REVIEW ARTICLE

Notch signaling: Its essential roles in bone and craniofacial development

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Notch signaling in the skeletal system

Introduction

The Notch receptors are a group of receptors involved in a variety of cellular functions. The gene got its name originally from Thomas Morgan’s seminal paper “The theory of the gene” when he noticed a Drosophila mutant with a notched wing. We now know that the Notch family encodes 5 receptors (Notch1-5) that have a variety of ligands. The Notch receptors are specifically involved in intercellular signaling; the receptors are located on the cell surface membrane and bind to ligands on neighboring cells. In brief, Notch receptors have multiple highly conserved domains including; Notch extracellular domain (NECD) that binds to ligands, a negative regulatory region (NRR), and a Notch intracellular domain (NICD) that is cleaved and translocates to the nucleus during signaling.

Notch signaling is highly conserved between species, underscoring its important role from invertebrates to humans. Notch signaling has been shown to be important in a variety of human diseases including congenital syndromes, such as Alagille’s, as well as acquired diseases, such as cancer. Researchers have recently shown increasing interest in the Notch pathway, a nod to a growing understanding of its importance. A quick PubMed search will show that publications with the keywords “Notch” and “cancer” have increased from 71 in 2000 to more than 700 in 2018. Notch has also been shown to be involved in skeletal development, homeostasis, and disease. Notably, Notch dysfunction in bone can lead to brachydactyly, spondylocostal dysostosis, and osteosarcoma; the mechanisms of which will be discussed further in this review.

This research shows that Notch is important in bones and skeletal development, and more recent research has also shown that Notch signaling contributes to the complicated coordination of craniofacial embryogenesis. We hope to summarize the important roles of Notch signaling, discuss the implications of Notch signaling in craniofacial development, the skeletal system, and in the context of disease and therapies.

A brief note on gene and protein nomenclature-in our review we reference genes and proteins using the nomenclature dictated by the HUGO Gene Nomenclature Committee, the International Committee on Standardized Genetic Nomenclature for Mice, and the Zebrafish Nomenclature Committee (ZNC). For example, the gene and protein Bone Morphogenetic Protein-9 will be referenced as follows: BMP9 (human protein), Bmp9 (mouse protein), bmp9 (zebrafish protein), BMP9 (human gene), Bmp9 (mouse gene), bmp9 (zebrafish gene).

Overview of Notch signaling

In this section we will describe the Notch receptors including their structure and downstream signaling. The mammalian Notch signaling pathway consists of four evolutionarily conserved single-pass transmembrane receptors (Notch1, 2, 3, 4) that bind to five canonical ligands through juxtacrine signaling.
repeats and the heterodimerization domain, and an intracellular domain (NICD) that is eventually cleaved, translocated to the nucleus, and responsible for activating transcription.2

Prior to expression in the cell membrane, the precursor receptor protein is cleaved in a ligand independent manner (S1 cleavage) at the heterodimerization domain by furin-like convertase in the golgi apparatus to convert the 300 kDa sequence into a 110–120 kDa sequence (including the NICD) that heterodimerizes with the 200 kDa NECT sequence, held together by Ca2+ dependent ionic bonds.12–14 This cleavage process is regulated by c-Src, and the c-Src mediated Notch1-Furin interaction is further induced by Tgf-α and Pdgf-BB.15 Post cleavage the NECT EGF region is glycosylated, most prominently through the addition of O-fucose, O-GlcNac, and O-glucose, regulating ligand affinity, recognition, and binding, as well as surface stability and expression.16 This series of glycosylations has been shown to be essential to proper receptor functioning and signaling through a series of enzymatic knockout experiments, most likely by ensuring proper tertiary structure for cleavage of the intracellular domain post ligand binding.17

Notch ligands

The canonical ligands, which are single-pass transmembrane proteins that all possess a Delta/Serrate/Lag-2 (DSL) domain and a conserved EGF-like repeat region, include three members of the Delta-like family (Dll1, Dll3, and Dll4) and two members of the Jagged family of Serrate homologs (Jag1, and Jag2).10 In the canonical pathway these ligands bind to and activate receptors on adjacent cells (trans-activation), but ligands are also able to interact with receptors on the same cell (cis-inhibition and cis-activation). Cis-inhibition (and its interplay with trans-activation) has been shown to be important in numerous processes, including neuroepithelial differentiation and postnatal human epidermis differentiation, with Dll1 acting as a trans-activator when expressed on an adjacent cell and acting as a cis-inhibitor when expressed on the same cell.18–20 Though less well understood and often masked by cis-inhibition, recent evidence has been uncovered the role of cis-activation, which potentially also plays a part in cell fate determination and patterning.21,22

In addition to the Delta and Serrate families of ligands, a range of other ECM-expressed proteins and micro-environmental factors have been shown to interact with the Notch pathway through a variety of mechanisms, including direct ECM-receptor interactions, indirect transcription mechanisms, interactions with other pathways, and biochemical and biophysical factors.10,23

Signal transduction and pathway

In the canonical ligand binding pathway, the receptor ligand interaction is followed by trans-endocytosis of the NECT of the receptor into the ligand cell, resulting in an outward force on the receptor that exposes the S2 site within the NRR region for cleavage by a series of ADAMs proteins, thereby freeing the NICD. Fig. 1 summarizes the Notch transduction pathway. Experiments have shown that sole ligand binding with no outward force from trans-endocytosis results in the NRR region remaining as a structural inhibitor, thus preventing S2 cleavage by ADAMs.12 Following S2 cleavage, the NICD is separated from the cell-membrane through an S3 cleavage by γSec (gamma secretase), which binds to the residual 12aa ectodomain formed by the S2 cleavage through the nicastrin subunit in the γSec protein.24,25

The newly freed NICD is then promptly translocated to the nucleus, where it forms a transcriptional regulatory complex with Rbpjκ and Maml to regulate and activate transcription of target genes.26 In the absence of NICD the Rbpjκ and Maml complex binds with transcription repressors, but the formation of the NICD-Rbpjκ-Maml complex dispels the repressors and recruits transcription activators. Following transcription, the Maml protein recruits CdK8, which phosphorylates NICD, and also recruits a series of proteins that ubiquitinate the NICD, ultimately resulting in its degradation, reverting the complex to a pre-NICD state of transcription repression.12

It is important to note that there has been sizeable evidence for Notch signaling pathways outside of the well-studied canonical one, most prominently in various cancers and immune system function. For example, γSec inhibition did not block all Notch functioning in cancer cells, Rbpjκ knockout mice showed that non-canonical Notch-4 signaling is involved in mammary gland tumorigenesis, and numerous studies have shown Th cell differentiation to be driven by Notch signaling, but independent of Rbpjκ.27

Notch downstream targets

The downstream targets of the Notch pathway are the families of Hes and Hey proteins, evolutionarily conserved basic helix-loop-helix (bHLH) proteins that typically act as transcription repressors.10 Of the seven Hes proteins (Hes1-7), all except Hes2 and Hes3 are targeted by the Notch pathway, as are all three of the Hey proteins (Hey1, Hey2, HeyL). Given the pleiotropic nature of Notch signaling and the wide swath of phenotypes it is involved in, it comes as little surprise that the Hes and Hey proteins have a range of functions.

Hes1, 3, and 5 have been shown to be involved in delaying differentiation of neuronal stem cells, both in adults and development. Specifically, mouse Hes1 has been observed to act through an oscillatory relationship with Achaete–scute homolog 1 (Ascl1), which activates the regulators of cell cycle progression.26 Further, human HES3 has been shown to increase malignancy in lung cancer.29 Similarly, human HES6 has been shown to not only act as an inhibitor of HES1 (and thus promote neuronal differentiation), but also promote aggressiveness in multiple cancers including prostate and colorectal.30–32 Hes4 drives osteogenesis and inhibits adipogenesis by inhibiting Twist-1.33 Hes7 plays a notable role in somitogenesis, and is controlled not only by the Notch pathway, but also the E-box and T-box pathways.34

The Hey proteins are required for vascular development, and also partake in a range of other functions.35 For example, HeyL plays a critical role in muscle stem cell
proliferation in overloaded muscles, and Hey2 has been tied to amyloid β-protein production in Alzheimer’s mice and increased malignancy for both hepatocellular and non-small cell lung carcinoma.36,37 Similarly, human HEY1 has also been shown to drive proliferation of various carcinomas and prevent differentiation of neural stem cells in the adult brain.38–40

Notch signaling crosstalk

The Notch pathway has interactions with many other important cell signaling pathways. In this section we will highlight Notch crosstalk with the NFκB pathway, as well as with some other oncogenic pathways, while Notch crosstalk with the Bmp, Hedgehog, and Wnt pathways will be discussed later. Fig. 2 provides an overview of the interacting signals involved in the Notch pathway. The full scope of NFκB signaling is beyond the scope of this paper, but in brief, the NFκB pathway is classically activated during inflammation but has also been shown to be important in cancer biology as well.41 The NFκB family consists of homo or heterodimers made up of p50, p65, c-Rel, p52, and RelB.42–44 These proteins are sequestered in the cytosol by IκB inhibitor proteins including IκB-α, IκB-β, IκB-ε, Bcl-3, as well as NFκB precursor proteins p100 (p52 precursor) and p105 (p50 precursor).43,44 For further reference the reader is referred to the comprehensive review by Osipo et al.44 Crosstalk between Notch and NFκB has been shown to be bidirectional and have a variety of mechanisms. One study showing the transcriptional regulation of Notch on NFkB, showed that Notch-1 induces NFkB2 promoter activity increasing the transcription of p100.45 In another direction, NFκB transcriptionally upregulates Jagged-1 and that NFκB and Notch-2 can have a synergistic effects, both in B cells.46,47 Other studies have also shown that Notch-1 and NFκB physically interact in the nucleus,48 and more recently a study showed that Notch and NFκB are novel coregulatory signals of miR-223, a cancer associated microRNA, and the tumor suppressor FBXW7 in T-ALL cells.49 Notch and NFκB pathways are obviously intertwined in complex ways and further exploration of this relationship may yield important cancer therapies and insights.

