endochondral ossification Gli3 represses chondrocyte differentiation by restricting the amount of proliferating chondrocytes (Koziel et al., 2005).

5.2.3 Abnormal brain morphology of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice (II)

Gli3 has a well-established role in CNS development (Tole et al., 2000; Blaess et al., 2008). We investigated if the abnormal brain morphology correlated with the calvarial anomalies at E16.5 (II). Compared to the Wt brain we detected the following macroscopic morphological changes; the olfactory bulbs had failed to develop completely, the dorsomedial telencephalon was truncated and the diencephalon extended more anteriorly. The midbrain was expanded and the cerebellum was larger extending more ventrally.

We found the calvarial bone anomalies to correlate with the abnormal brain morphology. The frontal bones normally reside superior to the cerebral hemispheres and the interfrontal suture forms between the two frontal bones superior to the falx cerebri that separates the cerebral hemispheres. Frontal sections across the frontal bone area of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice confirmed that the falx cerebri was absent from between the cerebral hemispheres. Frontal bones were developing lateral to the forebrain, but no clear interfrontal suture was detected and ossification was observed across the midline. The sutural architecture is completely lost in the interfrontal suture. The enlarged and ventrally further extending cerebellum also corresponds with the wider interparietal bone.

On one hand, initially it was thought that the CNS defects of craniosynostosis patients were ultimately caused by compression due to the premature suture fusion. On the other hand, it has been suggested that the craniosynostosis could be secondary to the brain defects and caused by mechanical pressure changes transmitted by the dura mater (Faro et al., 2006). Several recent studies have attempted to shed light to this controversy by looking at craniosynostosis syndromes caused by elevated activation of *Fgfr* mouse models. These syndromes are characterised by both craniosynostosis and CNS anomalies. Comparison of mouse models of the two major Apert mutations found no correlation between brain phenotypes and the extent or pattern of coronal suture fusion (Aldridge et al., 2010). Holmes and Basilico (2012) further showed that coronal suture synostosis occurs when Apert syndrome causing mutation is limited to the PM indicating aetiological independence from the CNC derived dura mater. These findings suggest surprisingly high level of independence in the development of these organelles. However, our findings indicate clear

correlation between these structures. This tempts us to speculate that the development of the brain and the skull are synchronised at a very early stage during patterning of the tissues. Ablating *Gli3* conditionally either from the CNS or the calvarial mesenchyme by using *Gli3*^{flox} mutant mice that harbour *loxP* sites flanking exon 8 of *Gli3* (*Gli3*$^{tm1Alj/J}$) would provide us with a valuable tool in studying the role of these individual organelles in calvarial development (Blaess et al., 2008).

Novel findings on the role of Gli3R in the midbrain during palatal development reveal an early link between brain and face development. Fuzzy (Fuz) is responsible for normal trafficking of the retrograde intraflagellar transport, carried out by IFT43, for example (Gray et al., 2009; Brooks and Wallingford, 2012). *Fuz* null allele (*Fuz*$^{-/-}$) mice obtain a high-arched-palate. The palate develops from the first branchial arch formed by the CNC that arises from the posterior mesencephalon and rhombomere 1, where Fgf8 controls neural crest cell number (Osumi-Yamashita et al., 1994; Creuzet et al., 2004). Distal tip of the primary cilia is lost in *Fuz*$^{-/-}$ mice leading to attenuated Gli3 processing and subsequently to absence of Gli3R (Tabler et al., 2013). Gli3R is necessary to suppress *Fgf8* expression in the midbrain (Aoto et al., 2002). Loss of Gli3R disturbs palate development in *Fuz*$^{-/-}$ mice from the initial stage onwards as hindbrain fates and *Fgf8* expression domain are expanded. Consequently, elevated numbers of neural crest cells migrate to the BA1, and subsequently form enlarged maxillae. Furthermore, *Fuz*$^{-/-}$ mice display craniosynostosis, which is also linked to increased Fgf signalling (Tabler et al., 2013). *Fuz*$^{-/-}$ mice are reported to have fusion of the coronal suture as do mice and humans with hyperactive Fgf signalling. Interestingly, although calvarial phenotype was not the focus of Zhang et al. (2011) study of the on *Fuz*$^{-/-}$ mice, they published an image of the skeletal stained E18.5 aged calvaria, where the interparietal bone and lambdoid suture area show high resemblance to *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. Excessive *Fgf8* transcription and increased amount of neural crest cells may also have an effect in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. Specifically the bones that arise from the CNC, the frontal and interparietal bones, showed increased ossification in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice.

