Suppressor of Fused restraint of Hedgehog activity level is critical for osteogenic proliferation and differentiation during calvarial bone development

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Hedgehog signaling plays crucial roles in the development of calvarial bone, relying on the activation of Gli transcription factors. However, the molecular mechanism of the role of regulated Gli protein level in osteogenic specification of mesenchyme still remains elusive. Here, we show by conditionally inactivating Suppressor of Fused (Sufu), a critical repressor of Hedgehog signaling, in Wnt1-Cre–mediated cranial neural crest (CNC) or Dermo1-Cre–mediated mesodermal lineages that Sufu restraint of Hedgehog activity level is critical for differentiation of preosteogenic mesenchyme. Ablation of Sufu results in failure of calvarial bone formation, including CNC-derived bones and mesoderm-derived bones, depending on the Cre line being used. Although mesenchymal cells populate to frontonasal destinations, where they are then condensed, Sufu deletion significantly inhibits the proliferation of osteoprogenitor cells, and these cells no longer differentiate into osteoblasts. We show that there is suppression of Runx2 and Osterix, the osteogenic regulators, in calvarial mesenchyme in the Sufu mutant. We show that down-regulation of several genes upstream to Runx2 and Osterix is manifested within the calvarial primordia, including Bmp2 and its downstream genes Msx1/2 and Dlx5. By contrast, we find that Gli1, the Hedgehog activity readout gene, is excessively activated in mesenchyme. Deletion of Sufu in CNC leads to a discernible decrease in the repressive Gli3 form and an increase in the full-length Gli2. Finally, we demonstrate that simultaneous deletion of Gli2 and Sufu in CNC completely restores calvarial bone formation, suggesting that a sustained level of Hedgehog activity is critical in specification of the osteogenic mesenchymal cells.

The mammalian skull vault (top of neurocranium), which is composed of the paired frontal bones and parietal bones, an interparietal bone, and junctions between calvarial bones called the sutures, is formed from cranial osteogenic mesenchyme derived from two distinct embryonic tissue sources, the cranial neural crest (CNC) and paraxial mesoderm (1–4).

Calvarial bones form by intramembranous ossification, which in mice begins with the condensation of mesenchymal progenitors at E12.5 when the frontal and parietal bone primordia become evident. Condensed mesenchymal progenitor (osteoprogenitor) cells then undergo vigorous proliferation and differentiate into osteoblast precursor cells and express the early osteoblast marker Runx2. As the next 2 days of development proceed, these progenitor cells progress to preosteoblasts and mature osteoblasts expressing Runx2, Osterix, type 1 collagen, bone sialoprotein (BSP), osteocalcin (OC), and osteopontin (OPN) and secreting bone matrix. The ossification centers are formed by direct bone matrix deposition forming bone plates between the brain and the epidermis. Osteoblast differentiation occurs in the margins of osteogenic centers (the osteogenic front), where osteoblasts invade into, and progenitor cells are recruited from, the surrounding mesenchyme. Calvarial bone plates grow until they are nearly approximated but remain unfused at the sutures (5, 6).

Hedgehog (Hh) signaling plays a pivotal role in calvarial patterning and ossification (5, 7, 8). The mutation and disruption of the Hh signaling network result in a variety of genetic disorders associated with the growth defects in the calvaria and the sutures (craniosynostosis), both in humans and in mice (5, 9, 10). Combinatorial data of null mutation phenotypes and distinct expression patterns suggest that Sonic hedgehog (Shh) may prevent suture fusion, whereas Indian hedgehog (Ihh) activates the intramembranous ossification and suture fusion, although there is disputability regarding whether Ihh and Shh function as negative or positive regulators of intramembranous bone development (8, 11–14). The Hh signaling is mediated mostly through Gli family transcription factors upon Hh activation (15). Ligand binding to the receptor Patched (Ptc) results in release of the transmembrane protein Smoothened (Smo) from Ptc inhibition, so that Smo transduces the signal intracellularly through interaction with Gli family of transcription factors, Gli1, Gli2, and Gli3 (5). Gli1 is transcriptionally regulated

