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UNDERSTANDING CRANIOSYNOSTOSIS AS A GROWTH DISORDER

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Abstract

Craniosynostosis is a condition of complex etiology that always involves the premature fusion of one or multiple cranial sutures and includes various anomalies of the soft and hard tissues of the head. Steady progress in the field has resulted in identifying gene mutations that recurrently cause craniosynostosis. There are now scores of mutations on many genes causally related to craniosynostosis syndromes, though the genetic basis for the majority of nonsyndromic cases is unknown. Identification of these genetic mutations has allowed significant progress in understanding the intrinsic properties of cranial sutures, including mechanisms responsible for normal suture patency and for pathogenesis of premature suture closure. An understanding of morphogenesis of cranial vault sutures is critical to understanding the pathophysiology of craniosynostosis conditions, but the field is now poised to recognize the repeated changes in additional skeletal and soft tissues of the head that typically accompany premature suture closure. We review the research that has brought an understanding of premature suture closure within our reach. We then enumerate the less well-studied, but equally challenging, non-sutural phenotypes of craniosynostosis conditions that are well-characterized in available mouse models. We consider craniosynostosis as a complex growth disorder of multiple tissues of the developing head, whose growth is also targeted by identified mutations in ways that are poorly understood. Knowledge gained from studies of humans and mouse models for these conditions underscores the diverse, associated developmental anomalies of the head that contribute to the complex phenotypes of craniosynostosis conditions presenting novel challenges for future research.

Keywords

skull development; FGFR; suture; osteogenesis; syndrome; non-syndromic; mouse models

Introduction

Craniosynostosis is a complex condition that always involves premature fusion of one or more cranial vault sutures. It affects approximately one in every 2000–2500 newborns[1,2], making it the second most common craniofacial anomaly after orofacial clefts. It occurs across all geographic regions, in all ethnic groups, and across socioeconomic statuses[3,4].

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Cranial sutures are fibrous joints consisting of two opposing osteogenic bone fronts separated by a mass of undifferentiated mesenchymal cells. Cranial vault sutures of modern mammals are evolutionarily derived from functioning cranial joints of the primitive, kinetic vertebrate skull, such as those found in extant fish[5]. The sutures in skulls of modern mammals allow temporary deformation during parturition or compression during trauma, inhibit bone separation for the protection of underlying soft tissues, and most importantly for craniosynostosis, serve as growth sites for cranial bones in the developing skull[6,7], allowing rapid bone formation and growth along the edges of the two opposing bones until they ossify and fuse postnatally. When sutures close prematurely, osseous unification of the two bones prevents growth at the fused suture and redirects it to other patent sutures, altering the global shape of the skull in predictable ways (Figure 1).

Research in genetics and developmental biology accomplished during the last two decades has focused on understanding how gene mutations alter the precise balance between cranial vault bone formation and patency at the suture. These data underlie our current understanding of cranial suture biology and the molecular and developmental pathogenesis of premature suture closure. In sum, the findings highlight the complex pathogenesis of craniosynostosis by identifying local aberrations in cell behaviors and tissue growth that could also contribute to a multitude of craniofacial developmental anomalies beyond premature suture fusion and reveal craniosynostosis conditions as complex clinical entities.

The study of craniosynostosis has a long history and many of these original ideas still permeate current craniosynostosis research. Hippocrates was the first to describe the relationship between the relative position of abnormally closed cranial sutures and the resulting shape of the head[8]. While Vesalius[9] and della Croce[10] provided elegant descriptions and illustrations of the cranial dysmorphology associated with craniosynostosis, our understanding of craniosynostosis was incomplete until Sömmerring and Gibson independently established the role of sutures as growth sites and deduced the impact of premature suture fusion on cranial morphology[11,12]. Otto developed Sömmerring's work and established the idea that premature suture fusion would restrict growth of the skull and redirect growth of the underlying soft tissues to alter the gross morphology of the cranium, an idea that was formalized in 1851 by Virchow[11,13]. Virchow proposed that when a cranial vault suture fused prematurely, growth of the vault was restricted in the direction perpendicular to the suture which led to expansion of the adjacent unaffected sutures and localized compensatory overgrowth in a direction parallel to the fused suture[14]. Jane and colleagues developed Virchow's ideas into an understanding of how a single fused suture causes compensatory growth at all neighboring sutures[15,16].

As part of his functional matrix hypothesis, Moss proposed that craniosynostosis is initiated by abnormalities of the early forming endochondral bones of the cranial base and that growth of the brain transmits biomechanical forces to later developing cranial vault bones through their attachments to the dura mater[17–19], the outermost meningeal layer that along with cranial vault bone periosteum is derived from the ectomeninx (Figure 2). Dura mater encloses the brain, lines the endocranial surface of cranial vault bones and forms fibrous dural reflections that in humans attach to the cranial base along the lesser wings of the sphenoid, at the crista galli of the ethmoid, and along the crest of the petrous portion of the

temporal bones[17]. According to Moss this physical coupling between the dura mater and the cranial base facilitates the transmission of information pertaining to brain expansion between the cranial base and the cranial vault, and in the case of craniosynostosis, could produce premature suture fusion[17]. Though descriptive, the importance of Moss' contribution is the recognition of the role of a changing system during growth and that all cells, including bone cells, grow in response to specific functional (biomechanical) demands of surrounding tissues.

Various classification systems have been developed to help make sense of the variation in craniosynostosis phenotypes. First, craniosynostosis can be characterized as simple or compound. Simple craniosynostosis involves fusion of a single suture, whereas compound craniosynostosis includes premature closure of two or more sutures[20]. Craniosynostosis can also be classified as primary or secondary. Primary craniosynostoses are conditions in which suture fusion is the consequence of a developmental disorder that directly targets the suture, while secondary craniosynostosis includes those conditions in which premature suture fusion is secondary to another known abnormality, often associated with central nervous system, metabolic, or hematological disorders and diseases[20,21]. Finally, craniosynostosis can be classified as syndromic - occurring as one of a suite of traits or symptoms that consistently co-occur and characterize a syndrome - or as non-syndromic (or isolated), where premature suture fusion is the most obvious phenotypic anomaly and the genetic cause is unknown.

Approximately 92% of craniosynostosis cases are non-familial and 85% are nonsyndromic[11,22]. The other 15% of cases are diagnosed with one of the nearly 200 known craniosynostosis syndromes, and about half of these follow a Mendelian pattern of inheritance[22–24]. The frequency of fusion of each of the cranial sutures varies, with the sagittal suture (Figure 3) being the one most often fused in cases of isolated craniosynostosis. The coronal suture (Figure 3A) is the suture that is the next most often prematurely fused, occurring in 20–30% of all cases of craniosynostosis, syndromic and isolated[25,26]. Females are more commonly affected in cases of coronal craniosynostosis, whereas males are more commonly affected in cases of sagittal craniosynostosis. Craniosynostosis of one coronal suture (unicoronal craniosynostosis) occurs twice as often as bicoronal craniosynostosis. Synostosis of the metopic suture had a reported prevalence of 6–7 in 100,000 live births in 2000, but this incidence may be increasing[27]. Finally, premature fusion of the lambdoid suture (Figure 3A) is relatively rare, estimated to represent about 1% of all craniosynostosis cases. Each case of craniosynostosis presents with fairly predictable changes in the shape of the cranial vault, along with associated variable dysmorphologies of the craniofacial complex (Figure 1).

The complex nature of craniosynostosis is reflected in the difficulty in obtaining satisfying therapeutic treatments. Despite the tailoring of surgical approaches to individual needs, modern surgical treatment for craniosynostosis conditions has remained relatively unchanged, involving endoscopic strip craniectomy and helmet molding in the simplest cases[28–30] and single or repeated reconstructive surgeries in the more complex cases[31,32], with little means to predict surgical outcome. That reconstructive surgeries commonly initiated during the first year of life do not uniformly provide satisfying results and need to

be repeated in some cases confirms that processes of postnatal growth and development are working against the morphological correction achieved by the surgeon and identifies craniosynostosis conditions as growth disorders[33]. Consequently, understanding prenatal and postnatal craniofacial development and growth is critical to developing therapeutics for this complex disease.

Cranial morphogenesis

The adult human skull is composed of twenty-two bones that form the neurocranium and the facial skeleton. Additionally, three separate bones that comprise the osseous portion of the inner ear are contained within the petrous portion of the temporal bone and the non-articulating hyoid bone lies just superior to the thyroid cartilage to assist in functions of the tongue, larynx, and pharynx. In general, single skull bones develop either through the formation of single “ossification centers” (islands of mineralizing tissue) or by the union of multiple distinct ossification centers. As individual bones expand and begin to take on their unique shape, they approach one another but remain separated by the formation of sutures (Figure 3A,C).

Process of ossification and cells that make bone

Islands of mineralizing tissue destined to become bones of the cranial vault begin to form from neural crest- or paraxial mesoderm-derived mesenchyme between 23 and 26 days[34] of human fetal development. In humans and several vertebrate model organisms, neural crest cells establish the anterior and lateral portions of the cranial vault, including the frontal bones and the squamous portion of the temporal bones, along with many bones of the face and the anterior segments of the osseous cranial base[35–38] (Figures 3, 4A). In humans and mice, the posterior portion of the cranial vault, including the parietal bones, the lateral aspects of the interparietal (when present, as in mice [Figure 3D]), and the squamous occipital in humans[35,36] are derived from paraxial mesoderm, along with the exoccipital and basioccipital portions of the occipital bone, and the petrous portion of the temporal bone (Figures 3, 4A). In mice, the mid-section of the interparietal receives contributions of neural crest cells. As streams of these two cell populations successfully migrate to their targets, they interact with local epithelial cells and locally secreted factors that induce the formation of cell condensations[39]. At the condensation stage, mesenchymal osteoprogenitor cells take one of two paths to osteogenesis: 1) mesenchymal cells differentiate directly into osteoblasts to form bone through intramembranous ossification; or 2) mesenchymal cells follow a chondrogenic path in which they differentiate into chondroblasts that form cartilage anlagen that are eventually replaced by bone through endochondral ossification[40] (Figure 4B). Angiogenesis is known to be necessary for endochondral bone formation and evidence is accruing to establish the critical role of angiogenesis during intramembranous bone formation, as growing vessel networks not only provide a source of circulating factors and cells to previously avascular sites, but their migrating endothelial cells are an active part of the regulatory network underlying bone formation[41].

In mammals, all cranial vault bones and the majority of the facial bones form intramembranously (Figures 3, 4B). In intramembranous ossification, no cartilage precursor

is formed, and cells of the mesenchymal condensation pass through a pre-osteoblast stage and differentiate directly into osteoblasts[42] (Figure 5). Intramembranous ossification of the cranial vault bones is initiated by condensation of mesenchymal cells between the dermal epithelium and the forming meninges (Figure 2A). As these cells differentiate along an osteogenic path, they first synthesize and secrete collagen type I fibers in a random fashion[43,44], along with bone sialoprotein and osteocalcin[45], followed by polarized bone matrix[46]. Once the initial bone forming matrix (osteoid) is established, osteoblasts deposit hydroxyapatite to mineralize the matrix and continue to deposit osteoid along the periphery of the forming bone. As the osteoid expands and mineralizes, osteoblasts become trapped within the bone matrix where they either undergo apoptosis or differentiate to function as osteocytes[47] (Figure 5). Intramembranous ossification of the facial bones occurs primarily within condensations of osteogenic mesenchyme of the facial prominences and the maxillary and mandibular prominences of the first pharyngeal arch[48].