Koyabu et al. noted that in many species the interparietal bone fuses early to adjacent bones; either the supraoccipital or the parietal (Koyabu et al., 2012). Furthermore, they suggested that enlargement of the brain in relation to the body size among species coupled to the earlier fusion of the interparietal bone may be caused by changes in Tgfβ and/or Fgf signalling. Both pathways contribute to brain development and increased signalling of both pathways cause premature suture fusion. The same criteria apply for Hh signalling. Mounting evidence suggests that increasing Hh signalling by disruption of Hh-repressors causes suture fusion and, interestingly, in mice this specifically affects the interparietal bone.

5.3 Interaction of Gli3 and Fgf signalling during calvarial development (I, III)

Gli3 participates in tissue patterning and differentiation by regulating Fgf pathway. Gli3 controls patterning of the isthmus and cerebellum by restricting *Fgf8* expression domain (Blaess et al., 2008). During mammary gland development Gli3 induces and patterns the mammary placodes by activating Fgf10, which in turn stimulates canonical Wnt signalling through Fgfr2IIIb. Veltmaat et al. were able to rescue abnormal mammogenesis in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice by application of exogenous Ffg10 (Veltmaat et al., 2006). We therefore investigated if Fgf signalling acted downstream of Gli3 in the developing calvaria.

5.3.1 Role of Fgf signalling pathway in the calvaria (III)

Fgf signalling is involved in several stages of calvarial ossification. Activating mutations of *FGFR1, -2* and *-3* cause premature fusion of the coronal suture (reviewed by Passos-Bueno et al., 2008). Several Fgf ligands are expressed in the calvaria. In study III we investigated the role of Fgfr2 splice variants; Fgfr2IIIb and Fgfr2IIIc, during craniofacial development. Fgfr2IIIb and its ligand Fgf10 govern tooth and palate development, while Fgfr2IIIc and an important ligand Fgf2 have a role in calvarial development. We show in study III that both receptor variants of Fgfr2 and their ligands *Fgf2* and *Fgf10* are, however, all expressed in the frontal bone primordia of Wt calvaria at E13.5. Although, in physiological situation Fgf2 is the predominant ligand in the calvaria, which signals through the receptor Fgfr2IIIc, in pathological situations Fgf10 is present and can utilize the ectopically expressed receptor Fgfr2IIIb.

5.3.2 Fgf2 rescues craniosynostosis in the *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ calvaria (I)

To examine if Fgf signalling acted downstream of Gli3 in the developing calvaria we applied Fgf2 and Fgf10 impregnated beads in the lambdoid suture of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice aged E15.5 in tissue culture. Fgf2 was, indeed, able to prevent the lambdoid suture synostoses of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice, while Fgf10 or BSA-control beads had no effect. Fgf2 was able to reduce elevated osteoblastic progenitor proliferation in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ lambdoid suture to a normal level. We also showed by *in situ* hybridization that Fgf2 additionally prevented osteoblastic differentiation by restoring *Twist1* expression in the lambdoid suture. Our results suggest that in the lambdoid suture Gli3 inhibits osteoblast differentiation by activating Fgf2, which in turn represses Runx2 activation by inducing *Twist1* expression.

The effects of Fgf signalling on osteoblast differentiation depend on the differentiation stage targeted. In immature calvarial osteoblasts Fgf2 decreases calvarial mesenchymal proliferation and inhibits osteoblastic differentiation, while Fgf applied on OFs, on the other hand, accelerates suture closure as the cells targeted are already partially differentiated into osteoblasts. Rice et al. (2000) have previously shown that Fgf2 has an inhibitory effect on calvarial ossification by activating *Twist1* expression.

We suggest that in the lambdoid suture Gli3 interacts, either directly or indirectly, with Twist1 to inhibit osteoblastic differentiation as Twist1 binds to the Runt DNA-binding domain of the Runx2 protein to inhibit its function (Bialek et al., 2004). Gli3 and Twist1 are known to co-operate also during limb patterning as *Twist1*$^{+/-}$;*Gli3*$^{+/Xt\text{-}J}$ compound mutant mice display a more severe polydactyly than that seen in either *Twist1*$^{+/-}$ or *Gli3*$^{+/Xt\text{-}J}$ single mutant mice (O´Rourke et al., 2002). Furthermore, Twist1 is also required to inhibit *Shh* expression in the anterior limb bud (Zhang et al., 2010). This suggests that Twist1 may also have a role in restricting Hh ligand (Ihh) activation during calvarial ossification.