Other oncogenic pathways that Notch has been recently shown to interact with include inflammatory cytokines such as IL-1 and Leptin. Leptin is classically known as a regulator of food intake, but has also been implicated as an inflammatory marker. It has also recently been shown to be important in tumorigenesis, especially breast cancer.50,51 Leptin is the product of the Ob gene and is a protein that signals by binding to one of its six extracellular receptors (obRa-obRf) in a 1:1 fashion.52 Intracellularly Leptin has

Figure 1  Notch signaling overview. This figure summarizes the signal transduction pathway of Notch. (1) Furin cleaves the precursor receptor in the golgi apparatus and the NECD heterodimerizes with the NICD. (2) NECD is glycosylated. (3) Notch ligands attached to the same cell are able to inhibit (cis-inhibition) Notch receptors. (4) Notch ligands on adjacent cells activate (trans-activation) the Notch heterodimer. (5) NECD is trans-endocytosed by the ligand cell. (6) S2 site exposed on the NRR region is cleaved by ADAMs proteins. (7) NICD is freed from the cell membrane through S3 cleavage by γSec. (8) NICD translocates to the nucleus where it forms a transcriptional regulatory complex with Rbpjk and Maml. (9) NICD is ubiquinated and degraded reverting the complex to state of repression.
been shown to signal through a variety of cascades including Jak/Stat, Mapk, Irs1, and Soc3. One study showed that in breast cancer cell lines, Leptin upregulated Notch receptors, ligands, and target genes. It was also demonstrated that the tumorigenic effects of leptin were abrogated when Notch signaling was blocked by DAPT, and that Notch activation by Leptin was blocked when IL-1 signaling was blocked by antibody.56 The same group recently published a study showing that Notch blockade similarly decreased the effects of Leptin on pancreatic cancer cells.57 These studies provide persuasive evidence of Leptin–Notch interaction, but the mechanism remains unclear. Although the evidence on the Leptin–Notch axis is evolving, it holds promise as an important part of cancer biology.

**Notch in bone and the skeletal system**

The human skeleton consists of bone derived from mesoderm, except for the skull, which is developed from neural crest along with mesoderm. During embryogenesis, the skeletal system is formed via the coordinated action of chondrocytes and osteoblasts, which form cartilage and bone, respectively.56 Cartilage generated by chondrocytes is later ossified to form bone via endochondral ossification (eg. limb long bones). Bone can also be formed directly from mesenchymal condensation via intramembranous ossification (eg. skull bones). This ossification process continues postnatally until the mid 20s.59 In the adult skeleton, bone homeostasis continues via the coupled action of osteoblasts and the bone-resorbing osteoclasts. The Notch signaling pathway has emerged as an important component of both skeletal development and homeostasis, and as a possible therapeutic target for many clinical problems.58

**Skeletal development**

In embryogenesis cartilage is formed from condensed mesenchymal tissue that has differentiated into chondrocytes.60 In this early stage, most of the skeleton is cartilaginous until it is later replaced by bone via ossification. Notch signaling has been shown to play a role in both chondrogenesis, osteoclastogenesis, and osteoblastogenesis.61,62 During cartilage and bone development in embryogenesis, a common precursor cell, the mesenchymal progenitor cell, condenses and differentiates into an osteochondroprogenitor cell. Osteochondroprogenitor cells can then differentiate into either osteoblasts or chondrocytes, depending on the input they receive from transcription factors. The transcription factor SRY-box transcription factor 9 (Sox9) induces chondrocyte differentiation, whereas the runt-related transcription factor 2 (Runx2) induces osteoblast differentiation. The stages of differentiation in the chondrocytic lineage are the osteochondroprogenitor cell, condensed mesenchyme, proliferating chondrocytes, prehypertrophic chondrocytes, and finally hypertrophic chondrocytes. Rapid mitosis occurs in the stages before hypertrophic chondrocytes are formed, which occurs during endochondral ossification.

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**Figure 2** Summary of signaling crosstalk discussed in current review. This figure highlights the interaction between the different pathways discussed in the review. BMP activates the SMAD protein which is thought to interact with the intracellular NICD. Hh signaling activates GlI/2 which causes transcription of Hes1, a shared target with Notch. Leptin signaling activates SOCS3 which downstream upregulates transcription of the Notch receptor. Wnt signaling causes transcription of the Runx2 transcription factor, which is inhibited by Hey1.
During normal chondrogenic differentiation, NICD is not expressed in the proliferative zone, but is instead expressed in the prehypertrophic and hypertrophic cartilage zones, the zone at which chondrocytes stop mitosis and begin to hypertrophy. Inhibition of Notch signaling during prehypertrophic and hypertrophic chondrocyte differentiation leads to an increase in chondrocyte proliferation, which ultimately results in decreased bone formation.63,64 The predominant Notch receptor carrying out this role in chondrocyte differentiation is the Notch-2 receptor.63 The mechanism by which Notch regulates chondrocyte differentiation is through inhibition of the collagen type II promoter (Col2a1), the gene encoding the major cartilage matrix protein.65 Once the Notch-related transcription factors Hes1 and Hey1 are activated, they bind to Sox9 enhancer, suppressing Sox9 gene expression.66 However, Sox9 is co-expressed with Col2a1, so Col2a1 expression is also suppressed. Furthermore, downregulation of Notch-related transcription factors increases the expression of these chondrogenic transcription factors.64,66 These studies show that Notch signaling decreases chondrogenesis tipping the scale in favor of osteogenesis.

The second major role that Notch signaling plays in skeletal development is in osteoblastogenesis. During osteoblastogenesis, osteoblast precursors proliferate and expand, eventually mineralizing as mature cells. The stages of differentiation in the osteoblastic lineage are the osteochondroprogenitor cell, perichondral cells, preosteoblasts, and finally osteoblasts. Depending on the differentiation stage of osteoblasts, Notch signaling may variably have a suppressive or inductive effect. Overall, NICD overexpression suppresses the differentiation of early-stage precursors, promotes the proliferation of intermediate osteoblast-lineage cells, and inhibits mature osteoblast formation.68 In the early stage of osteoblastogenesis, the key molecular switch for osteoblastic commitment is the transcription factor Runx2.69 Downstream of RBPjκ, Hey transcriptional suppressors inhibit Runx2 activity, thereby inhibiting the differentiation of osteochondroprogenitor cells into perichondral cells (early stage).70,71 Inhibition of early-stage precursors is mediated by Notch-2 through the action of RBPjκ.70 However, this effect is reversed in the differentiation of perichondral cells into preosteoblasts (intermediate stage), as NICD expression during this stage promotes proliferation characterized by excessive immature woven bone and fibroblast cells within the marrow cavity.72,73 In this stage RBPjκ is also the main mediator of Notch’s function, as the effect of NICD is abolished upon deletion of RBPjκ.72 In the late stage, where preosteoblasts differentiate into mature osteoblasts, Notch signaling once again exhibits an inhibitory function.72 These results suggest that Notch signaling suppresses the early and late stages of osteoblastogenesis but induces the intermediate stage.

Skeletal homeostasis

Bone is a dynamic tissue that is continually adapted to preserve skeletal size, shape, structural integrity, and mineral homeostasis.74 The process of skeletal homeostasis is achieved via bone remodeling. Bone remodeling is the tightly regulated process of resorption of existing bone by osteoclasts, followed by the formation of new bone by osteoblasts.75 This process is driven by osteocytes, which detect and respond to hormonal and mechanical stimuli to coordinate the function of osteoclasts and osteoblasts.76 Multiple coordinated cellular and molecular events work simultaneously to regulate bone remodeling and thereby influence skeletal homeostasis.74 Notch signaling has been shown to be important for the proper differentiation and function of osteoblasts and osteoclasts.