In endochondral ossification, aggregated mesenchymal cells differentiate into chondroblasts and form cartilage that is later replaced by bone[49]. Initially, chondroblasts condense and subsequently differentiate into chondrocytes that proliferate rapidly to form an avascular cartilage “model” (anlagen) in roughly the shape of the forming element[50]. Unlike bone, these cartilage models can grow by accretion and interstitially as chondroblasts proliferate. Once differentiated, chondrocytes eventually stop dividing and increase in volume to become hypertrophic chondrocytes, some dying by apoptosis. As these large chondrocytes die, the surrounding cells, some of which are delivered by invading blood vessels, differentiate into osteoblasts that produce bone matrix and eventually replace the cartilage with bone[34,51]. Although intramembranous and endochondral ossification have traditionally been thought to be separate processes, new research has shown that hypertrophic chondrocytes can survive and directly differentiate into osteoblasts, showing evidence for a “chondrocyte-to-osteoblast continuum,” contributing to endochondral bone formation and postnatal bone repair[52,53] (Figure 5).

Osteocytes form inter-cellular communication networks that convert mechanical signals delivered to bone into biomechanical signals that contribute to the regulation of bone formation and turnover executed by osteoblasts and osteoclasts during bone modeling and remodeling[54]. During bone modeling, defined as either bone formation by osteoblasts or bone resorption by osteoclasts on a given surface, the skeletal elements acquire their overall morphology and mass and go through the shape changes necessary for growth, but resorption and formation are not coupled at this stage[50,55]. Bone remodeling, by contrast, renews and rebuilds the skeleton and involves bone resorption and formation that occur sequentially and in a coupled manner on a given surface[50,54].

The cranial base and facial skeleton in humans

Though the focus of craniosynostosis research is on the premature fusion of cranial vault sutures, isolated analysis of bones of the cranial vault does not acknowledge the fundamental connection of the vault with the rest of the skull. The osseous cranial base is composed of parts of the occipital bone that surround the foramen magnum, much of the sphenoid, the petrous portion of the temporal bones, and parts of the ethmoid and the vomer. Due to its

positioning beneath the brain, the cranial base provides a structural connection between the cranial vault (above) and facial skeleton (below).

The cranial base forms as an element of the chondrocranium, a portion of the endoskeleton that functions to protect the embryonic brain and three principal sense organs. The complex development of the chondrocranium has been covered elsewhere[56–58]. Generally speaking the chondrocranium serves as an embryonic a template that is replaced by bone later in development. The significance of the chondrocranium to head development has been attributed to the interstitial growth of cartilage, which enables rapid production of complex structures as well as their continued growth during embryonic development of the head[56].

Cartilaginous joints called synchondroses form between some of the bones of the cranial base and serve as important growth sites. Cranial synchondroses consist of two endochondrally ossifying bone fronts closely bonded by a specialized hyaline growth cartilage. Cranial base synchondroses make substantial contributions to anteroposterior expansion of the skull as the brain grows.

The facial skeleton forms from a series of distinct facial primordia including the frontonasal process, the paired maxillary processes, and the paired mandibular processes, together with certain elements of the more caudally forming pharyngeal arches[59]. Early in facial development, neural crest cells migrate from the border of the neural and surface ectoderm towards their facial targets physically interacting with head ectoderm, mesoderm and endoderm and exchanging signals to shape a series of complex units of the developing face including skeletal precursors.

The development of facial bones is exceedingly complex because of the number of bones involved and because their anlagen develop within facial processes that dynamically grow outward, rotate, and fuse in response to tissue interactions under the direction of a multitude of morphogens[48,60–62]. Facial bones are primarily ossified intramembranously and can form from the coalescence of several ossification centers (e.g., maxillae), or from single centers (e.g., zygomatic bones). Individual bones of the upper and midface meet at sutures that when patent contribute to facial growth but less is known of the properties of facial sutures (but see[63]).

The cranial vault in humans

In humans, each frontal bone ossifies from a single center, which appears between 6 and 7 weeks *in utero*[34]. The two human frontal bones abut at the metopic suture (Figure 3A), which is composed of cells of neural crest origin (Figure 4C), and is the first cranial vault suture to undergo fusion, typically beginning during infancy at approximately 9 months, with fusion usually completed by 2–4 years of age[34]. Unlike humans, the anterior portion of the murine interfrontal suture (the murine equivalent of the human metopic suture) remains open throughout life as do most murine cranial sutures[64] (Figure 3C). Human parietal bones appear approximately 7–8 weeks *in utero*[34], and when fully formed, the left and right parietal bones border the sagittal suture, which is composed of cells derived from both neural crest and paraxial mesoderm cells (Figure 4C). Growing frontal and parietal bones

overlap at the coronal suture, which is also derived of both neural crest and paraxial mesoderm[35] (Figure 4C).

Other bones that contribute to the human cranial vault are:

- the squamous portion of the temporal bone, which starts to mineralize intramembranously in the 7th or 8th week *in utero*[34];
- the intramembranous lateral surface of the greater wing of the sphenoid bone, which joins with the alisphenoid (formed in mice by intramembranous and perichondral ossification as an extension from the ala temporalis cartilage) to ossify about 9–10 weeks during human fetal developmental[34];
- the squamous portion of the occipital bone which has intramembranous (the most superior part of the squama that corresponds with the murine interparietal bone (Figure 3B)) and endochondral (the supra-occipital) contributions[65]. By the 3rd month of fetal development, these two ossification centers expand as they mineralize further and fuse to form the squamous occipital[65,66].

At birth in humans, the occipital bone is represented by four mineralized bodies: the squamous occipital (fused supra-occipital and interparietal), the left and right ex-occipital elements incorporating the occipital condyles, and the basioccipital. These latter portions of the occipital bone form endochondrally and surround the foramen magnum, but all occipital elements fuse into a single occipital bone postnatally. The parietal and occipital bones meet at the lambdoid suture where they overlap.

Increase in size of the cranial vault is primarily driven by the growing brain[67–69]. In humans, the brain reaches two-thirds of its adult size within the first two years of life, achieving adult size between 6–10 years of age[70]. Sutures are important growth sites, allowing the cranial vault bones to add tissue along the sutural edges to accommodate, protect, and keep pace with the growing brain. However, because sutures form and close at different times, the amount of growth accomplished by different cranial vault sutures is not uniform[71]. For example, the human metopic suture is invariably fused by 4 years of age so that the majority of subsequent sutural growth of the anterior cranial vault occurs primarily at the sagittal and coronal sutures[71]. Since bone is added at the osteogenic fronts of opposing bones of a suture, the direction of growth accomplished by a suture is perpendicular to the orientation of the respective suture (e.g., the orientation of the sagittal suture is anteroposterior, so growth local to this suture increases the mediolateral dimension of the skull) and continues throughout brain growth[71,72]. Although bone is added at the osteogenic fronts contributing to size increase, cranial vault bones continually adjust their shape while increasing their size through modeling and remodeling of their inner and outer surfaces to maintain a consistent fit with the surface of the growing brain and other neighboring soft tissues.

When a cranial vault suture fuses prematurely, growth arrest occurs at the fused suture and further growth occurs local to still-patent sutures altering growth trajectories and producing changes in cranial vault shape (Figure 1). It is assumed that the rapidly growing brain experiences localized compression when a suture closes prematurely that results in increased

intracranial pressure, especially in individuals with multiple premature suture closure[73–75], though direct measures of intracranial pressure are rare.

Mesenchymal cells of the suture provide an engine for cranial vault growth

Cranial vault sutures are fibrous joints that consist of two osteogenic bone fronts and an intervening cellular mass of undifferentiated mitotic mesenchymal cells (Figure 6), all of which are bounded by the surface of the osteogenic layer (superficial) and the external surface of the dura mater (deep)[63]. Experimental studies on fetal and neonatal rat coronal suture development showed that removing the ecto-periosteal layer did not affect the development or maintenance of the coronal suture[76], but absence of dura mater in newly formed coronal sutures caused obliteration of the suture[77,78], indicating that signals from (or mediated through) the dura mater are required to maintain patency of the suture once the bone fronts on either side of the coronal suture overlap. However, the initial formation of the coronal suture may occur in the absence of dura mater, possibly in response to inductive signals from the nearby bone fronts[6,79,80].

The cellular composition of a suture is characterized by a mass of undifferentiated mesenchymal cells occupying the center of the suture, surrounded by a graduated series of cell types that are progressively more committed to an osteogenic fate, from pre-osteoblasts to differentiated osteoblasts, as one moves toward the cells defining the bone front[63,81] (Figure 5, 6C, 6D). A complex set of instructions emanating from the major cellular signaling pathways (WNTs, BMPs, FGFs, and others) interact to direct particular subpopulations of these cells to become osteoblast lineage cells and progress along an osteogenic fate[42]. Expression of *Runx2*, known as a master regulator for osteoblast differentiation, delineates cells that are committed to an osteogenic fate[82]. The undifferentiated and proliferating mesenchymal cells are key to the role of the suture in skull growth; they function to maintain separation between the dermal bones of the cranial vault and contribute to the regulation of growth of the skull as they differentiate towards an osteoblast fate. A constant population of undifferentiated mesenchyme is required to preserve suture patency. A subpopulation of these mesenchymal suture cells come to be osteoblast lineage cells that differentiate into osteoprogenitor cells and finally progress to mature osteoblasts that deposit and mineralize bone matrix, thus enabling localized bone growth[6].

Little is known of the basis (i.e., genetic, biomechanical, environmental) for normal suture closure that naturally occurs later in life in humans and most of what we know of the genetic mechanisms underlying suture closure comes from the study of premature suture fusion in mouse models for human craniosynostosis mutations. Gene expression patterns distinguish various sutures and suture subregions over space and time[83–86], and most of the current mechanisms considered causative for premature differentiation of osteoblasts, mineralization of sutural mesenchyme, and suture fusion represent aberrant genetic signaling, either occurring at the wrong place, at the wrong rate, or at the wrong time, leading to changes in the activities of cells of the suture (e.g., apoptosis, proliferation, differentiation).

Premature suture closure may be caused by disruption in any or a combination of these cell activities[6,87,88], and the pattern of disruption may be suture-specific[89], specific to

developmental time, and/or specific to processes regulated by the gene harboring the causative mutation or networks in which the gene interacts. For example, mutations in the fibroblast growth factor receptors (FGFRs) can cause accelerated cell differentiation at the osteogenic fronts, as well as within the suture matrix[90,91], while mutations in MSH Homeobox 2 (MSX2)[92] cause accelerated cell proliferation at the osteogenic bone fronts and reduced apoptosis in the suture[6,93].