5.4 Genetically reducing *Runx2* expression from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice prevents premature suture fusion (IV)

Abnormal expression pattern of *Runx2* was identified in the lambdoid suture of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice in the study I. *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ calvaria is characterised by excess ossification, while *Runx2*$^{+/-}$ mice have truncated calvarial bones. Runx2 and Gli family proteins are also known to interact during ossification. Runx2 has been shown to physically interact with Gli2, and Ihh promotes osteoblast

differentiation by initiating *Runx2* expression, which is mediated by Gli2 (Shimoyama et al., 2007). Gli3R, on the other hand, has also been shown to inhibit the function of Runx2 by competing for the same DNA binding site (competitive inhibition) (Ohba et al., 2008).

This prompted us to investigate if Gli3 is necessary in restraining Runx2 function in the calvaria, and if Runx2 dosage is important in maintaining the correct balance of osteogenesis in OFs of calvarial bones. We generated *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{+/-}$ compound mutant mice to reduce the dosage of *Runx2* in the calvaria. Interestingly, lambdoid as well as interfrontal sutures remained patent in these mice at E18.5. The frontal, parietal and interparietal bones all showed more hypoplasia compared to *Runx2*$^{+/-}$ calvaria (IV). As anticipated, osteoblasts failed to differentiate in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{-/-}$ mice, similar to *Runx2*$^{-/-}$ mice.

Runx2 has a fundamental role in controlling lineage-specification as well as cell proliferation during ossification (Young, et al. 2007). We found that increased proliferation detected in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ lambdoid and interfrontal sutures was indeed reduced to a normal level in corresponding *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{+/-}$ sutures. Furthermore, ectopic expression of genes related to osteoblast differentiation: *Runx2*, *Dlx5* and *Oc*, seen in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ lambdoid and interfrontal sutures, was also absent in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{+/-}$ mice (IV).

It is noteworthy that premature activation of *Runx2* expression in cranial mesenchyme using the paired related homeobox 1 promoter, which directs the transgene expression to limb bud and cranial mesenchyme from E9.5 onwards, causes early onset of mineralization, as well as complete destruction of the calvarial morphology and craniosynostoses of multiple sutures and fontanelles by E18.5 (Maeno et al., 2011). Increased number of *RUNX2* alleles also in humans has been reported to cause multiple craniosynostoses involving the coronal, sagittal and lambdoid sutures (Varvagiannis et al., 2013; Greives et al., 2013).

5.4.1 Gli3 acts as a gatekeeper to control the differentiation of osteoprogenitors by regulating Bmp signalling cascade (IV)

Elevating Bmp signalling in the calvaria, either by deleting *Noggin* or by constitutively activating *BMP type IA receptor* in the CNC, causes craniosynostosis (Warren et al., 2003, Komatsu et al., 2013). Gli3R, on the other hand, has been shown to directly repress *Bmp2* transcription during osteoblast differentiation (Garrett et al., 2003). Evidently, Bmp2 and Bmp4 specifically induce activation of Dlx5, which in turn activates *Runx2-II* expression. As we found *Dlx5*, as well as *Runx2-II*, to be ectopically expressed in the affected sutures of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ calvaria, and removal of one allele of *Runx2* from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice normalized expression of both of these genes, we next investigated Bmp signalling in these mice (IV). We found *Bmp2* and *Bmp4* to be also ectopically expressed in the interfrontal and lambdoid sutures of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. Furthermore, higher phosphorylation of Smad1/5/8, which indicates elevated Bmp signalling, was detected in the corresponding locations. Ectopic *Bmp* expression, as well as elevated phosphorylation of Smad1/5/8, was normalized in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{+/-}$ sutures (IV).

Mounting evidence indicates that Gli3 is able to restrict Runx2 function, but the question that remains is how is *Runx2-II* isoform ectopically activated in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ calvaria? Our results indicate that Gli3 acts as a gatekeeper to restrict the progenitors of osteoblasts from differentiating in the calvaria by regulating Bmp-dependent activation of *Dlx5* and *Runx2-II*. Bmp signalling is required for CNC derived mesenchyme to commit to osteogenic pathway (Abzhanov et al., 2007). Dlx5 also regulates determination of CNC cell fate and in the calvaria CNC contributes to the affected frontal and interparietal bones. Although *Dlx5*$^{-/-}$ mice develop truncated calvarial bones in which *Runx2* is expressed, if both *Dlx5* and *Dlx6* are deleted, calvarial bones fail to form indicating redundancy in

Dlx-gene function (Depew et al., 2005). Just recently, Dlx5 has been shown to bind directly to an enhancer that specifically directs *Runx2-II* expression to osteoblast lineage cells. Furthermore, Smad1 as well as Dlx5 are part of the enhanceosome that activate this enhancer (Kawane et al., 2014). It is noteworthy that expression of *Dlx5* and *Bmp2* are both downregulated in the *Runx2*$^{+/-}$ calvaria and *Runx2*$^{-/-}$ mouse does not express *Dlx5* nor *Bmp2*, which could affect decreased expression of these genes in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Runx2*$^{+/-}$ mice. However, the significant finding is that ectopic activation of Runx2-II corresponds with the *Gli3* expression pattern. In study I we showed that *Gli3* expression is strongest in the interfrontal suture and at the lateral edges of the interparietal bone, and in study IV ectopic *Runx2-II* activation was specifically detected at these locations in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. Furthermore, by genetically reducing *Runx2* from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice we were able to specifically prevent the ectopic expression of *Runx2-II* at the interfrontal and lambdoid sutures.