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by Gli2 and Gli3 and functions as a transcription activator (16, 17), although it is dispensable during mouse embryogenesis (18, 19). However, it is involved in osteoblast differentiation during endochondral ossification (20, 21). Gli2 and Gli3 both have an N-terminal repressor domain and a C-terminal activator domain flanking the zinc fingers that can function as both activator and repressor, depending on Hh signal presence. In general, Gli2 remains as a transcription activator due to inefficient protein processing, whereas Gli3 mostly functions as a transcription repressor because of efficient degradation. In both human and mice, loss of function of GLI3 causes Greig cephalopolysyndactyly syndrome, which causes the premature fusion of metopic (interfrontal) and lambdoidal sutures and abnormal frontal bone morphology (9, 22–24). Deletion of repressor GLI3 results in excessive osteoprogenitor proliferation and differentiation, strongly implicating GLI1 activity and regulation in the control of calvarial bone development. Moreover, in vitro studies have shown that Gli2 mediates the Ihh control of osteoblast differentiation of mesenchymal cells through direct interaction with Runx2 expression (25, 26), suggesting the importance of Gli in the regulation of osteogenesis. However, it remains unclear whether there is a genetic requirement of GLI1 activity level for early development of the calvarial bone development and how the on/off switch of the activator/repressor Gli2 and Gli3 is conducted in osteogenic mesenchymal cell proliferation and differentiation of osteoprogenitor cells.

In mammals, Suppressor of Fused (Sufu) is a major repressor of Hh signaling by modulation of Gli2 and Gli3. In the absence of Hh signal activation, Sufu can sequester the full-length Gli2 and Gli3 proteins (GliA) to the cytoplasm, facilitating the formation of repressive Gli2/3 (GliR) and thereby regulating Gli at the protein levels (27–32). By contrast, full-length Gli2 and Gli3 remain as activators in the presence of high Hh levels, probably by dissociation with Sufu (33, 34). The Sufu null mutation in mice causes a global increase in up-regulation of Ptc1 (35, 36). Conditional deletion of Sufu in the limb bud mesenchyme resulted in the alteration of anterior/posterior patterning and polydactyly, associated with the reduction in repressive Gli2 and Gli3 (37, 38). The fact that expression of Shh and Ihh in the calvarial primordia region is detected in a relatively late stage around E16.5 raises the possibility that activity of Gli2R and Gli3R is required for regulation of osteoprogenitor differentiation and maturation in intramembranous bone development (5, 8, 39). In this study, by ablation of Sufu in neural crest-derived mesenchymal cells, we show the requirement of Sufu regulation of Gli2 and Gli3 for calvarial bone formation during development. Sufu deletion in CNC lineage causes activation of Hh signaling in the mesenchyme for the calvarial bone primordia, resulting in the failure of developmental progression of osteogenesis beyond initial condensations in the calvarial mesenchyme via dysregulation of downstream molecules.

Results

Functional Sufu is required for skull vault development

In situ hybridization showed that Sufu is globally expressed in the calvarial mesenchyme (Fig. 1A and supplemental Fig. S1) that was consistent with immunofluorescence analysis results using the Sufu antibody (Fig. 1B), suggesting a role for this gene in the presumptive skull vault primordia. To investigate the potential function of Sufu in calvarial bone development, we examined mice with CNC-specific conditional deletion of Sufu using the Wnt1-Cre mouse (Sufufx/fx;Wnt1-Cre or Sufufx/fx;Wnt1-Cre) and mice with mesoderm-specific Sufu deletion using the Dermo1-Cre strain (Sufufx/fx;Dermo1-Cre). Inactivation of Sufu with Wnt1-Cre or Dermo1-Cre caused immediate postnatal lethality. Immunofluorescence and Western blot analyses demonstrated removal of Sufu in the calvarial mesenchyme of Sufufx/fx;Wnt1-Cre mutants (Fig. 1, C and D). Skeletal preparations with Alcian blue/Alizarin red staining showed that the Wnt1-Cre–mediated Sufu deletion resulted in specific loss of CNC-derived calvarial bones, such as the frontal bones and the central portion of the interparietal bone, as well as the facial bone.