Osteoclasts differentiate from macrophage precursors and have the unique ability of removing mineralized bone matrix. Osteoclasts are activated when their surface-bound receptor activator of nuclear factor kappa-B (RANK) is activated by a surface-bound receptor activator of nuclear factor kappa-B ligand (RANKL) on the osteoblast. Another key inducer of osteoclast activity is macrophage-colony-stimulating factor (M-CSf), which is also derived from osteoblasts. These two cytokines (RANKL and M-CSf) are critical for the differentiation and survival of osteoclast precursor cells.77 Notch signaling is an important regulator of osteoclastogenesis, and it can have either suppressive or inductive effects depending on the differentiation status of the osteoclast and the expression of certain ligands and receptors.68 For instance, studies have shown that bone marrow macrophages with Notch-1-3 deletions differentiate into osteoclasts more rapidly than the wild type in response to M-CSf.78 Macrophages with Notch-1-3 deletions are also much more sensitive to RANKL and M-CSf.79 In contrast, another study found that RANKL induced expression of Notch-2 in bone marrow macrophages during osteoclast differentiation.79 These results suggest that Notch signaling can act as either a stimulator or repressor of osteoclast differentiation based on the expression of certain ligands and receptors as well as the differentiation status of the cell.

Another major cell in the bone remodeling process is the osteocyte, which is a dendritic-like cell that is formed when an osteoblast becomes embedded in the matrix it has secreted. Osteocytes function in skeletal homeostasis by acting as mechanosensor cells that control the activity of osteoblasts and osteoclasts. They do so by synthesizing sclerostin, which antagonizes the activity of bone morphogenetic protein (Bmp), ultimately inhibiting bone formation.76,80 Notch plays a regulatory role in osteocytes by modulating both osteocyte differentiation and the mineralization mediated by osteoclasts.81 Both early and late osteocyte differentiation are influenced by Notch signaling. Through Hes1 activity, Notch inhibits the transmembrane glycoprotein E11, which is a critical protein in early osteocytogenesis. In the late differentiation stage of osteocytes, Notch both directly represses phosphorylated Akt and prevents the nuclear aggregation of β-catenin, thereby inhibiting osteocyte differentiation.82,83 In another study, Notch inhibition significantly affected terminal mineralization, leading to the spontaneous deposition of calcium phosphate on collagen fibrils, alteration of mineral crystal structure, and suppression of dendrite development in osteocytes.81 Overall, these studies show that Notch plays a regulatory role in the differentiation of osteocytes,
osteoclasts, and osteoblasts. Notch activity influences the properties of bone mineralization and is therefore an important mediator of skeletal homeostasis.

Abnormal Notch signalling in skeletal disorders

We have discussed above the development of the skeletal system and the role of Notch in the normal functioning of the skeletal system. Aberrant Notch signaling has been implicated in several genetic and sporadic disorders that affect the skeleton which are summarized in Table 1. Here we will examine a few specific examples of Notch signaling involvement in skeletal disorders.

Osteosarcoma is the most common primary bone malignancy and is the eighth most common primary tumor in children accounting for 2.4% of pediatric tumors.84 Death rates from osteosarcoma have been in decline but it is still associated with significant mortality, especially if there is metastatic spread, with a 27% 5-year survival rate with distant metastasis.85 Recent research has shown that aberrant Notch signaling is involved in osteosarcoma pathogenesis and may be useful as a therapeutic target. One study showed that NOTCH2 gene overexpression in human osteosarcoma cell lines, and another study showed that inhibition of Notch signaling led to arrest of the cell-cycle in G1.86 Other studies have shown that Notch is important in the metastatic potential of osteosarcoma. One study showed that inhibition of Notch signaling is associated with a reduction of aldehyde dehydrogenase activity, and an increase in Notch signaling led to an increase in invasiveness.87 Recently our lab has shown that BMP9 stimulated osteosarcoma growth is at least partially mediated by Notch signaling and that use of a dominant negative mutant of NOTCH-1 can inhibit the pro-oncogenic activities of BMP9.88 Furthermore, multiple recent studies have shown that the Notch pathway will be an important target for future osteosarcoma therapies.89–92

Brachydactyly is a limb malformation characterized by disproportionately short fingers and toes.93 There are many different forms of brachydactyly that include syndromic and non-syndromic forms. Studies have shown that at least one form of syndromic brachydactyly is associated with increased Notch signaling.94 In one particular study, cells from patients with brachydactyly with a loss of CHSY1 showed an increase in JAG1 and NOTCH receptor expression.94 Another study showed that loss of CHSY1 caused brachydactyly through BMP signaling, which supports the other results showing that Notch functions downstream of BMP.95,96 These results show that Notch signaling is important in limb development.

Spondylocostal dysostosis (SD), previously called Jarcho-Levin syndrome, is a disease of skeletal development and growth. It presents with a characteristic phenotype including a shortened thorax, rib malformations, scoliosis of varying severity, and vertebral anomalies.97 Multiple gene mutations and inheritance types have been associated with SD. The most commonly associated mutation is a delta-like 3 (DLL3) gene mutation resulting in truncated or nonfunctional protein products, which is an autosomal recessive form of the disease.98 A mouse model, pudgy mutant mice, with a phenotype similar to SD has also been produced through a DLL3 gene mutation, a functionally equivalent allele.99

DLL3 mutant gene products function as Notch ligands with a transmembrane domain.100 DLL3 gene products act as a dedicated inhibitor of Notch signaling.101 As with many other Notch-associated pathways, DLL3 has a cyclical effect and results in different Notch expression levels at various developmental time points.102 Specifically, DLL3 mutations result in less severe effects in the presomitic mesoderm, but cause large phenotypic defects.103 The skeletal defects in SD patients with a DLL3 gene mutation are the result of delayed somite formation, disrupted boundary formation, and altered anterior-posterior polarity.103 Interestingly, SD may also demonstrate an environment–gene interaction, as many Notch gene disorders demonstrate increased penetrance and worsened phenotypic severity in short-term gestational hypoxia models.104 Greater understanding of the many genes affecting Notch signaling, and especially the complicated oscillatory control of expression during development, may enhance our understanding of the role of Notch disruption in skeletal disorders.

Notch and craniofacial development

The role of Notch signaling in craniofacial development is a relatively new field with many unanswered questions. We found there to be a lack of literature reviewing the known role of Notch in the craniofacial system. In this section, we will briefly summarize the normal development of the human cranium and face, as well as discuss research which has linked Notch signaling to this complex developmental process.

| Table 1 | Table of skeletal disorders associated with Notch signaling. |
|---------|-------------------------------------------------------------|
| Disorder | Gene(s) mutated | Clinical Manifestation |
| Spondylocostal dysostoses96 | DLL3, MESP2, HES7, LFNG | Dwarfism, vertebral anomalies |
| Brachydactyly94 | CHSY1 | Short digits, stunted growth |
| Hajdu–Cheney syndrome11 | NOTCH2 | Osteoporosis, fibular deformities |
| Osteosarcoma96,92 | NOTCH2, JAG1, HEY1, and HEY2 | Decreased bone density |
| Alagille Syndrome49 | JAG1, NOTCH2 | Bile duct abnormalities, heart abnormalities, craniofacial abnormalities |
| Osteoporosis68 | JAG1 | Fractures, decreased bone mineral density |
| Adams-Oliver Syndrome69 | ARHGAP31, DLL4, NOTCH1, or RBPJ | Aplasia cutis, limb defects |
Overview of craniofacial development

Craniofacial development is a complex process that requires the coordination of multiple pathways to be successful. The full scope of craniofacial development is beyond this review, but herein we provide some relevant background.

Endoderm, mesoderm, ectoderm and its derivative neural crest cells make up the building blocks of all adult tissue and are derived from the trilaminar disc that the zygote forms after implantation. Neural crest cells (NCCs) have long been established as important players in craniofacial development, although only recently has their multipotent potential been shown in vivo. The face is formed by groups of migrated cranial neural crest cells (CNCCs) that interact with ectoderm and endoderm to form the five facial primordia; the frontonasal prominence, and the paired mandibular and maxillary processes. These processes form around the central depression, the stomodeum, that will become the mouth. They begin to form around the third week beginning with the mandibular processes, followed by the frontonasal prominence. The frontonasal prominence, derived from ectoderm, develops into two frontonasal processes each with a medial and lateral nasal process. The nasal processes will fuse to become the intermaxillary segment that develops the middle part of the nose, philtrum, premaxilla, primary palate, and a portion of the nasal septum. The mandibular processes, derived from the first pharyngeal arch, grow quickly from both sides and fuse in the midline creating the precursor to the lower jaw. The maxillary processes, derived from the first pharyngeal arch as well, grow inwards towards the nasomedial processes; the fusion of the maxillary process with the nasolateral processes create the lateral nostril border and the ala nasi. The fusion of the maxillary processes and the intermaxillary segment create the upper lip; the fusion of the palatal processes of the maxillary processes creates the secondary palate; and the fusion of the maxillary processes and medial nasal processes creates the primary palate. Failure during the development can lead to pathology such as craniofacial clefts, for which two theories of pathogenesis have been proposed. The classic theory proposes that it is a failure of fusion of the facial processes that leads to clefts, while the second theory proposes that a failure of mesenchymal penetration to support the epithelial walls leads to dehiscence and resulting clefts.