It has long been known that suture patency requires that a sufficient population of mid-suture mesenchymal cells remain undifferentiated to keep the two bone fronts separated, but there is more than one way for this cell population to be lost. Recent findings implicate GLI1+ cells of the suture mesenchyme as the main mesenchymal stem cell population for adult craniofacial bone growth and development that maintains suture patency, gives rise to the osteogenic fronts, periosteum, and dura mater, and regulates formation of adult craniofacial bones[87]. Reduction of GLI1+ cells in the sutures of postnatal day 7 *Twist1*$^{+/-}$ mice, a mouse model for Saethre-Chotzen syndrome, was associated with fusion of the coronal and fronto-premaxillary sutures and arrested overall skull growth[87]. These findings highlight the role of specific suture stem cells in maintaining suture patency and provide exciting information regarding a specific aspect of the more global aspects of craniosynostosis phenotypes. Individual bone shapes are known to be different from normal in mouse models for craniosynostosis. Defects in this stem cell population would impact the number of cells available to build individual bones, thus constraining directions and magnitudes of growth, potentially affecting the quality and shape of bones, and contributing to overall craniofacial dysmorphogenesis.

Genetic contributors to cranial vault suture biology in craniosynostosis conditions

The idea that craniosynostosis is a genetic disorder arose shortly after Octave Crouzon[94] originally described craniosynostosis in a mother and child as “hereditary” due to the observation that they exhibited the same phenotype. By the 1930s, the dominant inheritance pattern present in certain types of craniosynostosis was recognized by at least some researchers[95]. However, identifying specific mutations in genes responsible for craniosynostosis (Table 1) awaited the advances in molecular genetics that transpired over the last 30 years. The first gene implicated as causative for craniosynostosis syndromes was for the relatively rare Boston-type craniosynostosis, a fully penetrant, autosomal dominant disorder with varying degrees of craniofacial dysmorphology[92,96]. Boston-type craniosynostosis is caused by a mutation in *MSX2*, a conserved homeobox gene that is involved in regulation of inductive tissue interactions in embryogenesis[92]. Gain-of-function mutations in *MSX2* cause premature suture fusion, and the loss-of-function mutations in *MSX2* result in cranial vault ossification defects[97].

Since these early discoveries, causative mutations for craniosynostosis syndromes have been verified in at least seven genes (*FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *MSX2* and *RAB23*) but additional genetic variants associated with craniosynostosis syndromes (see Table 1) and isolated craniosynostosis continue to be implicated[98–100]. Because of the

knowledge of craniofacial sutures as growth sites, and the evident association of early closure with cranial vault morphology, most studies of gene action have focused on what happens within the suture when a causative mutation is present. Knowledge of the effects of these mutations on cell behaviors comes from the study of human cells from patients known to carry specific craniosynostosis-causing mutations and from the study of mouse models. A wealth of detailed information exists for each of the genes that carry mutations known to be causative for craniosynostosis, and with each new discovery, the complexity of the mechanisms involved and potential interaction among these factors in suture formation, maintenance, and fusion become more apparent. Here we provide a brief description of some of the mutations in genes known to cause craniosynostosis. Detailed explanations of the molecular signaling and interactions underlying how mutations in these major genes cause premature suture closure are available in several fine, in-depth reviews and the original reports cited within[22,101,102] (for *FGFR* see [45,103–105], for *TWIST1* see [84,105–108], for *MSX2* see [106,109], for *EFNB1* see [106,107], for *RAB23* see [101]).

Fibroblast growth factor receptors (FGFRs)

The most prevalent craniosynostosis syndromes (Apert, Crouzon, Pfeiffer, and Muenke syndromes) (Figure 7) are caused by mutations in genes encoding FGFR1, FGFR2 and FGFR3 (Figure 8) (Table 1). The FGF/FGFR pathway is a paracrine signaling pathway comprised of 18 ligands (FGF1-FGF10 and FGF16-FGF23) and four receptors (FGFR1-FGFR4) that play critical roles in cell functions and are active in many developmental processes as diverse and important as gastrulation, somitogenesis, and the development of the central nervous system, limbs, lungs, and vascular system[110–112]. Each of the FGFRs possess an extracellular region with three immunoglobulin-like (Ig) domains (D1-D3) for binding FGF ligands, a transmembrane domain, and a cytoplasmic tyrosine kinase domain[112,113]. Different FGFs show distinct binding affinities for the individual FGFRs based on unique sequences in the N- and C-terminal tails of the FGFs and the immunoglobulin (Ig) domains of the FGFRs, as well as variation in the timing and location of their expression[113,114]. FGF/FGFR binding, which is usually mediated by heparin sulphate glycosaminoglycan binding to the FGF, causes the FGFRs to dimerize and activate tyrosine kinase activity within the cell[113].

Identification of mutations in *FGFRs* that cause skeletal dysplasia like achondroplasia[115] and craniosynostosis[116–119] in humans revealed the importance of the FGF/FGFR signaling system in normal bone growth and development[45]. Craniosynostosis-causing sequence changes in the *FGFRs* are most commonly encoded in the extracellular portion of the gene, particularly in the linker region between Ig domains II and III[120], and they act to produce gain-of-function mutations that assist dimerization of the FGFRs[121]. These mutations either alter the binding affinity for FGFRs toward all FGFs or a specific subset of FGFs[121,122], or cause constitutive activation of the FGFR pathway, in which binding of an FGF ligand is not required to stimulate FGFR dimerization and intracellular activity[123,124]. The downstream consequence of FGFR activation in the suture is the onset of runt-related transcription factor 2 (RUNX2) expression, essential for the differentiation of osteoblasts. Expression of RUNX2 leads suture mesenchyme cells to differentiate into osteoblasts that will deposit bone and eventually unify the two osteogenic fronts of the suture[125,126].

TWIST1

Increased knowledge of the intracellular components of the FGF/FGFR pathway (Figure 8) has helped to identify additional craniosynostosis-causing genetic variants. Multiple genes that act as regulators or downstream effectors of the FGF/FGFR pathway have also been implicated in craniosynostosis syndromes. TWIST1, a basic helix-loop-helix transcription factor, is associated with Saethre-Chotzen syndrome (Figure 7) that typically includes craniosynostosis of the coronal suture (Table 1)[127–129]. A heterozygous loss-of-function mutation in TWIST1 results in increased osteogenic capability and causes suture closure through increased bone deposition[127–129]. TWIST1 is found in the developing suture mesenchyme and functions in all stages of osteoblast differentiation[80]. TWIST1 functions as a negative regulator of both FGFR2 and RUNX2[130,131].

TWIST1 and FGFR2—There are currently two non-mutually exclusive models for how TWIST1 regulates patency of the coronal suture. The first, established by Connerney et al., proposes that different TWIST1 dimers play a homeostatic role in regulating FGFR2 expression[84] (Figure 9). TWIST1 is able to form functional homodimers (T/T) as well as heterodimers with E proteins (T/E)[84]. One E protein that is known to heterodimerize with TWIST1 in the coronal suture, TCF12, has been implicated in coronal suture craniosynostosis[98] (see following). TWIST1 homodimers act as an FGFR2 agonist in cells of the osteogenic front, whereas TWIST1 heterodimers are found in suture mesenchyme cells, where they act as an FGFR2 antagonist[84]. The difference in TWIST1 dimer composition is mediated by ID (Inhibitor of DNA-binding/differentiation) proteins, which are expressed in the osteogenic front and bind preferentially with E proteins[84]. As a result, TWIST1 is forced to homodimerize in the osteogenic front because of decreased availability of E proteins[84]. In the suture mesenchyme, where ID proteins are absent, TWIST1 is able to dimerize with E proteins because of the lack of competition from ID. ID is upregulated by BMP signaling, which is itself upregulated by FGFR2 signaling, which downregulates expression of BMP antagonist noggin[84,108]. This interaction between FGFR2, BMP, and ID creates a homeostatic regulation of FGFR2 that restricts its expression to the osteogenic front and prevents fusion of the suture. In *Twist1*$^{+/-}$ mice, decreased expression of TWIST1 relative to ID results in increased formation of T/T dimers and decreased formation of T/E dimers, which increases the expression domain of FGFR2 into the suture, eventually resulting in suture fusion[108].

TWIST1, EFNB1 and MSX2—The second model for how TWIST1 regulates coronal suture patency concerns the interaction between TWIST1 and the EPH/EPHRIN cell-to-cell signaling pathway in the coronal suture (Figure 9). EPH/EPHRIN signaling operates through juxtacrine communicating junctions between membrane bound ligands (EPHRINs) and receptors (EPHs)[132]. While EPH/EPHRIN signaling is critical for multiple aspects of craniofacial development, including neural crest cell migration[133] and palatogenesis[134], its relevance to premature suture fusion lies in its interaction with TWIST1 and MSX2 in the maintenance of the boundary between neural crest-derived and mesoderm-derived cells at the coronal suture[106,107]. The coronal suture develops at a boundary between osteoblast lineage cells derived from neural crest that will form the frontal bone and those derived from mesoderm that will form the parietal bones[106]. TWIST1 and MSX2 function together to

maintain the integrity of this boundary, and EPH/EPHRIN signaling has an important downstream role in this process. Disruption of the boundary occurs when osteogenic neural crest cells invade the mesoderm-derived undifferentiated mesenchyme of the mutant coronal suture and establish osseous connections between the frontal and parietal bones[106] (Figure 10). In these experiments, haploinsufficiency of TWIST1 leads to expanded expression of MSX2 in the coronal suture, which coincides with reduced expression of EPHRIN-A2 and EPHRIN-A4 in the suture and the consequent invasion of osteogenic neural crest cells into the suture mesenchyme[106]. Further research showed that EPH/EPHRIN signaling acts downstream of TWIST1 to maintain the coronal suture boundary. Beyond this experimental research using mouse models, knowledge of the interaction of these genes in craniosynostosis is complemented by the discovery of coronal suture craniosynostosis-causing mutations in the human genes *MSX2*[92], the ephrinB1 human orthologue *EFNB1*[135,136], and the ephrinA4 human orthologue *EFNA4*[106].

Molecular Pathogenesis in Nonsyndromic Craniosynostosis

The genetic cause and molecular pathology for cranial suture closure is best defined in craniosynostosis syndromes. Relatively less is known of genetic causation in nonsyndromic craniosynostosis that represent 85% of all craniosynostosis cases, but that tide is beginning to turn as rare mutations are being identified in a small number of nonsyndromic craniosynostosis cases[22]. Individuals with nonsyndromic craniosynostosis rarely have abnormalities of the postcranial skeleton, and suture closure is considered an isolated anomaly. Subtle craniofacial anomalies in isolated craniosynostosis disorders are traditionally attributed to facial and cranial base growth disturbances that are caused by the premature closure of cranial vault sutures. Evidence is beginning to accumulate from humans and mice (see following section) to suggest that this assumption should be reevaluated. For example, three-dimensional quantitative analysis showed that the facial skeleton and cranial base are invariably different from normal in individuals with confirmed nonsyndromic coronal craniosynostosis[137]. Until the specific mechanisms by which a closing vault suture could cause facial and cranial base anomalies are defined, the idea that cranial vault suture closure causes change in facial and cranial base development resulting in the characteristic facial appearance of individuals with isolated craniosynostosis is a hypothesis requiring testing.