Figure 1. Sufu is required for the development of calvarial bones. A and B, in situ hybridization and immunohistochemical analyses showing the expression of Sufu in the development of presumptive frontal primordium. C and D, immunohistochecmistry and Western blot analyses showing the mesenchymal cell–specific removal of Sufu with Wnt1-Cre in the presumptive frontal primordium, respectively. E–L, skeletal staining with Alcian blue and Alizarin red shows the missing CNC-derived craniofacial bones, including the frontal bones and the central portion of the interparietal bone in neonatal Sufufx/fx;Wnt1-Cre (Sufufx/fx;Wnt1-Cre) mice compared with littermate wild type control mice (n = 8). (Wild-type control mice in this study did not carry the Cre allele). M–T, Alcian blue/Alizarin red staining showing mesoderm lineage–specific developmental defects of calvarial bones in neonatal Sufufx/fx;Dermo1-Cre (Sufufx/fx;Dermo1-Cre) mutant compared with littermate wild-type mice (n = 3). Scale bars, 200 μm (A–C) and 1 mm (E–T).
Sufu is required for calvarial bone development

bones (Fig. 1 (E–L) and supplemental Fig. S2); by contrast, ablation of Sufu with Dermo1-Cre caused specific defects in mesoderm-derived calvarial bones, including parietal bones and the lateral part of the interparietal bone (Fig. 1, M–T). These data suggest that an intrinsic function of Sufu is required for the calvarial bone formation. We thus focused our studies on the CNC-derived calvarial bone formation.

Mutation of Sufu in CNC does not affect migration but impedes condensation of CNC-derived mesenchymal cells

Formation of mesenchymal condensations at E12.5 is an early sign of intramembranous bone development in mice. However, scanning electron microscopy analysis showed that developmental hypoplasia of the facial/nasal processes occurred before E11.5 in Sufufx/fx mice, compared with the wild type (Fig. 2, A–H), indicating that Wnt1-Cre–mediated Sufu deletion does not affect CNC cell migration. To determine whether Sufu deletion impedes the formation of the mesenchymal condensations, we performed a series of histological analyses on the calvarial tissues between E12.5 and E14.5 throughout the process of the calvarial bone development (Fig. 2, I–N). Hematoxylin and eosin (H&E) staining on the embryonic head sections showed that the mesenchyme within the presumptive calvarial bone primordium appeared condensed in E12.5 Sufufx/fx;Wnt1-Cre mice carrying the R26R reporter allele (R26R;Sufufx/fx;Wnt1-Cre) between E11.5 and E13.5. X-galgal staining on sections of the embryonic head showed that the CNC cells could normally populate to the presumptive calvarial sites in the Sufu mutant (Fig. 2, A–H), indicating that Wnt1-Cre–mediated Sufu deletion does not affect CNC cell migration. To determine whether Sufu deletion impedes the formation of the mesenchymal condensations, we performed a series of histological analyses on the calvarial tissues between E12.5 and E14.5 throughout the process of the calvarial bone development (Fig. 2, I–N). Hematoxylin and eosin (H&E) staining on the embryonic head sections showed that the mesenchyme within the presumptive calvarial bone primordium appeared condensed in E12.5 Sufufx/fx;Wnt1-Cre, comparable with the wild type (Fig. 2, I and J). The mesenchymal condensation became evident at E13.5 in wild type but was much less in Sufufx/fx;Wnt1-Cre mutants (Fig. 2, K and L). Calvarial bone primordium underwent ossification in the wild type at E14.5, but it did not develop in Sufufx/fx;Wnt1-Cre mutants (Fig. 2, M and N). In the wild type, calvarial bone development was progressing toward differentiation after E13.5 and mineralization of mature osteoblasts at E14.5, as exhibited by von Kossa staining and the expression of OPN, the osteoblast marker involved in bone matrix mineralization (Fig. 2, O–T). However, neither of these markers was detectable in Sufufx/fx;Wnt1-Cre (Fig. 2, O–T), suggesting that there is no sign of mineralization of mature osteoblasts in the absence of Sufu. In summary, these data suggest that the failure of the calvarial bone formation in Sufufx/fx;Wnt1-Cre was attributed to interruption of mesenchymal cell condensation and differentiation to osteoblasts.