The bones of the skull vault are made up of both cranial mesoderm and neural crest cells. The mesoderm and neural crest cells go through epithelial-mesenchymal transformation and migrate to their locations in the primordial skull. Migrating mesenchymal cells form the bone primordia by condensing and forming membranes that respond to the growth of the underlying brain. These then expand apically and laterally to create the cranial sutures, which become the vital structures regulating skull growth. Bones of the skull form by intramembranous, rather than endochondral, ossification. This means that the bone is created directly from the progenitor mesenchyme rather than from cartilage. Intramembranous ossification starts in the bone primordia around the 7th or 8th week with the bone formation spreading centrifugally from the center of the future bones to form osteogenic fronts at the suture lines. The cranial base, however, is formed by endochondral ossification. The sutures are important in the regulation of skull growth - they remain patent in order to allow growth while at the same time they function as the site of new bone development. Skull expansion and bone development are at a delicate balance: too much osteogenesis leads to premature fusion while too little osteogenesis leads to suture agenesis.

All of these processes are controlled by interacting signaling pathways involving the bone and surrounding tissues. Although many of the underlying mechanisms and interactions have yet to be fully defined, the remainder of this review will cover some of the important known pathways as well as highlight the role of Notch signaling in this process.

Overview of craniofacial development signaling pathways and their crosstalk with Notch

Here we will discuss some of the important signaling pathways other than Notch that are involved in craniofacial development. Bmp, Wnt, and Hedgehog have all been proposed to involve crosstalk with the Notch pathway. Table 2 summarizes the multitude of pathways involved in craniofacial development.

| Pathway     | Examples of related processes or disorders                                      |
|-------------|--------------------------------------------------------------------------------|
| BMP         | Involved in facial, tooth, cranial suture, and palate morphogenesis.          |
| Hippo       | Involved in skull growth, odontogenesis                                      |
| FGF         | Disorders can cause craniosynostosis, skeletal dysplasias, many others       |
| Hedgehog    | Involved in palate, skull, and face development, disorders can cause holoprosencephaly, frontal bone dysplasias, many others |
| Endothelin  | Involved in epithelial-mesenchymal transition, disorders can lead to multiple craniofacial malformations. |
| TGFβ-3      | Involved in soft palate development, knockout can lead to cleft palate.       |
| Wnt         | Involved in fate determination of many cells, knockout can lead to brain, lip, and palate malformations. |
| ROCK/TAZ    | Involved in mechanical transductions and osteogenic differentiation           |
| Nell-1      | Involved in cranial suture patency and fusion as well as osteogenesis.       |
| Twist       | Involved in cranial suture morphogenesis, heterozygote loss causes coronal fusion. |
craniofacial development and some of the findings that have been associated with that pathway.

Bmps transduce signals by binding to Bmp receptors (BmpR). Ligand binding induces phosphorylation of the receptors, which then activate canonical signaling via receptor Smad.2,3 The Bmp signal activates Smad1, Smad5 and Smad8, which upon phosphorylation can associate with Smad4 (common Smad) into a heterodimeric complex that is further translocated to the nucleus where it activates transcription. The Bmp pathway has been shown to be vital for early mesenchymal stem cells as well as later in craniofacial bone structuring.124,125 Studies have shown that Bmp signaling and its downstream target Msx2 are important in the differentiation of osteogenic precursor cells in the initial phases of calvarial bone development, as well as in odontoblast formation.125,126 Others have shown that Bmp signaling affects the spatial formation of facial development, such as Bmp4 causing abnormal maxillae and mandible in mice,14 and Bmp5 and Bmp7 knockout mice having reduced branchial arch size.127 In particular, the Bmp and Notch pathways seem to intersect with regards to Runx2, a key transcriptional regulator of osteoblast differentiation. As we have discussed Runx2 is inhibited by Hes1 and can be upregulated by Bmps. Studies have shown that Runx2 knockout mice lacked intramembranous or endochondral ossification and the heterozygous phenotype mirrored the clavicular and cranial deformities of human genetic correlate cleidocranial dysplasia.128 Some reports propose that Notch signaling enhances Bmp-induced osteoblastogenesis. One study found that activating Notch signaling in MC3T3-E1 cells with adenovirally-overexpressed NICD stimulated Bmp2-induced osteoblastogenesis, which was shown via elevated ALP-expressing cells and newly formed calcified nodules.129 Interestingly there have been other reports, also in MC3T3-E1 cells, showing that if the cells overexpress Bmp2, stimulation of Notch targets suppress Bmp2 induced osteoblastogenesis.130 There is thus a complex relationship between the Bmp and Notch signaling pathways.

The Wnt pathway is another highly conserved pathway due its regulation of critical functions in the body from embryogenesis to adulthood. In the canonical pathway (Wnt/β-catenin), one of the Wnt ligands binds to the extracellular domain of the Frizzled G-protein receptor, lipoprotein receptor-related protein (Lrp), and neurotrophic tyrosine kinase, receptor-related 2 (Ror2), leading to the accumulation of β-catenin in the cytoplasm with its subsequent translocation into the nucleus to act as a transcription factor.4 Wnt3 and Wnt9b were found to be expressed in facial ectoderm at crucial stages of midfacial morphogenesis during mouse embryogenesis. Wnt3 was seen primarily in maxillary and midnasal ectoderm, while Wnt9b was highly expressed in midnasal, maxillary, and lateral nasal ectoderm.5 Studies in Zebrafish have also shown that wnt signaling is important in the dorsal-ventral patterning of the craniofacial skeleton and the wnt deficiency leads to lower jaw defects.131 Wnt action is thought to mostly occur by effects on neural crest cells in the developing craniofacial skeleton, and through its interactions with the BMP pathway.132 Wnt and Notch pathways overlap and interact during development, with studies showing their importance in the development of the ear and tongue.133,134 Notably activation of the Wnt pathway causes transcription of the Runx2 gene, and it has been shown that Hey1 inhibits Runx2 transcriptional activity on osteoblastic genes.135 One recent study showed that in the developing mouse cochlea, Wnt regulates Notch gene expression and antagonizes Bmp4, highlighting how the three pathways influence each other during development.136 In another study Notch overexpression inhibited osteoblastogenesis through an inhibition of the Wnt pathway.137 Interestingly, multiple recent reviews and studies have highlighted the Hippo pathway interaction with both the Notch and the Wnt pathway that occur during development.138–140

The Hedgehog pathway influences cell differentiation during early embryogenesis. The Hedgehog protein family consists of three ligands: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), of which only two are expressed within the craniofacial paradigm (Ihh and Shh).10 Hedgehog ligands bind to the responding cell via its 12-pass transmembrane receptor Patched (Ptc), which acts as a negative inhibitor of the protein SMO, another transmembrane protein. Shh binding to Ptc allows Smo to transduce the signal to the cytoplasm of the responding cell which leads to the activation of nuclear transcription factors.10 Shh is a key factor essential for early craniofacial development. Shh is present throughout the axial mesendoderm and is important in establishing portions of the eyelid and ventral forebrain. Specifically, Shh signals in three domains in development; the neuroectoderm of the ventral forebrain, the ectoderm of the facial midline, and pharyngeal endoderm.11 The fundamental nature of Shh in craniofacial development was demonstrated when Shh knockout mice presented with severe craniofacial defects including alobar holoprosencephaly and cyclopia.2,12 Similarly, Ihh is important for bone growth during maturation. In an Ihh deletion study, chondrocytes of mice carrying conditional and inducible null alleles of Ihh resulted in permanent defects in bone growth, inhibiting proliferation and promoting differentiation of chondrocytes, and ultimately leading to dramatic expansion of the hypertrophic zone and truncation of bone.12 Studies have shown crosstalk between Notch and Hedgehog signaling; for example, mice with a Jagged mutation showed a difference in the patterning of Shh expression.142 Also notably both Notch and Hedgehog pathways converge on the upregulation of the Hes1 transcription factor.142 Another study showed that Notch positively regulates Shh expression and that secreted Shh may be involved in the cell fate switch induced by Notch.143 More evidence of Hedgehog—Notch interaction comes from the enteric system, as one study showed that Hedgehog deficient mouse embryos showed Notch overactivity in the gut, and that recombinant Shh could override Notch-induced death of cultured mutant gut mesenchymal cells.144

As discussed, Notch is important on many different aspects of the developing cranium and face. These Notch-specific aspects of craniofacial development are highlighted are highlighted in Fig. 3, showing where studies have shown Notch functions so far. The developmental pathways of Notch, Bmp, Wnt, and Hedgehog have all been shown to interact together during patterning, ALS, hypoxia, and in carcinogenesis, and there have been many studies showing how these pathways interact. For further reading
on signaling pathway crosstalk we direct the reader to recent reviews discussing these pathways.\textsuperscript{142,145–148}

Abnormal Notch signaling in craniofacial disorders

Given the significant role of Notch signaling in craniofacial development described above, it follows that many studies have been done using transgenic mouse models to look at the phenotype of animals deficient in a certain part of the pathway, some of these findings are summarized in Table 3. The Notch pathway itself functions to coordinate the developing skeleton and face, with probably the most important example being Alagille syndrome. Alagille syndrome is an autosomal dominant disorder defined by abnormalities of the face, liver, heart, skeleton, eye, and, less frequently, kidney.\textsuperscript{149} Alagille is a relatively rare disorder, affecting 1:70 000 live births, and also is associated with bile duct and liver abnormalities in infants.\textsuperscript{150} Alagille syndrome is frequently associated with \textit{JAGGED1} (a ligand of Notch) mutations, with a minority of patients having a mutation in \textit{NOTCH2}.\textsuperscript{151} Alagille syndrome has been a model for studying Notch signaling and its downstream effects in humans, and many of the discussed studies arise from observations made in patients with Alagille syndrome.