Increasing evidence indicates that Hh signalling controls Bmp signalling. Lenton et al. (2011) have showed that loss of *Ihh* leads to a reduction of Bmp signalling in the calvaria. This is in concordance with our results concerning the increased Bmp signalling in the absence of *Gli3*, as when Ihh is not present the amount of Gli3R compared to Gli3FL is increased, and so Bmp signalling is hindered. In the limb bud Gli3 also regulates digit patterning by influencing Bmp signalling pathway. Gli3 inhibits the expression of the Bmp antagonist *Gremlin1* to allow the proliferating progenitors to exit toward Bmp-dependent chondrogenic differentiation (Lopez-Rios et al., 2012).

5.5 Evidence that Gli3R – Runx2 – Ihh –feedback loop controls intramembranous ossification of calvarial bones (V)

We and others have identified Ihh as the only Hh ligand present during embryonic calvarial bone development (I; Jacob et al., 2007; Kim et al., 1998). We thus wanted to study the interaction of Ihh and Gli3 during calvarial development.

5.5.1 Genetically reducing the dose of *Ihh* does not alter the *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ calvarial phenotype (V)

Absence of *Ihh* causes delayed calvarial ossification (Abzhanov et al., 2007; Lenton et al., 2011). Ihh has been shown to activate *Runx2* expression during ossification (Shimoyama et al., 2007), while in study IV we were able to rescue craniosynostosis in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice by reducing *Runx2* dosage. This prompted us to investigate whether by deleting *Ihh* from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice we could mimic the effect of partial *Runx2* deletion. We performed skeletal staining on *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$, *Ihh*$^{-/-}$ and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice aged E16.6, E17.5 and E18.5. Calvarial bones of *Ihh*$^{-/-}$ mice were smaller and the calvarial sutures wider compared to the Wt samples at all stages studied. Surprisingly, additional deletion of *Ihh* from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice had no effect on the calvarial phenotype. Lambdoid and interfrontal sutures fused prematurely in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice and morphology as well as size of calvarial bones was similar to that in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice (V).

We also studied osteoblast differentiation in the frontal bones at a tissue level by looking at expression patterns of early osteoblast markers; *Runx2* and *Osx* and late osteoblast markers; *Ibsp* and *Oc* in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$, *Ihh*$^{-/-}$ and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice at E15.5. Osteoblasts differentiated normally in all of these mice as all markers were expressed. All of the markers were also detected in the ectopic bones of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice already at this early stage. This also infers that the molecular toolbox used to control osteoblast development and therefore bone development of the

heterotopic bone is normal (V). These sections also confirmed that the frontal bones of *Ihh*$^{-/-}$ mice were small and interfrontal suture was wide compared to the Wt sample.

The analogous phenotype of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice prompted us to examine *Ptch1* and *Gli1* expression in the calvaria of *Ihh*$^{-/-}$, *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ and *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice to establish the level of Hh signalling (V). Expression of *Ptch1* and *Gli1* is considered the read-out of Hh signalling. mRNA of *Ptch1* and *Gli1* was detected in the frontal bones of Wt mouse at E15.5. In *Ihh*$^{-/-}$ calvaria *Ptch1* and *Gli1* were not expressed, confirming that Ihh is the only Hh ligand present in the calvaria at this stage. In *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice *Ptch1* and *Gli1* expression was comparable to the Wt, although they were ectopically expressed also in the ectopic bones seen in the interfrontal suture. Interestingly, in the double mutant mice, *Ptch1* and *Gli1* mRNA were not detected indicating that Hh signalling does not have a role in the aetiology of craniosynostosis during the stage of osteoblast differentiation.