Sufu deletion disturbs proliferation but not apoptosis of osteogenic mesenchymal cells in the calvarial bone primordium

For intramembranous bone formation, initially condensed mesenchymal cells undergo vigorous proliferation prior to differentiation and mineralization (5, 6) (Fig. 3). To understand the cellular mechanism causing a defect in the differentiation of preosteogenic mesenchymal cells in the Sufu mutant, we examined cell proliferation using BrdU labeling. Immunofluorescence and statistical analyses showed a significant decrease in the rate of the BrdU-labeled cells within the presumptive calvarial bone primordium in Sufufx/fx;Wnt1-Cre between E12.5 and E14.5, compared with wild type (Fig. 3, A–G). We also applied immunofluorescence staining using antibody against cyclin D1, a marker for proliferation, on the sections. We found that the rate of cyclin D1-positive cells was significantly decreased in the Sufufx/fx;Wnt1-Cre mice (Fig. 3, A–G) through all detected stages, consistent with the BrdU labeling results. To determine whether the failure of calvarial bone formation resulted from an increase of abnormal cell death within the primordium, we performed a TUNEL assay on the histological sections. Cell apoptosis signal of mesenchymal cells within the calvarial bone primordia in Sufufx/fx;Wnt1-Cre embryos and littermate control mice (R26R;Sufufx/fx;Wnt1-Cre, n = 3), I–N, H&E staining showing the condensation of mesenchymal cells in the frontal primordium both in littermate wild type and Sufufx/fx;Wnt1-Cre (SufuWnt1Cre) mutant (n = 3). O–R, von Kossa staining showing the mineralization of frontal bones in E14.5 in littermate wild type versus Sufufx/fx;Wnt1-Cre (SufuWnt1Cre) samples (n = 3). S and T, in situ hybridization showing the expression of mineralization marker OPN in the wild-type frontal bone but not in the littermate Sufu mutant (n = 2). Arrowhead, the presumptive frontal primordium. Scale bars, 1 mm (A and B) and 200 μm (C–T).
genesis but not for cell survival. Decreased mesenchymal cell proliferation may account for the developmental defect of the calvarial bone in the Sufu mutant.

**Sufu is required for specification of osteo-mesenchyme within calvarial bone rudiment**

To understand whether and how the aberrant differentiation of preosteoblasts contributes to the failure of calvarial bone formation in Sufu<sup>lox/lox</sup>; Wnt1-Cre embryos, we examined expression of Runx2 and Osterix (Sp7) in E12.5 and E13.5 embryos. Expression of Runx2, a core transcription factor and specific marker for preosteoblast (40), was initiated at late E12.5 in the osteogenic condensations and enhanced in preosteoblasts at E13.5 in wild type (Fig. 4, A and C). Osterix is a zinc-finger-containing transcription factor that is necessary for osteoblast differentiation and bone formation, and acts downstream of Runx2 (41). Osterix transcripts appeared in the preosteoblasts at E13.5 in the wild type (Fig. 4, E and G). However, neither Runx2 nor Osterix expression was recognized in the mesenchymal cells within the presumptive calvarial bone primordium at both stages in the Sufu mutant (Fig. 4, B, D, F, and H). Immunofluorescence analyses with antibodies against Runx2 and
Osterix did not show the protein products specific to the mesenchymal condensations of the presumptive calvarial bone primordia in Sufu\textsuperscript{fl/fl}; Wnt1-Cre mice (Fig. 4, I–P), consistent with our \textit{in situ} hybridization data. Taken together, these data suggest that Sufu is essential for the specification and differentiation of the initially condensed mesenchyme to Runx2/Osterix-expressing osteoblast precursors, and the dysregulation of these osteo-marker genes in conditional ablation of Sufu accounts for the failure of calvarial bone formation.