Alagille syndrome is associated with characteristic facies; researchers have shown that this may be in part because the Jagged-Notch pathway is responsible for limiting the dorsal extent of ventral genes in the face.\textsuperscript{152} The study showed that in transgenic Zebrafish, jag1 misexpression lead to repression of ventral gene expression and dorsalization of the ventral hyoid and mandibular skeletons. They also showed that loss of function mutation in \textit{jagged1b} caused the opposite phenotype with dorsal expansion of ventral gene expression and partial transformation of the dorsal hyoid skeleton to a ventral morphology. These results taken together with other findings demonstrated that the Notch signaling pathway provides vital instructions for the basic patterning of the face during development.\textsuperscript{152}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{The role of Notch signaling in craniofacial development. This figure highlights the regions discussed in the review in which Notch has shown to be important in the developing face and skull, notably in the mandible, ear, and cranial suture.}
\end{figure}
As previously discussed, the cranial sutures that form between the osteogenic fronts of the developing bones are vital structures in the development of the skull. Investigators have shown that the Notch pathway plays an important part in regulating the patency of sutures, with mutations leading to premature fusion known as craniosynostosis. Briefly, the coronal suture is formed between the neural crest derived frontal bone and the mesodermal derived parietal bone, and the boundary between them is defined by their respective osteogenic fronts separated by a non-osteogenic suture mesenchyme in the middle. A collection of studies including Merrill et al 2006, Yen et al 2010, and Ting et al 2009 showed that the loss of definition in this osteogenic-nonosteogenic boundary caused by defective Notch signaling may be the underlying mechanism in some instances of premature fusion in the coronal suture.

As discussed, Jagged is a ligand of Notch receptors. In humans, JAGGED1 mutations cause Alagille syndrome. While craniosynostosis is not a common symptom in Alagille syndrome, there have been reports of a conserved form of craniosynostosis in some patients. Fig. 4 shows the 3D reconstructions of a CT scan of a patient with Alagille Syndrome with fusion of all sutures except the coronal suture. A Sagittal T1 magnetic resonance image shows cerebellar tonsils hanging down below the foramen magnum. (B) Preoperative 3-dimensional cranial tomography reconstruction shows findings indicating that only the coronal suture is open and other sutures were fused. (C) Postoperative cranial tomography reconstruction image shows the details of craniotomy performed and the fused lambdoid suture. Reprint permission was granted by the authors and publisher (Yilmaz et al 2012, Pediatric Neurology, License number: 4775991160929).

| Knockout   | Phenotype                                                      | Reference          |
|------------|----------------------------------------------------------------|--------------------|
| Notch-1−/− | Lethal at E9, somitogenesis is disturbed                       | Conlon et al.180    |
| Notch-2−/− | Lethal at E11.5, cardiovascular and kidney defects             | McCright et al. 181|
| Notch-3−/− | Normal viability and fertility                                 | Krebs et al. 182    |
| Notch-2−/−, Notch-3−/− | Lethal at E11.5, severe vascular abnormalities    | Wang et al. 183     |
| Notch-4−/− | Normal viability and fertility                                 | Krebs et al. 184    |
| Notch-4−/−, Notch-1−/− | Lethal at E10.5, severe defects in angiogenic remodeling      | Krebs et al. 184    |
| Jag1−/−    | Lethal at E10, defects in remodeling of the embryonic and yolk sac vasculature | Xue et al. 185      |
| Jag2−/−    | Perinatal lethality, cleft palate due to fusion of unelevated palatal shelves with tongue, limb and thymic defects | Jiang et al. 141    |
| Dll1−/−    | Lethal at E12, defect in patterning due to loss of compartmentalization of somites Hrabé de Angelis et al. 186 |
| Dll4−/−    | Lethal at E10.5, major defects in arterial and vascular development | Gale et al. 187     |
widely in the Jagged1 mutants and expressed within the suture mesenchyme leading to a more osteogenic phenotype. This was supported by experiments showing that Hes1, a downstream target of Notch involved in osteogenesis, was detectable in the Jagged1 mutant sutures. The study further showed that Jagged1 has a epistatic relationship with Twist1, a widely studied transcription factor in which mutations cause Saethre-Chotzen syndrome and craniosynostosis. Interestingly Notch and Twist have both been shown to be highly expressed in mandibular condylar cartilage of mice as well, showing that they may work together in craniofacial development in locations other than the cranial suture. These results provide convincing evidence that Notch signaling plays an important role in the development of the cranium.

Some studies have shown that patients with Alagille syndrome may have hearing loss, which raised the possibility that there were defects in both the neural formation of the ear but also the formation of the ossicles. One study showed that mice with Jagged1 or Notch2 deleted in neural crest cells showed stapes defects. The authors of this study further showed that the defects in the stapes in mutant mice were similar to the defects in the stapes seen in patients with Alagille syndrome. They had previously shown that Jagged1 loaded onto a scaffold was able to induce osteogenesis in human mesenchymal stem cells. Specifically, Jagged1 was loaded onto a collagen scaffold and injected the polymer into appendicular (femoral) and craniofacial (skull) surgical defects. They had previously shown that Jagged1 loaded onto a scaffold was able to induce osteogenesis in human mesenchymal stem cells. The study showed that the scaffold loaded with Jagged1 led to a recovery of 43% of the bone volume in the craniofacial defect, 67% more than the vehicle control, and compared to a Bmp2 group the bone remained more in line with the original defect. It was concluded that Jagged1 could provide a useful tool in the future to help repair bone defects.

This research provides background in which Notch signaling and Jagged can be used as a growth factor in the tissue engineering triad of scaffold, cells, and growth factors. We ourselves have shown that the upregulation of Notch signaling may be a successful bone tissue engineering strategy by using Imiquimod, a Notch activator, to synergize Bmp9-induced osteogenesis. Notch signaling has been shown to play an important role not only in the patterning of the skeleton but also in its homeostasis and regeneration. Many studies have shown both gain-of-function and loss-of-function Notch mutations causing disparate and complex skeletal disorders. Further research can help develop new therapies to treat these disorders. The future of Notch research will also help further clarify its role in craniofacial development so we can better understand the abnormalities that occur when there is a deficiency. Craniofacial abnormalities remain a common occurrence, occurring in 1:1600 births according to WHO, and research in this field is far from complete. Further understanding of this pathway and others can also help us identify the subtle interplay that occurs during development to produce the variations that we see in normal and abnormal development of the face and skull.

Conflict of Interests

The authors declare that no competing interests exist.

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References

1. Morgan TH. The theory of the gene. Am Nat. 1917;51(609): 513–544.
2. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. J Cell Sci. 2013;126(10):2135–2140.
3. Kidd S, Lieber T, Young MW. Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes Dev. 1998;12(23):3728–3740.
4. Kopan R, Ilagan MaXG. The canonical notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137(2): 216–233.
5. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol. 2006;7(9):678–689.
6. Artavanis-Tsinkas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999;284(5415):770–776.

7. Gridley T. Notch signaling and inherited disease syndromes. Hum Mol Genet. 2003;12 Spec (1):R9–R13.

8. Nowell CS, Radtke F. Notch as a tumour suppressor. Nat Rev Canc. 2017;17(3):145–159.

9. Siebel C, Lendahl U. Notch signaling in development, tissue homeostasis, and disease. Physiol Rev. 2017;97(4):1235–1294.

10. Zanotti S, Canalis E. Notch regulation of bone development and homeostasis, and disease. Hum Mol Genet. 1999;284(5415):770.

11. Gridley T. Notch signaling and inherited disease syndromes. Hum Mol Genet. 1999;284(5415):776.

12. Steinbuck MP, Winandy S. A review of notch processing with new insights into ligand-independent notch signaling in T-cells. Front Immunol. 2018;9:e1230.

13. Blaumueller CM, Qi H, Zagouras P, Artavanis-Tsinkas S. Intracellular cleavage of notch leads to a heterodimeric receptor on the plasma membrane. Cell. 1997;90(2):281–291.

14. Logeat F, Bessia C, Brou C, et al. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci Unit States Am. 1998;95(14):8108–8112.

15. Ma Y-C, Shi C, Zhang Y-N, et al. The tyrosine kinase c-src directly mediates growth factor-induced notch-1 and furin interaction and notch-1 activation in pancreatic cancer cells. In: Buday L, ed. PLoS ONE. 2012;7(3):e33414.