Shh has a fundamental role in patterning of the head. Although *Shh* is not expressed in the calvarial tissue during the foetal period of the ossification phase, it is known that Shh affects early events of calvarial development as deletion of *Shh* from neural crest cells disrupts frontal bone development (Jeong et al., 2004). Furthermore, an interesting recent finding indicates that calvarial mesenchymal progenitor cells express *Gli1* transiently from E7.5 to E8.5 (Deckelbaum et al., 2010). It is thus probable, that calvarial development of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice is also affected by reduced repression of molecular pathways regulated by Shh signalling during early stages of head development. Calvarial phenotype of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Shh*$^{-/-}$ mice has not been published, but additional deletion of *Gli3* from *Shh*$^{-/-}$ mice has a considerable, positive impact on head development (Rallu et al., 2002; Litingtung and Chiang, 2000). Interestingly, additional deletion of the Hh co-receptor *Gas1* from *Shh*$^{+/-}$ mice (*Gas1*$^{-/-}$;*Shh*$^{+/-}$ mice) causes premature fusion of the coronal suture, which is not seen in *Gas1*$^{-/-}$ mice (Seppälä et al., 2007), indicating that reduction of *Shh* activity may also have an effect on suture patency. Heterozygous *Shh* mice have not been reported to have calvarial abnormalities.

Gli1 is a direct transcriptional target of Hh signalling. Our results indicate that absence of *Gli1* from the calvaria during prenatal calvarial ossification has no effect on embryonic suture patency as neither *Ihh*$^{-/-}$ nor *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice express *Gli1* in the embryonic calvaria (V). Interestingly, recent data by Zhao et al. (2015) show that ablation of *Gli1* in mice at one month of age postnatally leads to fusion of all calvarial sutures in two months. This is evidently due to the loss of MSCs originating from the postnatal sutures. From one month of age *Gli1* expression is restricted to the midsutural cells and Ihh, secreted from the OFs, regulate differentiation of these *Gli1*-positive MSCs. They further propose that craniosynostosis is caused by loss of these MSCs from the calvarial suture (Zhao et al., 2015). These data indicate that embryonic and postnatal regulation of suture patency differs profoundly, which further highlights the diverse aetiology of craniosynostosis.

5.5.2 Gli3R regulates osteogenic fate at the periphery of the osteogenic front (IV, V)

During endochondral ossification Gli3R does not function in physiological situation as Ihh has an indispensable role in inhibiting Gli3R formation. In pathological situations, i.e. in the absence of *Ihh*, Gli3R is responsible for many aspects of endochondral ossification defects (Koziel et al., 2005). During limb patterning, on the other hand, Gli3R functions independently, prior to Shh activation (te Welscher et al., 2002). The fact that additional deletion of *Ihh* from *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice did not affect the calvarial phenotype as well as absence of Hh signalling in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$;*Ihh*$^{-/-}$ mice corresponded to the findings concerning the limb bud patterning in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mice (te Welscher et al., 2002; Hill et al., 2009), prompted us to hypothesise that Gli3R has a significant role in the calvaria. Furthermore,

in the mouse model of PHS, a syndrome caused by mutations in *GLI3*, where only the level of Gli3FL is reduced, while Gli3R is unaffected, calvarial sutures remain patent (Böse et al., 2002).

As Gli3 isoform switching occurs at a post-transcriptional level the distribution of the isoforms is not possible to study at mRNA level. In study IV we found Gli3R to be the predominant isoform present in the calvaria by Western blot analysis using an antibody that recognises both the R- and the FL-isoforms. The presence of Hh ligand determines Gli3 isoform distribution and the expression pattern of *Ptch1* and *Gli1*, considered the read-out of Hh signalling, indicated that Gli3 is in the FL-form in the OFs. Early preosteoblasts that reside further away from *Ptch1/Gli1* expression domain, in the sutural mesenchyme, also expressed *Gli3* (I, V). These cells should obtain Gli3 in the R-isoform. In study V we confirmed this by comparing Gli3-isoform distribution in explants obtained from frontal bone OFs and interfrontal sutural mesenchyme, respectively, by Western blot analysis. Gli3R was present in higher levels in the sutural mesenchyme of the interfrontal suture compared to the OFs of the frontal bones (V).

Our results suggest that Gli3R functions prior to *Ihh* activation in the primary stage of osteoblast differentiation at the very edge of the mesenchymal condensations, where mesenchymal cells are committed to an osteogenic fate, in the periphery of the OFs. In these initial, very early preosteoblasts Gli3R restricts proliferation and refrains further differentiation possibly by activating *Fgf2* expression (I), repressing *Bmp2* expression (IV), as well as by restraining Runx2-I isoform from binding to DNA (Ohba et al., 2008).