Altered gene expression by loss of Sufu within calvarial mesenchyme

We next examined \textit{in situ} hybridization the expression of potential downstream targets of Sufu-dependent regulation in the calvarial mesenchyme of Sufu\textsuperscript{fl/fl}; Wnt1-Cre mice, in particular for several signaling molecules and transcription factors previously known for regulating the expression of Runx2 and Osterix in osteogenic differentiation. Dlx5 is an important mediator of calvarial osteoblast differentiation by positive regulation of Runx2 (42). Expression of Dlx5 was detected by \textit{in situ} hybridization in the osteogenic mesenchyme at E12.5 and significantly up-regulated at E13.5 within the calvarial bone rudiment of the wild type (Fig. 5, A and C). However, expression of Dlx5 was faintly detected in the calvarial mesenchyme of the Sufu mutant at E12.5 and decreased in E13.5 Sufu\textsuperscript{fl/fl}; Wnt1-Cre compared with the wild-type control (Fig. 5, A–D). Previous studies have shown that homeobox genes Msx1 and Msx2 are essential for the differentiation and proliferation of osteogenic cells within the calvarial rudiments (43). In a combination of Msx1/2 mutants, skull vault components, including the frontal bones, fail to form (44), indicating the requirement for regulation of CNC cell differentiation during calvarial bone development (44). At E13.5, both Msx1 and Msx2 were expressed in the calvarial osteogenic mesenchyme in wild type, but Msx2 transcripts within the preosteoblast rudiment were much stronger than those of Msx1 (Fig. 5, E and G). In contrast, no Msx1 transcript was detected by \textit{in situ} hybridization in the Sufu mutant (Fig. 5, E and G). In addition, in the calvarial mesenchyme of the Sufu\textsuperscript{fl/fl}; Wnt1-Cre mice, we observed dramatic down-regulation in expression of Bmp2 (Fig. 5, I and J), which is a well-known signaling molecule associated with Msx2 in calvarial bone development (39, 45). Together, these data suggest that Sufu-dependent Hh signaling
Sufu is required for calvarial bone development

Deletion of Sufu in CNC-derived mesenchymal cells perturbs the regulation of SHH activity via changing the protein stability of Gli2/3

Sufu is a critical negative regulator of Hh signaling by binding to the transcription factor GLIs. To investigate the effect of Sufu deletion on the Hh activity, we next detected the expression of Gli1, the target gene and readout of Hh signaling. During development of the skull vault in wild-type mice, expression of Gli1 was extensive in the CNC-derived cranial mesenchyme in E11.5 (Fig. 6A) and was dramatically down-regulated in the condensed mesenchyme for the calvarial primordia at E12.5. Interestingly, the transcripts of Gli1 were repeatedly detected in preosteoblasts at E13.5 (Fig. 6A). This dynamic expression pattern may suggest that the regulatory level of Hedgehog activity is critical for the condensed mesenchyme toward the preosteogenic fate. However, expression of Gli1 was retained and broadly detected in the CNC-derived mesenchyme throughout stages in the absence of Sufu (Fig. 6A), indicating that the level of Shh activity is abnormally up-regulated in the calvarial mesenchyme of Sufufx/fx;Wnt1-Cre. This is consistent with previously reported data for Sufu null mutation (35), suggesting that Sufu inhibitory regulation of Shh activity is essential for preosteogenic differentiation of the mesenchyme during intramembranous ossification.