16. Varshney S, Stanley P. Multiple roles for O-glycans in Notch signaling. FEBS Lett. 2018;592(23):3819–3834.

17. Jafar-Nejad H, Leonard J, Fernandez-Valdivia R. Role of glycans and glycosyltransferases in the regulation of notch signaling. Glycobiology. 2010;20(8):931–949.

18. Baek C, Freem L, G harming R, Sang H, Morin X, Tozer S. Mib1 prevents Notch Cis-inhibition to defer differentiation and preserve neuroepithelial integrity during neural delamination. PLoS Biol. 2018;16(4):e2004162.

19. Wang R, Liu K, Chen L, Aihara K. Neural fate decisions with cis activates and cis inhibit disorder in notch signaling. Bioinformatics. 2011;27(22):3158–3165.

20. Lowell S, Jones P, Le Roux I, Dunne J, Watt FM. Stimulation of human epidermal differentiation by Delta—Notch signaling at the boundaries of stem-cell clusters. Curr Biol. 2000;10(9):491–500.

21. Nandagopal N, Santal TA, Elowitz MB. Cis-activation in the Notch signaling pathway. elife. 2019;8:e37880.

22. Formosa-Jordan P, Ibaries M. Competition in notch signaling with cis enriches cell fate decisions. PLoS One. 2014;9(4), e95744.

23. LaFoya B, Munroe JA, Mia MM, et al. Notch: a multi-functional integrative system of microenvironmental signals. Dev Biol. 2016;418(2):227–241.

24. Shah S, Lee S-F, Tabuchi K, et al. Nicotinamide functions as a gamma-secretase-substrate receptor. Cell. 2005;122(3):435–447.

25. Sanchez-Irizarry C, Carpenter AC, Weng AP, Pear WS, Auster JC, Blacklow SC. Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. Mol Cell Biol. 2004;24(21):9265–9273.

26. Bray SJ, Gomez-Lamarca M. Notch after cleavage. Curr Opin Cell Biol. 2018;51:103–109.

27. Ayaz F, Osborne BA. Non-canonical notch signaling in cancer and immunity. Front Oncol. 2014;4,e345.

28. Hatakeyama J. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development. 2004;131(22):5539–5550.

29. Fang C, Jiang B, Shi X, Fan C. Hes3 enhances the malignant phenotype of lung cancer through upregulating cyclin D1, cyclin D3 and MAMP expression. Int J Med Sci. 2019;16(3):470–476.

30. Carvalho FLT, Marchionni L, Gupta A, et al. HES6 promotes prostate cancer aggressiveness independently of Notch signaling. J Cell Mol Med. 2015;19(7):1624–1636.

31. Xu Y, Liu X, Zhang H, et al. Overexpression of HES6 has prognostic value and promotes metastasis via the Wnt/beta-catenin signaling pathway in colorectal cancer. Oncol Rep. 2018;40(3):1261–1274.

32. Bae S, Bessho Y, Hojo M, Kageyama R. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Dev Camb Engl. 2000;127(13):2933–2943.

33. Cakouros D, Isenmann S, Hemmig SE, et al. Novel basic helix–loop–helix transcription factor Hes4 antagonizes the function of twist-1 to regulate lineage commitment of bone marrow stromal/stem cells. Stem Cell Dev. 2015;24(11):1297–1308.

34. Hayashi S, Nakahata Y, Kohno K, Matsu T, Bessho Y. Presomitic mesoderm-specific expression of the transcriptional repressor Hes7 is controlled by E-box, T-box, and Notch signaling pathways. J Biol Chem. 2018;293(31):12167–12176.

35. Fischer A. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev. 2004;18(8):901–911.

36. Fukuda S, Kaneshige A, Kaji T, et al. Sustained expression of HeyL is critical for the proliferation of muscle stem cells in overloaded muscle. elife. 2019;8, e48284.

37. Chen F, Zhao Y, Chen H. MicroRNA-98 reduces amyloid beta protein production and improves oxidative stress and mitochondrial dysfunction through the Notch signaling pathway via HEY2 in Alzheimer’s disease mice. Int J Mol Med. 2019;43(1):91–102.

38. Han C, Song Y, Lian C. MiR-769 inhibits colorectal cancer cell proliferation and invasion by targeting HEY1. Med Sci Mon Int J Exp Clin Res. 2018;24:9232–9239.

39. Brun M, Jain S, Monckton EA, Godbout R. Nuclear factor I represses the notch effector HEY1 in glioblastoma. Neoplasia. 2015;20(10):1023–1037.

40. Than-Trong E, Ortica-Gatti S, Mella S, Nepal C, Alunni A, Bally-Cuif L. Neural stem cell quiescence and stemness are molecularly distinct outputs of the Notch3 signaling cascade in the vertebrate adult brain. Development. 2018;145(10):dev16034.

41. Maniati E, Bossard M, Cook N, et al. Crosstalk between the Notch signaling pathway with cis enriches cell fate decisions. PLoS One. 2014;9(4), e95744.

42. Perkins ND. Regulation of NF-kappa B function. Cell. 2003;12 Spec(1):R9–R13.

43. Reit J, Jorgensen J, Leth-Mikkelsen A, et al. Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. EMBO J. 1999;18(10):2803–2811.

44. Buesa I, Sancenon R, Morier A, et al. Synergism between NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 2007;8(1):49–62.

45. Roberts SGE, Weinzierl ROJ, White RJ, Campbell KJ, Perkins ND. Regulation of NF-kappaB function. Biochem Soc Symp. 2006;73:165–180.

46. Osipo C, Golde TE, Osborne BA, Miele LA. Off the beaten pathway: the complex cross talk between Notch and NF-kappa B. Lab Invest. 2008;88(1):11–17.

47. Oswald F, Liptay S, Adler G, Schmid RM. NF-kappaB2 is a putative target gene of activated notch-1 via RBP-Jk. Mol Cell Biol. 1998;18(4):2077–2088.

48. Bash J, Zong W-X, Bangua S, et al. Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. EMBO J. 1999;18(10):2803–2811.

49. Moran ST, Cariappa A, Liu H, et al. Synergism between NF-kappaB and Notch2 during the development of marginal zone B lymphocytes. J Immunol. 2007;179(1):195–200.
48. Wang J, Shelly L, Miele L, Boykins R, Norcross MA, Guan E. Human notch-1 inhibits NF-κB activity in the nucleus through a direct interaction involving a novel domain. *J Immunol*. 2001;167(1):289–295.

49. Kumar V, Palermino R, Talora C, et al. Notch and NF-κB signaling pathways regulate miR-223/FBXW7 axis in T-cell acute lymphoblastic leukemia. *Leukemia*. 2014;28(12):2324–2335.

50. Cirillo D, Rachiglio AM, Montagna R la, Giordano A, Normanno N. Leptin signaling in breast cancer: an overview. *J Cell Biochem*. 2008;105(4):956–964.

51. O’Brien SN, Welter BH, Price TM. Presence of leptin in breast cell lines and breast tumors. *Biochem Biophys Res Commun*. 1999;259(3):695–698.

52. Devos R, Guisez Y, Heyden JV der, et al. Ligand-independent Notch signaling in bone homeostasis via RBPjk and Hey upstream of NFATc1. *PLoS Genet*. 2012;8(3),e1002577.

53. Bjørbaek C, Uotani S, Silva B da, Flier JS. Divergent signaling involves Sry-type high-mobility-group (SOX) transcription factors. *Rev Cell Mol Biol* 2000;8(5):309.

54. Bjørbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Divergent signaling of SOCS-3 as a potential mediator of leptin resistance. *Cell Biochem Biophys*. 2001;36(1):461–471.

55. Rutkovskiy A, Stensløkken K-O, Vaage IJ. Osteoblast differentiation at a glance. *Osteoporos Rep*. 2008;14(3):306–314.

56. Regan J, Long F. Notch signaling and bone remodeling. *Curr Osteoporos Rep*. 2013;11(2):126–129.

57. Dong Y, Jesse AM, Kohn A, et al. RBPjk-dependent Notch signaling in breast cancer. *Oncotarget*. 2017;8(11):19050–19063.

58. Yavropoulou MP, Yovos JG. The role of Notch signaling in bone homeostasis. *J Biol Chem*. 2008;14(3):299–305.

59. Tu X, Chen J, Lim J, et al. Physiological notch signaling maintains bone homeostasis via RBPjk and Hey upstream of NFATc1. *PLoS Genet*. 2012;8(3),e1002577.

60. DeLise AM, Fischer L, Tuan RS. Cellular interactions and differentiation through binding of notch signaling proteins HES-1 and Hey-1 to N-box domains in the COL2A1 enhancer site. *Arthritis Rheum*. 2008;59(7):2754–2763.

61. Staines KA, Prideaux M, Allen S, Buttle DJ, Pitsillides AA, Farquharson C. E11/Podoplanin protein stabilization through association with ALDH activity and an aggressive metastatic phenotype in murine osteosarcoma models. *Osteoarthritis Cartilage*. 2013;21(5):627–636.