Ihh, on the other hand, is expressed in the OFs of calvarial bones, but only by a subpopulation of cells: in more mature osteoblasts (Abzhanov et al., 2007; V). Ihh acts as a gatekeeper at the boarder of mature bone and the OF, like Abzhanov et al. (2007) postulated, from where it signals to the cells of the OF to allow them to differentiate into osteoblasts (Jacob et al., 2007; Lenton et al., 2011). We propose that Ihh does this ultimately by inhibiting the processing of Gli3 into the R-isoform. Our findings draw individual and somewhat contradicting findings together in building a unifying model of the role of Ihh during calvarial ossification.

Hh controls cell patterning and cell differentiation in many embryonic tissues by acting as a long-range morphogen. Briscoe and Thérond state in a recent review that Gli activity, controlled by Hh signalling, is responsible for patterning many tissues. It is necessary to regulate both the level and the timing of Gli activity to influence when and where genes are activated. Different Hh ligand concentrations result in a Gli activity gradient, which has been postulated to contribute to tissue patterning activity. The level of the R to FL ratio of Gli proteins activates different genes. The duration of Hh signalling is also important (Briscoe and Thérond, 2013).

Several mouse models that have recently been published also underline the fundamental role of Gli3R in calvarial morphogenesis. Feng et al. (2013) introduced an ENU-induced recessive mouse model, *Ptch1*^{DL}, in which DL represents a hypomorphic allele of *Ptch1*. These mice show very similar calvarial phenotype as *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. Reduction of Ptch1 function leads to decrease in Gli3R as Hh signalling is activated in the absence of Hh ligand. *Fuz*$^{-/-}$ mouse is another example of reduced production of Gli3R causing craniosynostosis (Tabler et al., 2013). Loss-of-function mutations in *RAB23* also cause craniosynostosis in humans (Jenkins et al., 2007). Rab23 is a negative regulator of Hh signalling possibly by precisely promoting the generation of Gli3R (Eggenschwiler et al., 2006). Regard et al. (2013) have shown that deletion of *Gnas* from mice causes heterotopic ossification. *Gnas* encodes $G\alpha_s$, which is a physiological activator of PKA, also an inhibitor of Hh signalling. They show that Hh signalling activation due to loss of $G\alpha_s$ signalling is both necessary and sufficient for heterotopic ossification through the intramembranous mechanism and also speculate that intramembranous bone formation in the calvaria may be promoted by ligand-independent activation of Hh signalling (Regard et al., 2013).

Transcriptional control of *Gli3* in the calvaria remains elusive. In the spinal cord as well as the retina Wnt signalling has been shown to repress Hh signalling by activating *Gli3* expression (Alvarez-Medina et al., 2008; Yu et al., 2008; Borday et al., 2012). Furthermore, primary cilia have also been linked to Wnt signalling. Brugmann et al. (2010) found that the loss of *Kif3a* caused truncated primary cilia on CNC cells associated with downregulated ß-catenin-dependent Wnt signalling, as well as reduced *Gli3* expression levels. They further speculated if primary cilia would integrate Wnt and Hh signals, thereby regulating *Gli* expression (Brugmann et al., 2010). Interestingly, it has also been proposed that Wnt activation is required for calvarial suture patency, while Wnt inhibition results in ectopic bone formation within the suture mesenchyme and suture closure (Behr et al., 2010).

5.5.3 Runx2 is able to activate *Ihh* expression in the calvaria (V)

During chondrocyte maturation Runx2 enhances chondrocyte proliferation by directly inducing *Ihh* expression (Yoshida et al., 2004). Gli3R, on the other hand, interacts with Runx2 at many levels to inhibit its function (Ohba et al., 2008; I; IV). This prompted us to examine the possibility that Runx2 would activate *Ihh* expression also during calvarial intramembranous ossification as *Runx2* expression precedes *Ihh* expression in the differentiating osteoblast lineage. We isolated E15.5 calvarial primary osteoblasts and restricted *Runx2* expression in these cells by anti-Runx2 siRNA while inducing osteoblast differentiation. We found Ihh protein levels significantly downregulated in these cells compared to the control group (V).

We propose that in the OFs of calvarial bones Ihh-Gli3-Bmp2-Dlx5-Runx2-Ihh feedback loop regulates ossification. Gli3R restricts osteoblast differentiation in the sutural margin of the OF by repressing bone specific *Runx2-II* by inhibiting *Bmp2* expression. Mature osteoblasts at the border of mature bone and the OF express *Ihh*, which acts as a morphogen to the cells of the OF. Ihh reduces the amount of Gli3R in these cells and thus allows *Bmp2* expression that further activates Runx2-II by Dlx5-dependent manner, while also activates proliferation. Runx2-II, on the other hand, activates *Ihh* expression in the final stages of osteoblast differentiation.