The facility of new genomic tools and technologies is bound to elevate the number of genes that can be readily identified with craniosynostosis conditions. Genome wide association studies (GWAS), whole exome, or even whole genome sequencing are certain to increase the number of craniosynostosis loci identified, and it is likely that many of the new variants will be members of already identified networks. For example, a GWAS[99] identified two susceptibility loci for nonsyndromic sagittal craniosynostosis near *BMP2* and *BBS9*. Sharma et al.[98] recently used exome sequencing to identify 38 heterozygous *TCF12* mutations in a relatively large sample of unrelated individuals with coronal craniosynostosis. They also showed that the dosage of TCF12-TWIST1 heterodimers is critical to coronal suture morphogenesis, a result consistent with previous work on Saethre-Chotzen syndrome.

More recently, mutations in ETS2 repressor factor (*ERF*)[138] and zinc finger protein of cerebellum 1 (*ZIC1*) have been found to cause coronal craniosynostosis. Heterozygous mutations in the *ERF* gene were implicated in both syndromic and nonsyndromic craniosynostosis, Chiari malformation, and language delay[138]. ERF binds close to regulatory sites controlled by RUNX2, and reductions in dosage of ERF can cause disruptions in RUNX2 activity, leading to changes in osteoblast differentiation in suture cells and premature suture fusion[138] (Figure 8). Individual family members with heterozygous mutations in the third exon of *ZIC1* have a distinct phenotype that includes severe coronal craniosynostosis and variable learning disabilities[100]. Through the use of animal models, it is proposed that the effect of ZIC1 on cells occurs during early formation of the supraorbital regulatory center (early frontal bone) via a complex of signaling networks ultimately resulting in premature closure of the coronal suture and learning disabilities.

Beyond sutures: Craniofacial dysmorphogenesis in mouse models for craniosynostosis

Compared to cranial vault sutures, relatively little is known about how the mutations that recurrently cause craniosynostosis affect cranial cells and tissues to result in midfacial retrusion and other non-sutural phenotypes. The complexity of midfacial development and the diversity of mutations associated with craniosynostosis means that individual phenotypic contributions to generalized clinical entities associated with craniosynostosis conditions (e.g., midfacial retrusion, hypertelorism, cranial base diminution, palate deformities) may differ depending upon the specific mutation other genetic contributors and environmental inputs[139]. Evidence from studies of humans and mice is beginning to accumulate suggesting that a prematurely closed or dysfunctional suture could be necessary, but not sufficient, to produce the additional complex craniofacial phenotypes that typically define craniosynostosis conditions.

Many genes are conserved across mammals, and the proximate functions of most of those genes are likely conserved, as well[140]. Because humans share key developmental mechanisms with most other mammals, mouse models for many of the more common craniosynostosis syndromes have served as the primary workhorse of craniosynostosis research and have been used primarily to identify the aberrant genetic signaling leading to changes in the activity of osteoblast lineage cells of the suture. But these murine models mimic additional craniofacial anomalies that are known to occur in craniosynostosis conditions (Table 1). A number of excellent reviews have summarized the specific aberrant cellular and molecular mechanisms underlying premature suture closure in these animal models that have brought us to a clearer understanding of suture biogenesis[102,141,142]. Here we provide examples of the less well-studied non-sutural craniofacial phenotypes that characterize mouse models for craniosynostosis conditions. At this point the descriptions are primarily morphological but the availability of animal models provide a tool for analysis of additional aspects of cranial dysmorphogenesis that are worthy of study.

Mouse models for FGFR-related syndromes

Total disruption of FGFR1 and FGFR2 results in early embryonic lethality[143,144]. To overcome lethality, conditional knockout mice have been created and used to study the role of FGFR signaling pathways in the regulation of bone formation throughout development[145,146]. The *Fgfr1*$^{P250R/+}$ Pfeiffer syndrome mouse model[147], carrying the murine P250R mutation (equivalent to human FGFR1 P252R mutation), provides valuable insight into the role of FGFR1 in intramembranous bone development[116,147–150]. The phenotype of *Fgfr1*$^{P250R/+}$ Pfeiffer syndrome mice exhibit premature fusion of the inter-frontal, sagittal and coronal sutures and a skull that is shortened antero-posteriorly, laterally widened, and vertically heightened[147]. Accelerated osteoblast proliferation and increased expression of genes related to osteoblast differentiation local to the vault sutures suggests that bone formation is locally increased at the suture[147]. Accelerated osteoblast differentiation associated with early expression of RUNX2 suggested that RUNX2 is downstream of FGFR signaling and supported earlier evidence that FGFR1 is involved in osteoblast differentiation[71,151]. While change in osteoblast activity that is localized to the sutures provides an explanation for early closure of the frontal and sagittal sutures, it does not account for changes that produce a relatively wide skull or midfacial retrusion, clearly evident in these mice. The authors attribute midfacial retrusion to primary craniosynostosis of the facial sutures, but also suggest that FGF signaling within the pharyngeal arches could also affect differentiation or proliferation of osteoblast lineage cells thereby contributing to maxillary retrusion[149].

Mouse models for Apert syndrome have been developed on outbred[152,153] and inbred[154,155] genetic backgrounds. Models carrying dissimilar *Fgfr2* mutations but on similar inbred backgrounds allow direct comparison of the effects of the mutation without the confounding influence of uncommon background genes. The *Fgfr2*$^{+/S252W}$ and *Fgfr2*$^{+/P253R}$ mouse models mimic many of the human craniofacial phenotypes associated with Apert syndrome and have been key in revealing that mutations in FGFR2 cause craniosynostosis through distinct mechanisms[142,152–156]. Study of *Fgfr2*$^{+/S252W}$ Apert syndrome mice show that fusion of the coronal suture is associated with an upregulation of osteogenesis through increased proliferation and failure of mechanisms that inhibit differentiation of cells of the sutural mesenchyme, driving the frontal and parietal bones into a single osteogenic domain[155]. In the *Fgfr2*$^{+/P253R}$ mice[155], abnormal osteoblastic proliferation, differentiation and osteogenesis, but no increased apoptosis, was observed in the coronal sutures.

Quantitative analysis of craniofacial phenotypes in mouse models for Apert syndrome show the brain, cranial vault, facial skeleton, and cranial base to be dysmorphic[157–159]. Consistent with early clinical investigations suggesting abnormal cartilage development of the cranial base as primary in craniosynostosis[160], study of the *Fgfr2* P253R mutation under the control of the *Col2a1* promoter enhancer that limits the effects of the mutation to chondrocytes, led to the suggestion that the overall craniofacial dysmorphology of this mouse model is not simply a result of coronal suture closure, but that a primary disturbance in growth of the cranial base and precocious endochondral ossification also contribute[157].

Quantitative study of three-dimensional data from micro-computed tomography (μCT) images show the facial skeleton, rather than the cranial vault to be the most affected region

of the skull in newborn *Fgfr2*$^{+/S252W}$ and *Fgfr2*$^{+/P253R}$ Apert syndrome mice[88,159]. Facial dysmorphology is more severe in *Fgfr2*$^{+/S252W}$Apert syndrome mice relative to *Fgfr2*$^{+/P253R}$ mice, and is localized to the posterior palate where abnormal cellular proliferation, differentiation, and apoptosis is observed in the maxillary-palatine suture[88]. Analysis of additional facial sutures by μCT show early fusion (by E17.5) in *Fgfr2*$^{+/S252W}$ and *Fgfr2*$^{+/P253R}$ Apert syndrome mice contributing to differences in prenatal craniofacial growth patterns between mice carrying the *Fgfr2* mutations and their unaffected littermates whose facial sutures remained patent[161]. In both *Fgfr2*$^{+/S252W}$ and *Fgfr2*$^{+/P253R}$ Apert syndrome mouse models, the 3D morphology of the skulls and select cranial soft tissues of mutant mice were statistically significantly different from those of their unaffected littermates at E17.5, a time at which their coronal sutures are patent[161]. Since facial and cranial base dysmorphology preceded coronal suture fusion that invariably occurs by birth in *Fgfr2*$^{+/S252W}$ and *Fgfr2*$^{+/P253R}$ Apert syndrome mice, it was proposed that fusion of the coronal suture is one of many dysmorphic features in these mouse models for Apert syndrome, rather than the cause of the global craniofacial dysmorphology[159]. However since changes in cellular and molecular mechanisms of the nascent coronal sutures were documented in these mouse models as early as E12.5–E13.5[162], it is possible that cellular changes of the suture prior to fusion could change the growth potential of neighboring tissues and contribute to craniofacial dysmorphogenesis nonlocal to the suture.

The *Fgfr2c*$^{C342Y/+}$ Crouzon syndrome mouse[163] exhibits many of the characteristic traits of Crouzon syndrome, including coronal suture fusion, domed shaped cranial vault, eye proptosis, midfacial retrusion, and occasional cleft palate. Quantitative analysis of 3D μCT and magnetic resonance microscopy images reveal local differences in skull morphology, coronal suture patency, brain shape but not brain size, and significant reductions in nasopharyngeal and eye volumes between *Fgfr2c*$^{C342Y/+}$ mice and unaffected littermates at birth, providing an expanded catalogue of clinical phenotypes in this mouse model caused by changes in FGF/FGFR signaling[164]. *Fgfr2c*$^{C342Y/C342Y}$ mice show limb abnormalities such as joint fusions and broad first digits and lung and trachea defects, which are recorded in some human patients with Crouzon syndrome[163].

Heterozygous mutants of another Crouzon syndrome mouse model, *Fgfr2*^{W290R}, present features similar to patients with Crouzon syndrome, exhibiting mild craniofacial anomalies at birth that become more severe with age[124]. On the basis of nasal cartilage malformation and missing presphenoid, these authors proposed that midfacial defects in some craniosynostosis syndromes are a direct result of the mutation on patterning and development of the midface rather than an indirect effect of other craniofacial dysmorphogenesis[164]. Malformed digits found in individuals with a severe form of Pfeiffer syndrome that carry the W290R mutation[165] are similar to anomalies found in the limbs of *Fgfr2*$^{W290R/W290R}$ mutant mice[166,167]. These homozygous mice exhibit constitutive FGFR2 activation with typical tissue-specific patterns, but increased expression of the IIIb and IIIc isoforms in many of the defective organs[167].

The *Fgfr3*$^{P244R/+}$ Muenke syndrome mouse model carries the murine equivalent of the human Muenke syndrome mutation (*FGFR3* P250R)[168] and has been informative in the study of craniofacial variation and the physiological mechanisms underlying hearing loss in

patients with this syndrome[169]. Some human individuals with Muenke syndrome have phenotypic features that are not clearly distinguishable from other craniosynostosis syndromes including craniofacial dysmorphology, abnormalities visible on hand and foot radiographs, brachydactyly, and sensorineural hearing loss[170]. Others have few or no anomalies other than coronal craniosynostosis, and still others have only macrocephaly or normal head shape[171]. Because of the extreme phenotypic variation, clinical diagnosis of Muenke syndrome may be difficult and is verified by the presence of the *FGFR3* P250R mutation.

Initial analysis of the *Fgfr3*$^{P244R/+}$ mouse model backcrossed to generate congenic lines in four different strain backgrounds provided a phenotypic profile for adult mice, paralleling a number of key traits found in human patients with Muenke syndrome but the skull phenotype was dependent on genetic background and sex, with males more often affected[168]. Homozygous mutants (*Fgfr3*$^{P244R/P244R}$) are viable and their adult cranial phenotypes include a domed skull shape that shows premature fusion of the facial sutures, but an unfused coronal suture, short cranial base, and loss of hearing, but adult heterozygous (*Fgfr3*$^{P244R/+}$) mice have been reported with no overt skull abnormalities[71,169]. On the other hand, phenotypes of rounded skull and shortened snout occur in adult *Fgfr3*$^{P244R/+}$ Muenke syndrome mice, even when the coronal suture remained patent, a result consistent with the presence of skull dysmorphogenesis in prenatal Apert syndrome mice, prior to coronal suture fusion[168,169].