62. Wang J, Shelly L, Miele L, Boykins R, Norcross MA, Guan E. Human notch-1 inhibits NF-κB activity in the nucleus through a direct interaction involving a novel domain. *J Immunol*. 2001;167(1):289–295.

63. Dong Y, Jesse AM, Kohn A, et al. RBPjk-dependent Notch signaling in breast cancer. *Oncotarget*. 2017;8(11):19050–19063.

64. Engin F, Yao Z, Yang T, et al. Dimorphic effects of Notch signaling in bone homeostasis. *Nat Med*. 2008;14(3):299–305.

65. O'Brien SN, Welter BH, Price TM. Presence of leptin in breast cell lines and breast tumors. *Biochem Biophys Res Commun*. 1999;259(3):695–698.

66. Devos R, Guisez Y, Heyden JV der, et al. Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. *J Biol Chem*. 1997;272(29):18304–18310.

67. Bjarbaek C, Uotani S, Silva B da, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem*. 1997;272(51):32686–32695.

68. Bjarbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of leptin resistance. *Cell Mol. Cell Biol*. 1999;36(1):461–471.

69. Szanto I, Kahn CR. Selective interaction between leptin and insulin signaling pathways in a hepatic cell line. *Proc Natl Acad Sci Unit States Am*. 2000;97(5):2355–2360.

70. Guo S, Gonzalez-Perez RR. Notch, IL-1 and leptin crosstalk outcome (NILCO) is critical for leptin-induced proliferation, migration and VEGF/VEGFR-2 expression in breast cancer. *PloS One*. 2011;6(6),e21476.

71. Harbuzariu A, Rampoldi A, Daley-Brown DS, et al. Leptin-Notch signaling axis is involved in pancreatic cancer progression. *Oncotarget*. 2016;8(5):7740–7752.

72. Yavropoulou MP, Yovos JG. The role of Notch signaling in bone development and disease. *Norm Athens Greece*. 2014;13(1):24–37.

73. Keibl F, Mall FP. *Manual of Human Embryology*. J. B. Lippincott Company; 1912.

74. Delise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage*. 2000;8(5):309–334.

75. Karlsson C, Lindahl A. Notch signaling in chondrogenesis. *Int Rev Cell Mol Biol*. 2009;275:65–88.

76. Zanotti S, Smerdel-Ramoya A, Stadmeyer L, Durant D, Radtké F, Canalis E. Notch inhibits osteoblast differentiation and causes osteopenia. *Endocrinology*. 2008;149(8):3890–3899.

77. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ. Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis*. 2002;33(2):77–80.

78. Dong Y, Jesse AM, Kohn A, et al. RBPjkappa-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Dev Camb Engl*. 2010;137(9):1461–1471.

79. Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA. Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. *Biochem J*. 2001;360( Pt 2):461–470.

80. Hosaka Y, Saito T, Sugita S, et al. Notch signaling in chondrocytes modulates endochondral ossification and osteoarthritis development. *Proc Natl Acad Sci U S A*. 2013;110(5):1875–1880.

81. Grogan SP, Olee T, Hiraoka K, Lotz MK. Repression of chondrogenesis through binding of notch signaling proteins HES-1 and HEY-1 to N-box domains in the COL2A1 enhancer site. *Arthritis Rheum*. 2008;58(9):2754–2763.

82. Staines KA, Prideaux M, Allen S, Buttle DJ, Pitsillides AA, Farquharson C. E11/Podoplanin protein stabilization through inhibition of the proteasome promotes osteocyte differentiation in murine in vitro models. *J Cell Physiol*. 2016;231(6):1392–1404.

83. Shao J, Zhou Y, Lin J, et al. Notch expressed by osteocytes plays a critical role in mineralisation. *J Mol Med Berli Ger*. 2018;96(3–4):333–347.

84. Staines KA, Prideaux M, Allen S, Buttle DJ, Pitsillides AA, Farquharson C. E11/Podoplanin protein stabilization through inhibition of the proteasome promotes osteocyte differentiation in murine in vitro models. *J Cell Physiol*. 2016;231(6):1392–1404.

85. Shao J, Zhou Y, Xiao Y. The regulatory roles of Notch in osteocyte differentiation via the crosstalk with canonical Wnt pathways during the transition of osteoblasts to osteocytes. *Bone*. 2018;108:165–178.

86. Ottaviani G, Jaffe N, Bruelard DS, Bielack S, eds. *Pediatric and Adolescent Osteosarcoma*. *Cancer Treatment and Research*. Boston, MA: Springer US; 2010:3–13.

87. Survival Rates for Osteosarcoma. https://www.cancer.org/cancer/osteosarcoma/detection-diagnosis-staging/survival-rates.html. Accessed January 10, 2020.

88. Tanaka M, Setoguchi T, Hirotsu M, et al. Inhibition of Notch pathway prevents osteosarcoma growth by cell cycle regulation. *Br J Canc*. 2009;100(12):1957–1965.

89. Mu X, Isaac C, Greco N, Huard J, Weiss K. Notch signaling is associated with ALDH activity and an aggressive metastatic phenotype in murine osteosarcoma cells. *Front Oncol*. 2013;3:e143.

90. Li P, Zhang W, Cui J, et al. Targeting BMP9-promoted human osteosarcoma growth by inactivation of notch signaling. *Curr Cancer Drug Targets*. 2014;14(3):274–285.

91. Ma Y, Ren Y, Han EQ, et al. Inhibition of the Wnt-β-catenin and Notch signaling pathways sensitizes osteosarcoma cells to
chemotherapy. Biochem Biophys Res Commun. 2013;431(2):274–279.
90. Won KY, Kim YW, Kim H-S, Lee SK, Jung W-W, Park Y-K. MicroRNA-199b-5p is involved in the Notch signaling pathway in osteosarcoma. Hum Pathol. 2013;44(8):1648–1655.
91. Cao Y, Yu L, Dai G, et al. Cinobufagin induces apoptosis of osteosarcoma cells through inactivation of Notch signaling. Eur J Pharmacol. 2017;794:77–84.
92. Engin F, Bertin T, Ma G, et al. Notch signaling contributes to the pathogenesis of human osteosarcomas. Hum Mol Genet. 2009;18(8):1464–1470.
93. Temtamy SA, Aglan MS. Brachydactyly. Orphanet J Rare Dis. 2008;3:e15.
94. Tian J, Ling L, Shboul M, et al. Loss of CHSY1, a secreted FRINGE enzyme, causes syndromic brachydactyly in humans via increased NOTCH signaling. Am J Hum Genet. 2010;87(6):768–778.
95. Li Y, Laue K, Temtamy S, et al. Temtamy preaxial brachydactyly syndrome is caused by loss-of-function mutations in the spondylocostal dysplasia/pudgy gene Dll3 and initiation of early somite boundaries. Nat Genet. 1998;19(3):274–278.
96. Maisenbacher MK, Han J-S, O'brien ML, et al. Molecular analysis of congenital scoliosis: a candidate gene approach. Childs Nerv Syst. 2012;28(1):23–31.
97. Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dyn Off Publ Am Assoc Anat. 2000;219(4):438–441.
98. Bonilla-Claudio M, Wang J, Bai Y, Klysik E, Selever J, Martin JF. Bmp signaling regulates a dose-dependent transcriptional program to control facial skeletal development. Development. 2012;139(4):709–719.
99. Feng J, Jing J, Li J, et al. BMP signaling orchestrates a transcriptional network to control the fate of mesenchymal stem cells in mice. Dev Dyn Engl. 2011;144(14):2560–2569.
100. Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. J Cell Biochem. 2011;112(12):3491–3501.
101. Naka K-I, Yasuda M, Watanabe N, et al. Stimulation of osteoblastic cell differentiation by notch. J Bone Miner Res. 2002;17(2):231–239.
102. Minamizato T, Sakamoto K, Liu T, et al. CCN3/NOV inhibits BMP-2-induced osteoblast differentiation by interacting with BMP and Notch signaling pathways. Biochem Biophys Res Commun. 2007;354(2):567–573.
103. Garcia-Castro MI, Marcelle C, Bronner-Fraser M. Ectodermal wnt function as a neural crest inducer. Science. 2002;297(5582):848–851.
133. Zhu X-J, Yuan X, Wang M, et al. A Wnt/Notch/Pax7 signaling network supports tissue integrity in tongue development. J Biol Chem. 2017;292(22):9409–9419.

134. Jayasena CS, Ohyama T, Segil N, Groves AK. Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. Development. 2008;135(13):2251–2261.

135. Žamurović N, Cappellen D, Rohner D, Susa M. Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. J Biol Chem. 2004;279(36):37704–37715.

136. Munnamalai V, Fekete DM. Notch-Wnt-Bmp crosstalk regulates radial patterning in the mouse cochlea in a spatiotemporal manner. Development. 2016;143(21):4003–4015.