As osteoblasts differentiate normally in the absence of *Ihh*, it is clear that other parallel pathways control osteoblast differentiation in the OF. Our model also fails to explain what sustains Gli3R function once the OFs of the opposing bones have come close enough so that Ihh can diffuse across the suture. Is suture patency maintained simply by many parallel inhibitory factors, such as Gli3R, Twist1, Axin2, Noggin that all act in integrated fashion? Or is suture mesenchyme non-osteogenic and completely independent from the OFs that need to be apoptopically eliminated prior suture fusion? Is this suture mesenchyme missing from sutures that fuse prematurely?

It is noteworthy that Msx2 also controls *Ihh* expression during chondrocyte maturation (Amano et al., 2008). *Msx2* is expressed in the OFs of calvarial bones and may co-operate with Runx2 in regulating *Ihh* expression. Increased activation of *Msx2* in humans and mice also causes craniosynostosis (Liu et al., 1995; Liu et al., 1999).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this thesis was to unravel the role of Hh signalling, specifically of the transcription factor Gli3 and Hh ligand Ihh in the regulation of calvarial bone development. In addition, we also set out to reveal interactions of Hh signalling pathway with other molecular regulators of calvarial development.

We found that Hh signalling pathway participates in calvaria development and elevated Hh signalling causes craniosynostosis in mice. Recent findings indicate that specifically Gli3R has a critical role in calvarial development (IV; V; Tabler et al., 2013). We have shown that deletion of *Gli3* in mice causes craniosynostosis of the lambdoid as well as the interfrontal suture (I and II). Gli3R maintains suture patency by several mechanisms (Figure 8). *Gli3* is expressed in differentiating osteoblasts from early until late maturation stages. At the edge of osteogenic condensation, between the sutural mesenchymal cells and the OF, the mesenchymal progenitor cells and early preosteoblasts express *Gli3*. As *Ihh*, expressed by more mature osteoblasts, does not reach these cells, Gli3 is in the R-isoform. Gli3R inhibits osteoblast differentiation from proceeding by activating Fgf2, which further activates *Twist1* expression (I). Twist1 inhibits Runx2 function in early preosteoblasts (Bialek et al., 2004). Ohba et al. have also shown that Gli3R is able to inhibit Runx2 function directly by competing for the same DNA binding site on the osteocalcin promoter (Ohba et al., 2008). Gli3R also inhibits *Bmp2* expression in these cells, which leads to attenuation of *Dlx5* and osteoblasts specific *Runx2-II* expression in the early stages of osteoblast differentiation (II). Ihh, expressed at the border of mature bone and OF, acts as a long-range morphogen and reaches the cells of the OF, confirmed by *Ptch1* and *Gli1* expression. Gli3 is in the FL-form in these cells and Gli3R is not there to inhibit osteoblast differentiation from proceeding further (V). Our results also suggest that Runx2 activates *Ihh* expression in the mature osteoblasts (V) and Ihh-Gli3-Bmp2-Dlx5-Runx2-Ihh feedback loop regulates ossification of calvarial bones in the OFs.