A quantitative study of craniofacial morphology of an independently derived *Fgfr3*$^{P244R/+}$ mouse at E17.5 and at birth (P0), revealed many subtle craniofacial anomalies in heterozygous and homozygous mutants, along with premature fusion of the coronal and some facial sutures. Phenotypic variation and dysmorphology were greater in homozygous mice relative to heterozygotes[172]. Decreased alkaline phosphatase activity and RUNX2 expression was observed in the developing mandibles of *Fgfr3*$^{P244R/P244R}$ embryos indicating that the mutation may repress ossification of the mandible. This localized process may also account for additional subtle skull anomalies of the facial skeleton and cranial base but needs to be further investigated.

Murine models of Saethre-Chotzen syndrome

The *Twist1*$^{+/-}$ mouse, with only one functional copy of the *Twist1* gene, mimics the clinical phenotype observed in Saethre–Chotzen syndrome[173,174,] including coronal suture abnormalities and additional cranial and postcranial traits. Analyses of these models has provided an explanation of coronal suture closure based on the principal of developmental boundaries[87,106,107]. The separation of cells into distinct populations is accomplished by special mechanisms of cell affinity that are fundamental to development[175]. Boundaries are commonly formed between cell populations by cells that can simultaneously maintain the separation of cells while influencing the patterning of surrounding cells producing progressively finer subdivisions of a tissue[175,176]. As discussed previously, cells of the coronal suture serve as a boundary between the domains of neural crest-derived cells destined to form the frontal bone and of mesoderm-derived cells that will form the parietal and prevent neural crest cells from crossing into the suture[177]. Disruptions in this boundary

in *Twist1*$^{+/-}$ mutant coronal sutures has been shown to result in premature coronal suture closure caused by neural crest cells crossing into the domain of undifferentiated mesoderm of the suture[87,106,107], due to either the cells' failure to correctly interpret boundary signals or an anomalous ability to receive signals encouraging migration into the sutural mesenchyme.

Twist1$^{+/-}$ mice showed reduced levels of TWIST1 that favors chondrogenesis and thereby induce endochondral ossification in the coronal suture between postnatal days 9 and 15[178]. At postnatal day15, in addition to premature fusion of the coronal suture, *Twist1*$^{+/-}$ mutant mice have a consistent pattern of craniofacial dysmorphology affecting all major regions of the skull. The cranial vault is high (acrocephalic) and wide (brachycephalic), the facial skeleton is flat and wide, and the cranial base is shortened, not unlike the cranial morphology of children with Saethre-Chotzen syndrome[179]. The authors recognize these differences to at least partially be the direct result of the TWIST1 haploinsufficiency on the developing craniofacial skeleton[179].

In summary, the consistent finding of anomalies of the facial skeleton, cranial base and select cranial soft tissues in human craniosynostosis conditions and in mouse models carrying the craniosynostosis-causing mutations demonstrates a strong relationship among these developmental phenotypes. Focus on the suture has provided in-depth knowledge of how these mutations change cellular activities local to the suture causing premature closure and cranial vault dysmorphogenesis. There is no doubt that premature closure of a vault suture will change cellular and tissue relationships in a growing head, and in this way, may contribute secondarily to dysmorphogenesis occurring nonlocal to the suture. But the causative mutations are on genes known to function extensively throughout development, so it seems probable that the primary effects of these mutations are not limited to the suture or to the cranial vault and can be contributing directly to the dysmorphogenesis of the facial skeleton and cranial base as well as additional tissues and organs of the head.

Perspective: acknowledging complexity to push the field forward

Currently there are three chief explanations for premature closure of cranial vault sutures that have been proposed from the scientific experiments reviewed here: 1) deviations in the activity of osteoblast lineage cells within the suture; 2) defects in boundary formation; and 3) reduction in suture stem cell populations. The specifics of the experiments that provide evidence for each of these three explanations are unique but all evidence clearly demonstrates how changes in instructions delivered to cells fundamental to developmental processes (i.e., proliferation, differentiation, apoptosis) cause sutures to close prematurely. Viewed in this way, each of the three explanations can be considered as varied aspects of a larger process. Knowledge of the diverse molecules associated with changes in suture patency is critical to understanding the mechanisms that link genes with sutural phenotypes and developing effective therapies, however, there is more to craniosynostosis than a closed suture. We propose an expanded definition of craniosynostosis from one that focuses uniquely on suture closure to one that includes additional craniofacial anomalies. Doing so will serve as an important tool to further our understanding of the genetic heterogeneity and developmental processes that underlie the spectrum of craniosynostosis phenotypes.

Analyses of human and murine craniosynostosis phenotypes reveal complex dysmorphology of many tissues of the head signifying that this condition comprises disruption in developmental mechanisms that are not limited to a suture, but encompass disturbances in overall cranial growth. Though our current knowledge of the processes involved in suture maintenance and premature closure is wonderfully detailed, these data provide only part of the picture. We have only a vague understanding of how the developmental basis of the well-defined sutural phenotypes might relate to associated anomalies of the cranial base and facial skeleton, or to soft tissue anomalies of the brain, nasopharynx, eyes, and other organs that commonly co-occur. Mutation-driven changes in the signaling of morphogens currently known to affect cranial vault suture patency by changing osteoprogenitor cell behavior might also directly alter the development of the facial skeleton and chondrocranium. The co-morbidity of premature cranial vault suture fusion with cranial soft tissue anomalies suggests that craniosynostosis mutations may affect additional developmental mechanisms that directly target non-osteogenic tissues (e.g., affecting the morphology of the forming facial prominences thereby changing relationships among developing facial organs, spaces, and bones), but does not negate the possibility that these tissues also respond to the effects of prematurely closing cranial vault sutures. Fine-grained analyses of the molecular underpinnings of additional craniosynostosis anomalies, as well as growth of the head as a unit, are needed.

Mechanistic explanations for premature suture closure can help build hypotheses about how the signaling genes that cause craniosynostosis syndromes might also affect cells that are not in the osteoblast lineage. Since many craniosynostosis causing mutations are on genes that are active at many times and in many places during head development, disruption in the function of these pathways has the potential to establish additional cellular changes that impact mechanisms of cell affinity underlying non-osseous tissue boundaries, and cranial morphogenesis generally. For example, cranial soft tissue structures that form prior to cranial osteogenesis could be primary targets of aberrant FGF/FGFR signaling (e.g., FGF-dependent pharyngeal endoderm of the primitive pharynx; FGF-dependent presumptive midbrain and first rhombomere) contributing fundamentally to the pathogenesis of non-sutural cranial phenotypes by modifying the physical arrangement or activities of cell populations, thus altering the location or nature of boundaries. Even subtle changes in physical arrangements of growing tissues could severely affect a cell's ability to respond to local signaling cues (physical or biochemical) during morphogenesis.

Decreasing mesenchymal stem cell (MSC) populations in the suture is the newest explanatory mechanism for craniosynostosis, and it opens novel avenues of research in overall craniofacial morphogenesis. Diminished suture stem cell populations are not only associated with premature suture closure, but also with change in volume of select cranial bones, cranial bone quality, cranial bone turnover, and overall skull size[87] providing a potential mechanism not just for localized changes such as early closure of a particular suture, but for the simultaneous disruption in individual bone development. The crucial role that MSCs play in bone formation further evidences the need to understand craniosynostosis conditions as an overall growth disorder, and not strictly as the loss of patency at one or more cranial sutures. Sutures are dynamic structures that change in size, shape, cellular makeup, and genetic signaling patterns throughout their initiation, growth, and fusion. MSCs

contained within the suture are similarly dynamic, as a single cell may be tasked with maintaining suture patency, depositing bone matrix, and directing bone modeling and remodeling as it differentiates along the osteogenic path.

The discrete nature of premature suture closure provides a reason for success in the investigation of causal factors. Unambiguously causal factors, like the mutations on genes that we have discussed (above and Table 1), are those that generate repeated errors of development and disease phenotypes. Premature suture closure may arise frequently in the presence of specific mutations, but may not in the presence of other mutations. The finding that the *Fgfr3* P244R mutation has different effects on different strains of laboratory mice[168] and that the human equivalent (the human P250R mutation on *FGFR3* causative for Muenke syndrome) produces remarkable phenotypic variation[171,180] is evidence of this fact and could be further studied to clarify the basis for variation in the development of craniosynostosis phenotypes. The interaction of underlying genetic variants with environmental exposures also contributes to unique aberrations of development so that studies of gene-environment interaction will undoubtedly play an important role in explaining observed phenotypic variation in craniosynostosis. Several environmental risk factors have been suggested in the development of craniosynostosis[181,182] including parity, prematurity, intrauterine constraint, and maternal tobacco or nitrosatable drug use, but overall, our knowledge of these effects and their individual and collective interaction with genes remains inconclusive. These complex relationships between environment and genetic mechanisms will need to be addressed, perhaps most efficiently through systems biology approaches, as we inch closer to identifying the major molecular contributors to those cases of craniosynostosis whose genetic cause is currently unknown.

An expanded definition of craniosynostosis that includes additional associated craniofacial anomalies opens new doors for research and will eventually allow a greater understanding of these complex growth disorders. However, an expanded definition does not refute the clinical and developmental importance of premature cranial vault suture closure or diminish the significance of the discoveries that we have reviewed here. The accumulated knowledge that we have presented about the closure of sutures is critical in the development of effective therapies, but as of this date, there is no evidence that targeting and preventing premature suture closure will ameliorate the accompanying craniofacial anomalies, though this may turn out to be the case (see [183]). Equally challenging will be the study of more subtle, but pervasive changes that affect cranial soft tissues and bones of the facial skeleton and neurocranium in craniosynostosis conditions. As we move forward, evaluating the causative role of newly discovered craniosynostosis mutations, it is critical to remember that genes routinely function in the development of many tissues, and that a dynamic architecture of interacting factors –genetic and nongenetic- underlies the development of any complex trait[184]. Beyond the significant strides by many researchers whose work we have reviewed, that architecture remains largely unknown.