137. Deregowski V, Gazzero E, Priest L, Rydziel S, Canalis E. Notch signaling in the skeletal system. 2017;138(15):3225.

138. Manderfield LJ, Aghajanian H, Engleka KA, et al. Hippo transduction, Hippo, Wnt, and TGF-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits beta signaling pathways in bone morphogenic protein 2-expressing osteogenic cells. Biochim Biophys Acta BBA - Mol Cell Res. 1863(2):303–313.

139. Morgan JT, Murphy C, Russell P. What do mechano-transduction, Hippo, Wnt, and TGFβ have in common? YAP and TAZ as key orchestrating molecules in ocular health and disease. Exp Eye Res. 2013;115:1–12.

140. Rayon T, Menchero S, Nieto A, et al. Notch and hippo converge on Cdx2 to specify the trophectoderm lineage in the mouse blastocyst. Dev Cell. 2014;30(4):410–422.

141. Jiang R, Lan Y, Chapman HD, et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev. 1998;12(7):1046–1057.

142. Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Judisch GF, El-Khoury GH. Four generations of arteriohepatic dysplasia. Hepatology. 1982;2(4(supl.)):467–474.

143. Le Caignec C, Lefevre M, Schott JJ, et al. Familial deafness, congenital heart defects, and posterior embryotoxon caused by cysteine substitution in the first epidermal-growth-factor loop of the notch signaling pathway. Genes Dev Camb Engl. 2015;142(17):2962–2971.

144. Lópeze SL, Paganell A, Siri MVR, Ocaña OH, Franco PG, Carrasco AE. Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in Xenopus. Development. 2003;130(10):2225–2238.

145. Kim T-H, Kim B-M, Mao J, Rowan S, Shviddasani RA. Endodermal Hedgehog signals modulate Notch pathway activity in the developing digestive tract mesenchyme. Dev Cell Eng. 2011;138(15):3225–3233.

146. Ma X, Drannik A, Jiang F, Peterson R, Turnbull J. Crosstalk between Notch and Sonic hedgehog signaling in a mouse model of amyotrophic lateral sclerosis. Neuroreport. 2017;28(3):141–148.

147. Nwab Kamdje AH, Takam Kamga P, Tagné Simo R, et al. Developmental pathways associated with cancer metastasis: notch, Wnt, and Hedgehog. Cancer Biol Med. 2017;14(2):109–120.

148. Takebe N, Harris PJ, Warren RQ, Ivy SP. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nat Rev Clin Oncol. 2011;8(2):97–106.

149. Katoh M. Networking of WNT, FGF, notch, BMP, and hedgehog signaling pathways during carcinogenesis. Stem Cell Rev. 2007;3(1):30–38.

150. Li L, Krantz ID, Deng Y, et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet. 1997;16(3):243–251.

151. Mcdaniell R, Warthen DM, Sanchez-Lara PA, et al. Notch2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet. 2006;79(1):169–173.

152. Zuniga E, Stellabotte F, Crump JG. Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face. Dev Camb Eng. 2010;137(11):1843–1852.

153. Merrill AE, Bochukova EG, Brugger SM, et al. Cell mixing at a neural crest-mesoderm boundary and defective ephrin-Eph signaling in the pathogenesis of craniosynostosis. Hum Mol Genet. 2006;15(8):1319–1328.

154. Yen H-Y, Ting M-C, Maxson RE. Jagged1 functions downstream of Twist1 in the specification of the coronal suture and the formation of a boundary between osteogenic and non-osteogenic cells. Dev Biol. 2010;347(2):258–270.

155. Ting M-C, Wu NL, Roybal PG, et al. EphA4 as an effector of Twist1 in the guidance of osteogenic precursor cells during calvarial bone growth and in craniosynostosis. Development. 2009;136(5):855–864.

156. Kamath BM, Stolle C, Bason L, et al. Craniosynostosis in Alagille syndrome. Am J Med Genet. 2002;112(2):176–180.

157. Yilmaz S, Turhan T, Mutluer S, Aydogdu S. The association of Alagille syndrome and craniosynostosis. Pediatr Neurol. 2013;48(2):146–148.

158. Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A. Signalling downstream of activated mammalian Notch. Nature. 1995;377(6547):355–358.

159. Howard TD, Paznekas WA, Green ED, et al. Mutations in TWIST1 cause Alagille syndrome and craniosynostosis. Am J Med Genet. 1997;15(1):36–41.

160. Krantz ID, Piccoli DA, Spinner NB. Alagille syndrome. J Med Genet. 1997;34(2):152–157.

161. Labrecque DR, Mitros FA, Nathan RJ, Romanchuk KG, Judisch GF, El-Khoury GH. Four generations of arteriohepatic dysplasia. Hepatology. 1982;2(4(supl.)):467–474.

162. Le Caignec C, Lefevre M, Schott JJ, et al. Familial deafness, congenital heart defects, and posterior embryotoxon caused by cysteine substitution in the first epidermal-growth-factor–like domain of Jagged 1. Am J Hum Genet. 2002;71(1):180–186.

163. Teng CS, Yen H-Y, Barske L, et al. Requirement for Jagged1-Notch2 signaling in patterning the bones of the mouse and human middle ear. Sci Rep. 2017;7(1):1–11.

164. Youngstrom DW, Senos R, Zondervan RL, et al. Intraoperative delivery of the Notch ligand Jagged-1 regenerates appendicular and craniofacial bone defects. NPJ Regen Med. 2017;2, e32.

165. Dishowitz MI, Zhu F, Sundararaghavan HG, Ifkovits JL, Judisch GF, El-Khoury GH. Four generations of arteriohepatic dysplasia. Hepatology. 1982;2(4(supl.)):467–474.

166. Le Caignec C, Lefevre M, Schott JJ, et al. Familial deafness, congenital heart defects, and posterior embryotoxon caused by cysteine substitution in the first epidermal-growth-factor–like domain of Jagged 1. Am J Hum Genet. 2002;71(1):180–186.

167. Teng CS, Yen H-Y, Barske L, et al. Requirement for Jagged1-Notch2 signaling in patterning the bones of the mouse and human middle ear. Sci Rep. 2017;7(1):1–11.

168. Dishowitz MI, Zhu F, Sundararaghavan HG, Ifkovits JL, Judisch GF, El-Khoury GH. Four generations of arteriohepatic dysplasia. Hepatology. 1982;2(4(supl.)):467–474.

169. Le Caignec C, Lefevre M, Schott JJ, et al. Familial deafness, congenital heart defects, and posterior embryotoxon caused by cysteine substitution in the first epidermal-growth-factor–like domain of Jagged 1. Am J Hum Genet. 2002;71(1):180–186.

170. Sun Z, da Fontoura CSG, Moreno M, et al. FoxO6 regulates Hippo signaling and growth of the craniofacial complex. Plaš Genet. 2018;14(10),e1007675.
171. Moosa S, Wolnik B. Altered FGF signalling in congenital craniofacial and skeletal disorders. *Semin Cell Dev Biol*. 2016;53:115–125.

172. Jiang Y, Zhang S, Mao C, et al. Defining a critical period in calvarial development for Hedgehog pathway antagonist-induced frontal bone dysplasia in mice. *Int J Oral Sci*. 2019;11(1):e3.

173. Clouthier DE, Williams SC, Yanagisawa H, Wieduwilt M, Richardson JA, Yanagisawa M. Signaling pathways crucial for craniofacial development revealed by endothelin-A receptor-deficient mice. *Dev Biol*. 2000;217(1):10–24.

174. Taya Y, O’Kane S, Ferguson MW. Pathogenesis of cleft palate in TGF-beta3 knockout mice. *Dev Camb Engl*. 1999;126(17):3869–3879.

175. Fu J, Ivy Yu H-M, Maruyama T, Miranda AJ, Hsu W. Gpr177/mouse Wntless is essential for Wnt-mediated craniofacial and brain development. *Dev Dyn Off Publ Am Assoc Anat*. 2011;240(2):365–371.

176. Yamada W, Nagao K, Horikoshi K, et al. Craniofacial malformation in R-spondin2 knockout mice. *Biochem Biophys Res Commun*. 2009;381(3):5972–5984.

177. Krebs LT, Xue Y, Norton CR, et al. Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genes N Y N*. 2003;37(3):139–143.

178. Carver EA, Oram KF, Gridley T. Craniosynostosis in Twist heterozygous mice: a model for Saethre-Chotzen syndrome. *Anat Rec*. 2002;268(2):90–92.

179. Conlon RA, Reaume AG, Rossant J. Notch1 is required for the coordinate segmentation of somites. *Dev Camb Engl*. 1995;121(5):1533–1545.

180. McCright B, Lozier J, Gridley T. Generation of new Notch2 mutant alleles. *Genes N Y N*. 2000;44(1):29–33.

181. Krebs LT, Xue Y, Norton CR, et al. Notch signaling is essential for vascular morphogenesis in mice. *PloS One*. 2012;7(5),e37365.

182. Hrabě de Angelis M, McIntyre J, Gossler A. Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature*. 1997;386(6626):717–721.

183. Gale NW, Dominguez MG, Noguera I, et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A*. 2004;101(45):15949–15954.