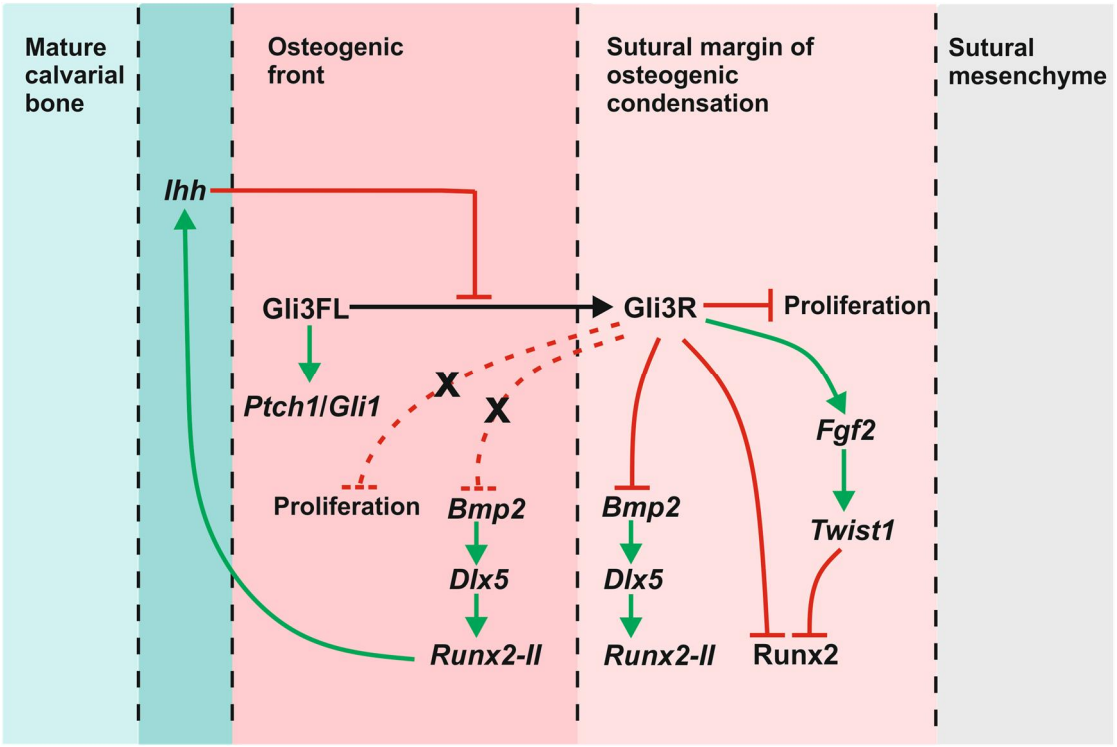

Figure 8. Schematic diagram of the role of Hedgehog signalling during calvarial intramembranous ossification (refer to the text for details).

The studies included in this thesis have focused on the role of Hh signalling in the stages of calvarial intramembranous ossification from E12.5 onwards when osteoblastic markers are first detected. We have provided evidence as to why calvarial bones of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice are large in size and how Hh signalling regulates ossification in the OFs. During this thesis project new intriguing findings concerning the earlier role of Hh signalling in determining calvarial mesodermal cells, as well as the effect of Gli3R in cranial neural crest cell population size point to an earlier role of Gli3 in calvarial patterning. Possibly we could find answers to questions that this thesis did not address: Why do only certain sutures fuse in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice? Our data indicate that *Gli3* expression is stronger in the interfrontal suture and at the lateral edges of the interparietal bone at E15.5 compared to other calvarial sutures, which may of course have an effect. It is, however, also noteworthy that the lambdoid and interfrontal sutures are the only sutures where all the components that form the suture are exclusively derived either from PM or CNC, respectively (Jiang et al., 2002; Yoshida et al., 2008; Figure 1). The coronal and sagittal sutures that remain patent are composed of both PM and CNC derived tissues.

Gli3 mutant mouse could serve as a good model to clarify more universal questions concerning craniosynostosis. What happens to the sutural mesenchymal tissue in craniosynostosis? Is the pressure of the overgrowing calvarial bones enough to eliminate the sutural mesenchyme or does the sutural mesenchymal tissue fail to differentiate in the first place? What makes two bones fuse? Do they fuse simply when two bone ends come into close enough proximity? Research on calvarial bone development has also focused, to a large extent, on frontal and parietal bones and the coronal suture that unites them. The details on interparietal bone development and how lambdoid suture is established are scarce. *Gli3* mutant mouse provides an excellent model to study earlier stages of interparietal bone development in more detail.

Head development involves the integrated formation of the calvaria, facial skeleton, cranial base, and the brain, all of which express *Gli3*. Furthermore, all of these tissues suffer varying degree of malformation in craniosynostotic syndromes as well as in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ mice. It is not, however, known how craniosynostosis may depend on changes elsewhere in the affected skull, and how changes in the major subdivisions of the skull contribute to the final dysmorphic phenotype. Lineage-specific or developmental stage-specific expression of *Gli3* would allow us to assess the influence of these separate regions on calvarial suture fusion during embryonic development. This would also circumvent the lethality of *Gli3* null allele mice at birth and would allow assessment of postnatal growth. Thorough understanding of how development of different tissues of the head is integrated could have a huge impact on management of pathological conditions involving the head, such as craniosynostosis.

In study V we found new evidence that Runx2 would regulate expression of *Ihh* during calvarial development. Our data is preliminary and requires more thorough investigation, but if this is the case, it is interesting as during endochondral osteoblast differentiation the situation is exactly the opposite: Ihh governs transcriptional control of *Runx2* (Kronenberg, 2003). On the other hand, during earlier stage of endochondral ossification, during chondrogenesis, Runx2 specifically activates *Ihh* expression (Yoshida et al., 2004). In fact, intramembranous ossification may relate to endochondral chondrogenesis more intimately than yet realised and comparison of these processes could entail interesting novel findings concerning intramembranous ossification.

Results of this thesis have provided valuable knowledge on the role of Gli3 and Hh signalling during calvarial intramembranous ossification. Increasing evidence shows that aberrant Hh signalling is behind human craniosynostosis and specifically Gli3R plays a pivotal role. Basic research on understanding the cause of the disease is a vital first step in creating more elaborate treatment options for patients in the future.

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