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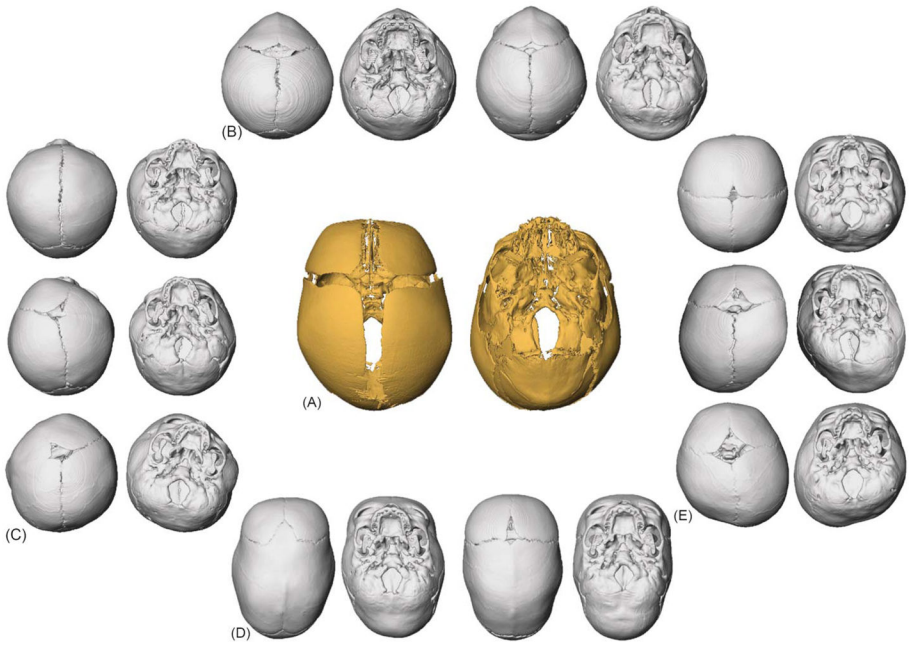

Figure 1.

Three-dimensional (3D) reconstructions of computed tomography (CT) images of human infants depicting different types of single-suture isolated craniosynostoses. Views are superior (left) and inferior (right) with face towards the top and occiput towards the bottom. (A) unaffected individual; (B) two examples of metopic craniosynostosis; (C) bicoronal craniosynostosis (top), right unicoronal craniosynostosis (center), left unicoronal craniosynostosis (bottom); (D) two examples of sagittal craniosynostosis; (E) bilateral lambdoid craniosynostosis (top), Right unilateral lambdoid craniosynostosis (center), and left unilateral lambdoid craniosynostosis (bottom). Images from our craniofacial database modified from [112].

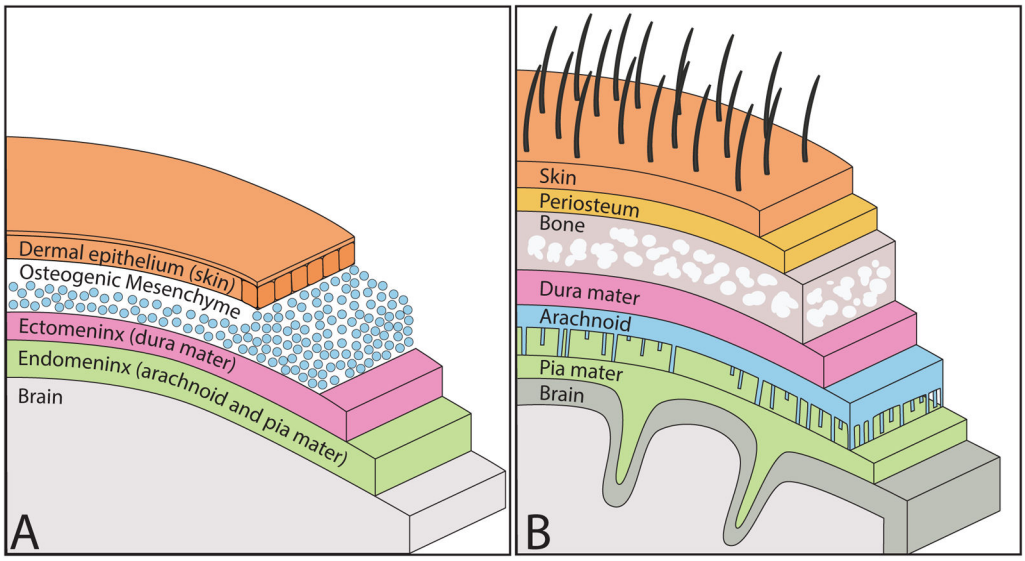

Figure 2.

Coronal section showing layers from dermal epithelium to brain during osteogenesis (A) before bone formation and (B) corresponding layers after bone formation.

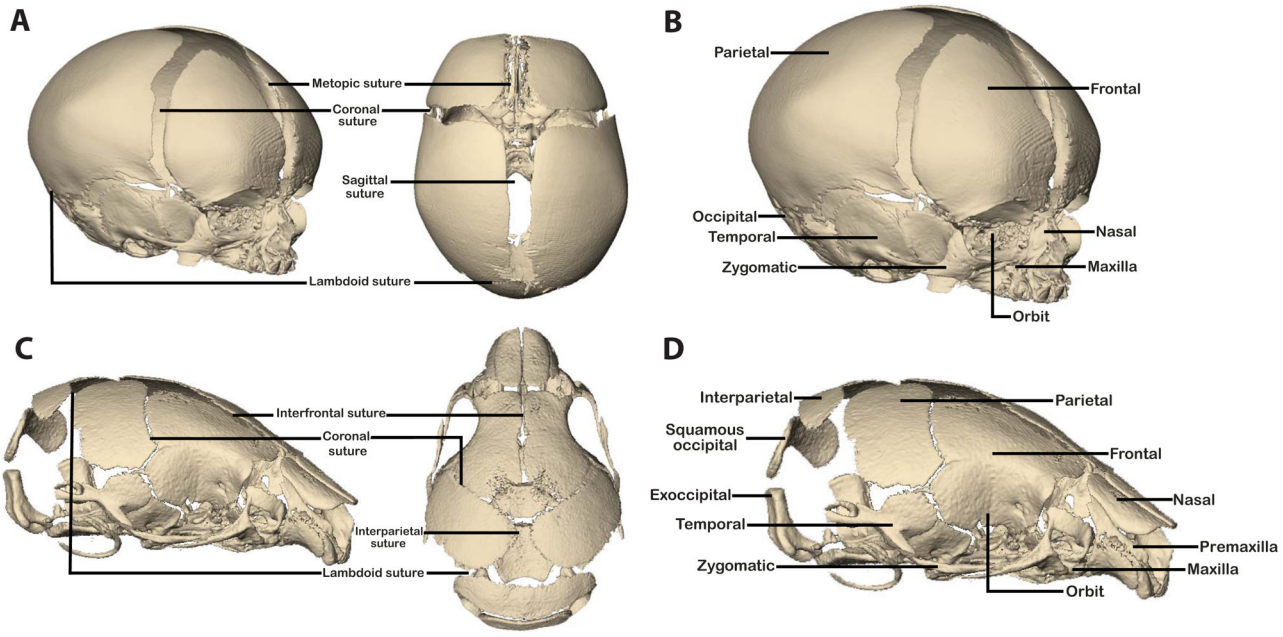

Figure 3.

3D reconstructions of CT images of a neonatal human cranium (A & B) and newborn mouse cranium (C & D) illustrating corresponding cranial bones and sutures of the two species. In panels A and C, an oblique lateral view is shown at left and a superior view at right with face to towards top and occiput towards bottom of page. The human sagittal and metopic sutures (A) correspond to the murine interparietal and interfrontal sutures (C), respectively. Selected corresponding cranial bones in the neonatal human (B) and mouse (D) skull are shown. Labels for the various facial bones in these species can be found elsewhere[46,263]. The interparietal bone in mice is analogous to the most superior segment of the squamosal portion of the occipital bone in humans. The premaxilla is a separate bone in mice but is fused with the maxilla prior to birth in humans.

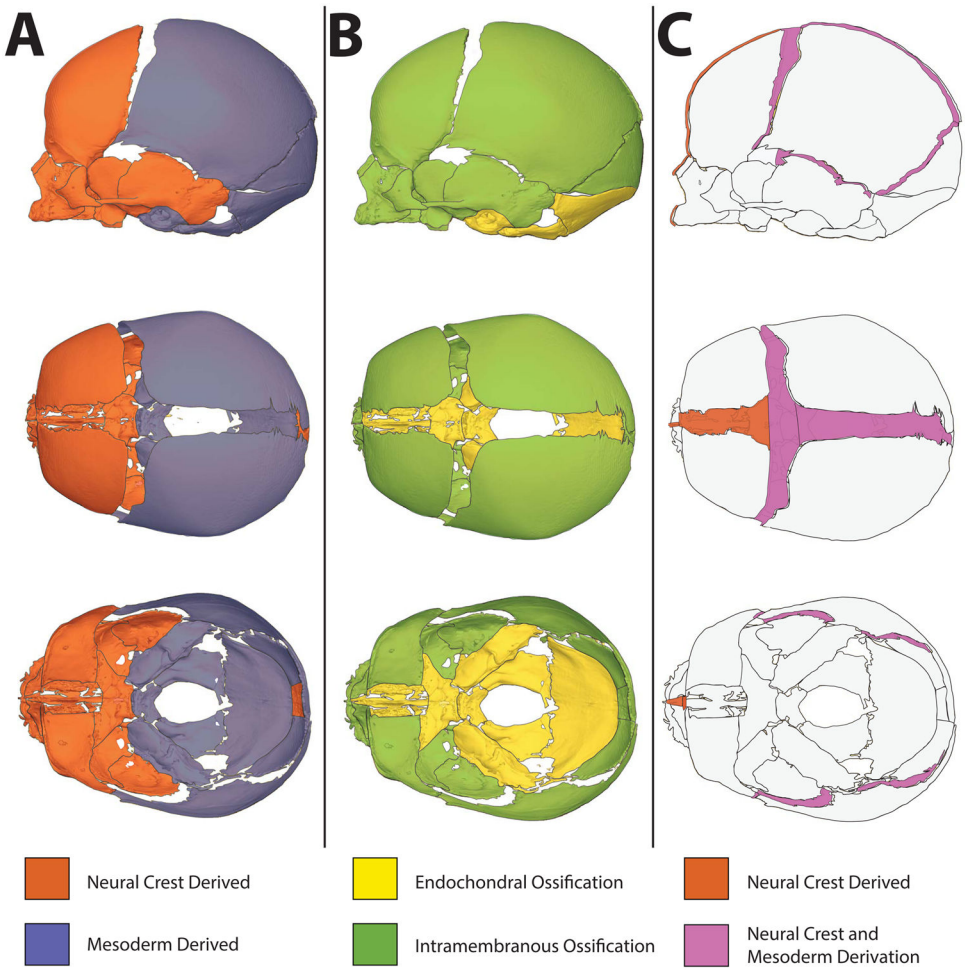

Figure 4.

3D reconstruction of CT images of a human neonatal cranium. Left lateral view at top, superior view at middle, bones of the cranial vault removed to show endocranial surface at bottom. Face to the left, occiput to the right in all views. Crania are labelled according to A) cellular origin of cranial bones: neural crest in orange, mesoderm in blue; B) ossification type: intramembranous ossification in green, endochondral ossification in yellow; and C) cellular origin of cranial suture mesenchyme; neural crest in orange, mixed neural crest and mesoderm origin in fuchsia.