We sought to identify the means by which Sufu modulates Hh signaling within the calvarial mesenchyme by clarifying the expression of Gli2/3 at the protein level. Gli2 primarily functions as a full-length activator, and Gli3 mainly acts as a repressor by an N-terminal truncated form in Hh signaling, and binding of Sufu to Gli2 or Gli3 can inhibit their processing and keep the stability of the full-length forms. To distinguish the effect of Sufu deletion on these processes, Western blot analyses were performed with protein extracted from embryonic calvarial samples. We found that the Gli2 full-length form was intensified, concomitant with a significant decrease in its truncated repressor form, in the Sufufx mutant compared with the wild type (Fig. 6B). By contrast, Gli3 existed primarily in the truncated repressor form, and the Gli3 repressor (Gli3R) in the CNC-derived mesenchyme was significantly decreased in E12.5 and E13.5 Sufufx;Wnt1-Cre mutant samples, in comparison with wild type (Fig. 6B). We did not detect a concomitant increase of Gli3 full-length form (Fig. 6B).

Overall, these findings, together with in situ hybridization data showing the abnormal activation of Gli1 expression in calvarial mesenchyme, suggest that within the calvarial mesenchyme, the inactivation of Sufu reduces Gli2 and Gli3 repressor forms and increases the activator form of Gli2, resulting in an abnormal high Hh activity.

Failure of calvarial bone development in Sufufx;Wnt1-Cre mutant can be restored by compound mutation of Sufu and Gli2 in CNC

The evidence above suggests that loss of Sufu in the CNC-derived mesenchyme interferes with Sufu regulation of Gli2 protein, resulting in unbalanced elevation of Hh output critical for differentiation of osteoprogenitor cells. To test this proposal in vivo, we generated Wnt1-Cre–mediated compound conditional deletion of Sufu and Gli2 (Fig. 7M). Skeletal preparations revealed that the calvarial bone formation in mice carrying Sufufx;Gli2fx/fx;Wnt1-Cre allele was comparable with that of wild-type control (Fig. 7, A–C), indicative of rescue of intramembranous bone ossification. von Kossa staining showed that mineralization of osteoblasts within the calvarial bone occurred in the double mutant at E14.5 of Sufufx;Gli2fx/fx;Wnt1-Cre (Fig. 7, D–F). Immunofluorescence analyses showed reactivation of cyclin D1 and Runx2 in the calvarial bone in the E14.5 double mutant (Fig. 7, G–I). In situ analysis showed that the expression of Gli1 was decreased in Sufufx;Gli2fx/fx;Wnt1-Cre mutants, compared with Sufufx;Wnt1-Cre (Fig. 7, J–L). It was further confirmed by qRT-PCR analysis that the expression of Hh signaling targets and downstream genes in Sufufx;Gli2fx/fx;Wnt1-Cre is comparable with wild-type control (Fig. 7N). In contrast, simultaneous deletion of Gli3 in Sufufx;Gli2fx/fx;Wnt1-Cre background failed to restore the calvarial bone formation but led to more severe craniofacial defects (supplemental Fig. S5). Taken together, these data may suggest that unbalanced Hh activity from loss of Sufu can be genetically neutralized by simultaneous deletion of Gli2, providing evi-
Sufu is required for calvarial bone development

Discussion

In this study, by conditional inactivation of Sufu in CNC cells, we provide genetic evidence that the level of Sufu-regulated Hh activity is critical for osteogenic specification of mesenchyme in calvarial bone formation. Moreover, inactivation of Sufu in the osteogenic mesenchyme leads to interruption of the molecular network critical for developmental progress of the initial condensed mesenchyme toward an osteoblastic lineage. Finally, we show the complete restoration of calvarial bone formation by compound deletion of Sufu and Gli2 in the CNC. Our study suggests that Sufu acts as an inhibitor of full-length Gli2 activity and an activator of Gli3 repressor activity in mesenchyme differentiation of osteo-progenitors in intramembranous bone development (Fig. 8).