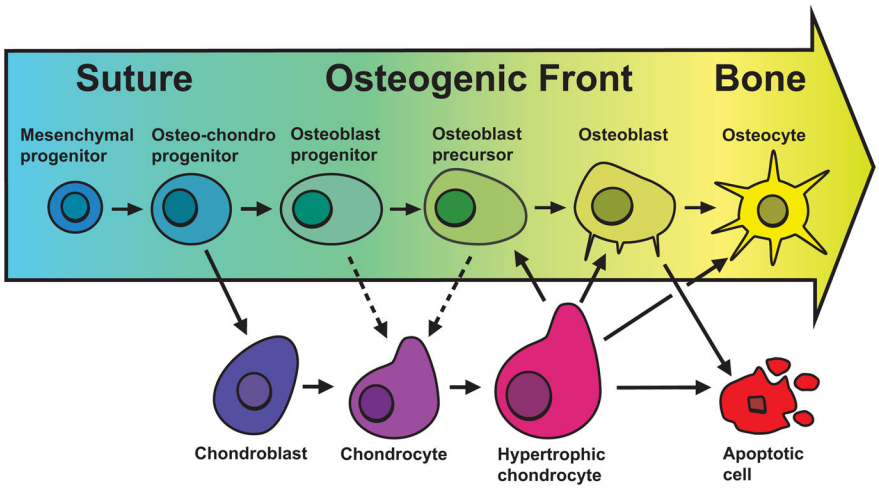

Figure 5.

Stages of bone and cartilage lineage cell differentiation. A patent suture represents a gradient of bone lineage cells that become more differentiated moving from mesenchymal progenitor cells of the mid-suture to the osteogenic front. Once osteoblasts become encased in bone matrix they can either differentiate into osteocytes that become enveloped into the forming bone or undergo apoptosis. Though the differentiation of osteoblast lineage cells (top row) are diagrammed according to their role in cranial vault bone development, similar paths are taken by these cells during endochondral ossification. Cells on the bottom row show the chondrocyte lineage, which is involved in endochondral ossification. Recent research shows that hypertrophic chondrocytes retain the potential to differentiate into osteoblast precursors, osteoblasts, and osteocytes[52,53]. Dashed lines show differentiation relationships that have not been confirmed in vivo. Diagram adapted from [42].

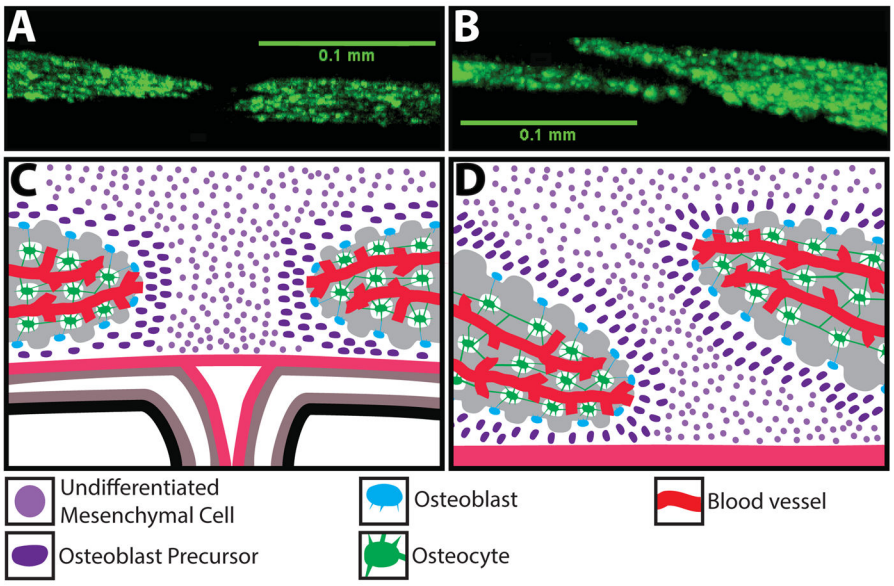

Figure 6.

Suture anatomy. A) Two-photon laser scanning microscopy (2PLSM) image of a murine inter parietal suture; bone labeled fluorescently with calcein, two days postnatal (P2), coronal section. B) 2PLSM image of a murine coronal suture at P2 with bone labeled fluorescently with calcein; para sagittal section, frontal at left, parietal at right. Note that the interparietal (sagittal) suture is an abutting suture and the coronal an overlapping suture. C) Cell composition of interparietal suture, with meningeal layers below (pink - dura mater, gray - arachnoid mater, and black - pia mater). D) Cell composition of coronal suture with dura mater below (pink).

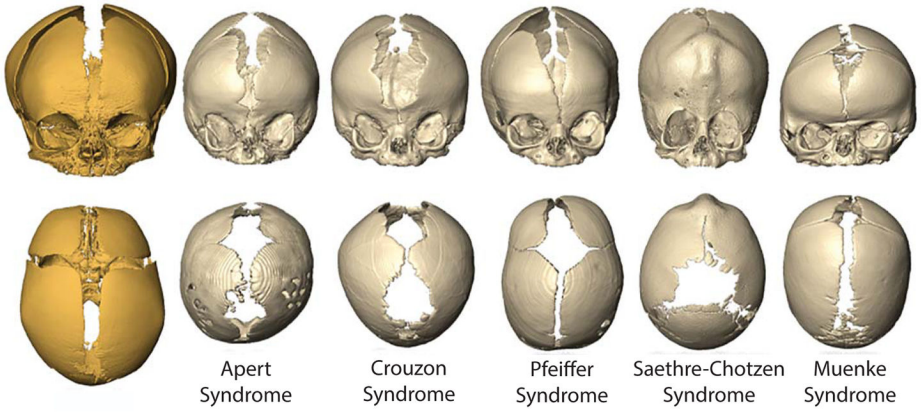

Figure 7.

3D reconstruction of CT images of a human neonatal cranium (anterior/facial view at top and superior/cranial vault view at bottom with face towards top and occiput towards bottom of page) of a typically developing infant (far left) and infants with different craniosynostosis syndromes. The common cranial features associated with the syndromes shown here include: bicoronal synostosis (Apert, Crouzon, Pfeiffer, Saethre-Chotzen and Muenke), metopic synostosis (Saethre-Chotzen), orbital dysmorphology (either hypertelorism or Harlequin deformity: Apert, Crouzon, Pfeiffer, Saethre-Chotzen, Muenke), and midfacial retrusion (Apert, Crouzon, Pfeiffer, Saethre-Chotzen and Muenke syndromes).

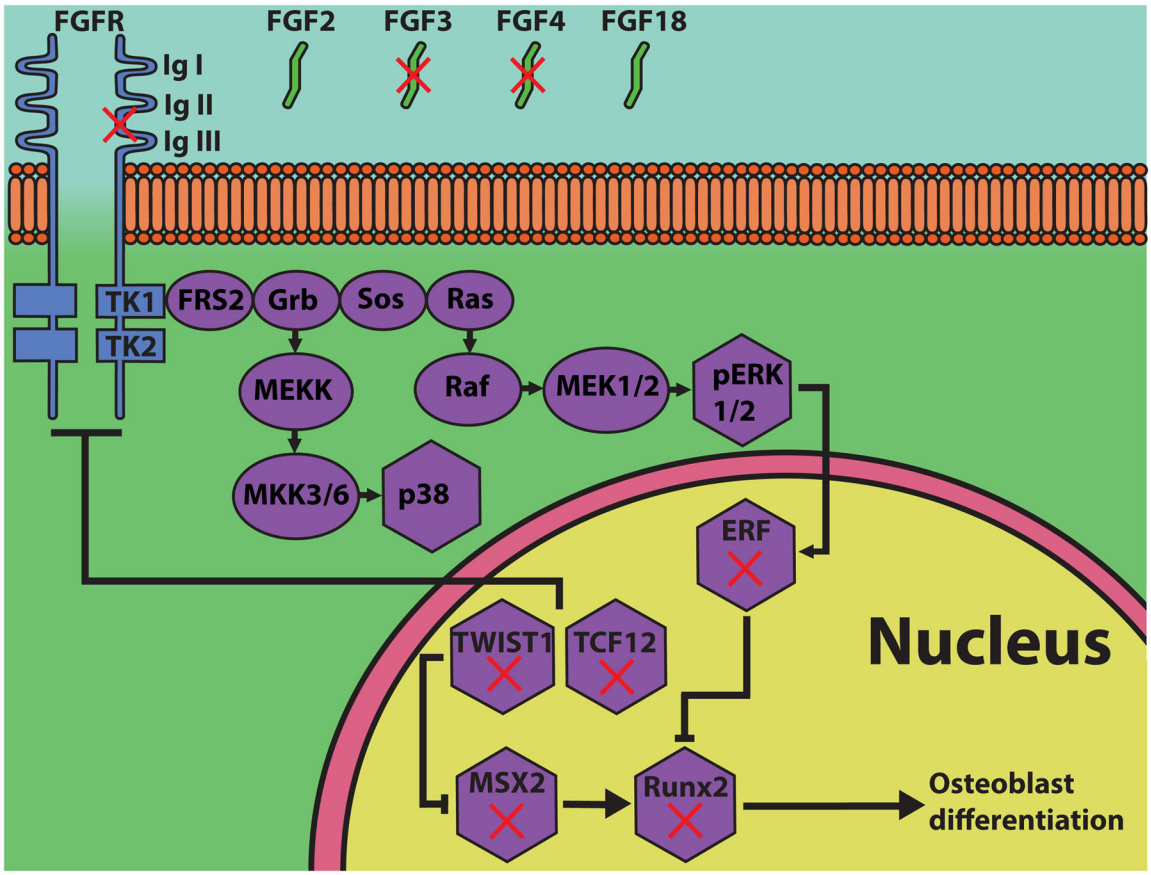

Figure 8.

Mutations of many genes of the FGF/FGFR pathway can cause craniosynostosis conditions. Red X's indicate genes with identified mutations that cause craniosynostosis[92,98,117,127,226–229]. Genes are colored according to function: FGF ligands (green), FGF receptor (blue), cell membrane (orange), downstream effectors of FGFR (purple). The end result of each of these mutations is to activate Runx2, which is necessary and sufficient for osteoblast differentiation. Additional unknown downstream contributors to craniosynostosis are indicated by the unlabeled hexahedron. Adapted from a figure presented by [229].

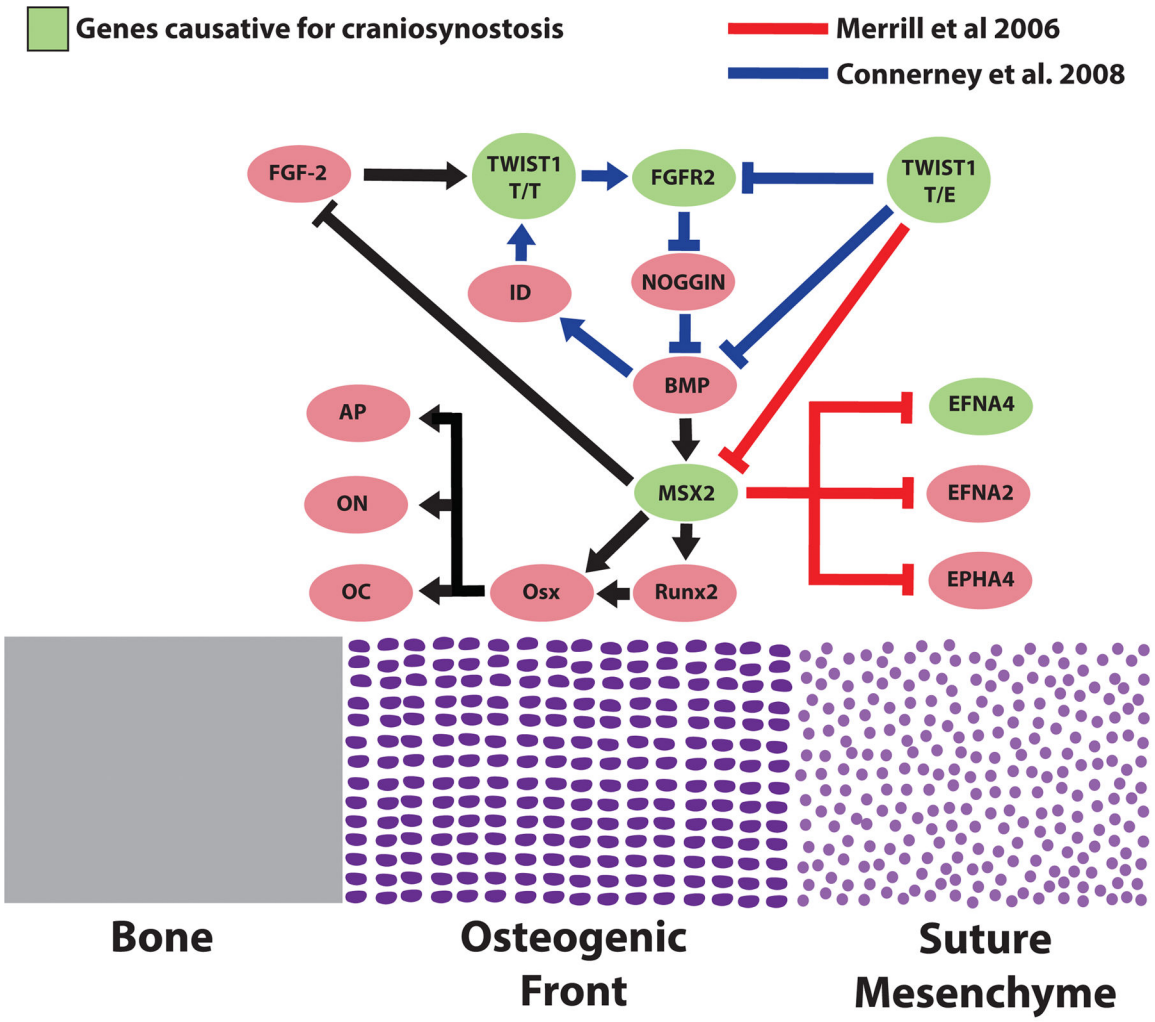

Figure 9.

Diagram of gene interactions in the suture showing the approximate relative locations of gene expression involved in maintaining undifferentiated state of suture mesenchyme cells and causing differentiation of osteoblast lineage cells along the osteogenic front[6,83,84,93,106–108,126,151,230–234]. A model proposed by Connerney et al., shows how TWIST1 heterodimers (T/E) and homodimers (T/T) regulate and are regulated by FGFR2 and BMP expression (blue lines)[84,108]. A second model proposed by Merrill et al., shows how TWIST1 regulation of MSX2 is responsible for EPH/EPHRIN regulation of the boundary between suture mesenchyme and the osteogenic front (red lines)[106,107]. Craniosynostosis is ultimately regulated by activation of RUNX2 and its downstream effectors. Genes known to cause craniosynostosis are colored green. Additional genes contribute to the processes diagrammed and other relationships among those genes included in the figure are possible.

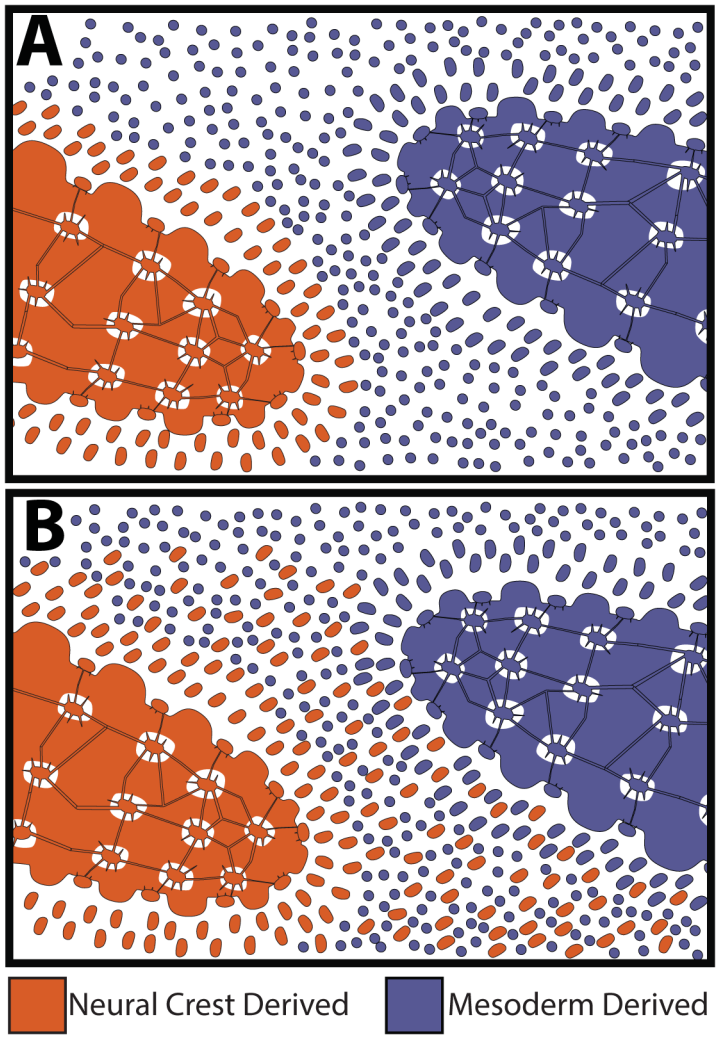

Figure 10.

Cellular origins of components of the coronal suture. A) Frontal bone (left) and parietal bone (right) border the mesoderm-derived mesenchymal cells of the coronal suture. B) Invasion of neural crest-derived osteogenic cells into mesoderm-derived mesenchymal cell population as a result of improper cellular boundary formation that leads to premature fusion of the suture.

Table 1

Craniofacial phenotypes and mutations associated with the better known craniosynostosis syndromes as presented by [22,72,101], with additional references added. Observed phenotypic features are those recorded in human patients and do not necessarily present in all patients diagnosed with the condition. The suite of phenotypes observed in any one patient can vary according to the mutation present, the individual's genome, environment, and other factors. The selected references include clinical observations and work on animal models carrying identified mutations.

Syndromes	Observed phenotypic features*	Genes	Mutations	Selected References
Apert	*Premature suture closure, brachycephaly, eye proptosis, midfacial retrusion, exorbitism, hypertelorism, heterotropia, high arched palate, cleft palate, structural brain anomalies, cognitive impairment, complex syndactyly*	*FGFR2*	*Ser252Trp*	119,185,186
			Ser252Phe	120
			Pro253Arg	119,153
			1119–2A G[f]	72
Crouzon	*Premature suture closure, brachycephaly, flat forehead, midfacial retrusion, eye proptosis, hypertelorism, mandibular prognathism, beaked nose, mild limb abnormalities, variable cognitive function*	*FGFR2*	*Cys342Tyr*	120,123,187,188
			Tyr105Cys	189
			Ser252Leu	120,122
			Pro253Leu	101
			His254Tyr	190,191
			Pro263Leu	192
			Ser267Pro	189
			Phe276Val	193,194
			Cys278Tyr	195
			Cys278Phe	120,187,196
			Ile288Asn	197
			Gln289Pro	187,188
			Trp290Arg	198
			Trp290Gly	194,199
			Lys292Glu	194,200
			Tyr308Cys	197
			Tyr328Cys	118
			Gly338Arg	188
			Gly338Glu	201
			Tyr340His	117,118,123,189,202,203
			Tyr340Ser	197
			Cys342Ser	117,187,188,204
			Cys342Tyr	188,198,205
			Cys342Arg	117,204,206
			Cys342Phe	187,189,207
			Cys342Trp	194,202,203,208
			Ala344Ala (splicing)	117,194,209,210

Syndromes	Observed phenotypic features*	Genes	Mutations	Selected References
			Ala344Pro	211
			Ser347Cys	202
			Ser351Cys	201,217
			Ser354Tyr	195
			Ser354Cys	117,120,188,194
			Ala344Gly	188
Crouzon syndrome with acantosis nigricans)	*Premature suture closure, brachycephaly, midfacial retrusion, acanthosis nigricans*	*FGFR3*	*Ala391Glu*	212–214
Pfeiffer	*Premature suture closure, brachycephaly, cutaneous syndactyly, hypertelorism, high forehead, midfacial retrusion, beaked nose, hearing loss, dental problems, brachydactyly, digit webbing, syndactyly, cloverleaf skull deformity, developmental delay, cognitive deficits*	*FGFR1 FGFR2*	*Pro252Arg*	116,187
			934CGC->TCT[SP >FS]	120
			Cys278Phe	187,196
			Trp290Cys(G->C)	215
			Trp290Cys(G->T)	72
			1119–3T->G^f	206
			1119–2A->G^f	206,216
			1119–1G->C^f	202
			Ala314Ser^f	206
			Asp314Ala	25
			Thr341Pro	205
			Cys342Tyr	187,205
			Cys342Arg	117,194,206
			Cys342Ser (G->C)	187,215
			Cys342Ser(T->A)	187
			Cys342Trp	202
			Ala344Pro	187
			Ser351Cys	217,218
			Val359Phe	187,219
			1263ins6^f	187
			940-2A->G	97,197,220
			940-2A->T	197,220
			G663E	97,150,189
Saethre-Chotzen	*Premature suture fusion, brachycephaly, high forehead, low frontal hairline, ptosis, hypertelorism, broad nasal bridge*	*TWIST1*	*Lys77Ser*	221
			Tyr103stop(308insA)	128,222
			Tyr103stop(C>A)	127
			Glu104stop	72,221
			Arg116Gln	129
			Gln119Pro	128
			Ser123stop	127

Syndromes	Observed phenotypic features*	Genes	Mutations	Selected References
			Ser123Trp	72,221
			Glu126stop	127
			Leu131Pro	127
			Ile134Met	129
			Ile135Met	223
			P139T	223
			405ins21[insAALRKII]	128
			416ins21[insKIIPTLP]	72,127,128,221
			417ins21[insKIIPTLP]	127,129
			Asp141Tyr	72,221
			K145G	128,222
			331delG (V111SfsX14)	223
			355delC (Q119fsX6)	224
			E126X	127,129,223
			A127dup	224
			P139dup	225
			P136S	223
			Y155X	224
			F158L	223,224
			Pro136Leu	221
Muenke	*+, premature suture closure, brachycephaly, orbital hypertelorism, midfacial retrusion, high arched palate, hearing loss, mild anomalies of the hands and feet, developmental delay*	*FGFR3*	*Pro250Arg*	171
Boston-type craniosynostosis	*Premature suture closure, frontal bossing, turribrachycephaly, cloverleaf skull deformity, vision problems, siezures*	*MSX2*	*Pro148His*	92

*features listed have been observed; data on frequency of each phenotype published elsewhere.

+according to 97, Muenke syndrome is clinically not diagnostic. Phenotypic appearance of patients carrying the FGFR3 mutation range from no characteristics to an appearance that overlaps with other craniosynostosis syndromes.