It is noted that Sufu-dependent Hh signaling activity probably targets the BMP2 signaling cascade during the regulation of preosteogenic mesenchyme. Previous studies have suggested that BMPs are required for commitment of CNC-derived mesenchyme to the osteogenic fate in intramembranous ossification, and Ihh negatively regulates the further differentiation of osteoblast precursors (11). We found that the osteogenic marker Bmp2 was diminished within the frontal bone-forming mesenchyme of Sufufc/fc;Wnt1-Cre mutant, suggesting that Sufu-dependent Hh signaling inhibits the commitment of mesenchymal progenitors to the osteogenic lineage by suppressing BMP signaling (11, 23). Consistently, the expression of osteogenic markers downstream of BMP signaling, such as Msx1/2 and Dlx5, is overtly suppressed in the Sufufc/fc;Wnt1-Cre mutant. Msx1 and Msx2 are required for differentiation in the CNC lineage within the frontal bone primordium by regulation of Runx2 (43, 44). In frontal bone development, Msx2 and Twist1 cooperatively control the proliferation and differentiation of the osteogenic mesenchyme (51). In addition, Msx1 and Dlx5 are both involved in the osteoblast differentiation by synergistic and positive regulation of Runx2 and Osterix (42). Previous data from in vitro analyses revealed that BMP2-induced Osterix expression is mainly mediated by Dlx5 and not by Runx2 (52). After identifying the above osteogenic marker genes whose expression had diminished upon conditional ablation of Sufu in the CNC-derived mesenchyme, we recognized that the absence of Sufu activity in the mesenchyme might have changed the regulation required for specifying preosteogenic mesenchyme to osteoblastic fate, in which excessive Hh signaling

Figure 6. Sufu mutation results in activation of Hh signaling and protein stability variation of Gli2/3. A, in situ hybridization showing up-regulation of Gli1 within the presumptive frontal primordium in Sufufc/fc;Wnt1-Cre (SufuWnt1Cre) mutants compared with littermate wild-type control (n = 3) during calvarial bone development. Dashed lines outline the presumptive frontal primordium. B, Western blot showing a discernable increase of full-length Gli2 (Gli2F) and significant decrease of truncated repressor forms Gli2 (Gli2R) and Gli3 (Gli3R) in mesenchymal cells within the frontal primordium in the Sufu mutant versus the wild type (n = 3). Each image shows a representative result of independent triplicated experiments. Expression levels were quantified from the band intensity as relative values of the target protein/actin expression ratios. *, p < 0.05; **, p < 0.01; ns, non-significant; Student’s t test. Error bars, S.D. Wt, wild type; M, Sufufc/fc;Wnt1-Cre.
Sufu is required for calvarial bone development

Hey proteins with Runx2 that down-regulates its transcriptional activity (53). Our data indicating that up-regulation of Notch ligand Jagged1 in the Sufu<sup>−/−</sup>;Wnt1-Cre mutant, together with the increased expression of Notch signaling target gene Hes1 in the calvarial bone primordia of Sufu<sup>−/−</sup>;Wnt1-Cre mutant, suggest that excessive Hh signaling inhibits the osteoblastic specification of mesenchymal progenitors through activating downstream Notch signaling (49).

The present results confirm and extend earlier works in long bone development suggesting that Hh signaling is required for osteogenesis in the context of temporal specificity. Hh signaling has proved an essential but transiently required component for initial specification of an osteoblast progenitor to a Runx2-expressing osteoblast precursor (54, 55), but Hh is not required once Runx2 and Osterix are expressed in the osteoblast precursor. Activation of Hh signaling in human mesenchymal stem cells at early stages, but not in differentiated osteoblasts, inhibits human osteoblast differentiation by decreasing Runx2 expression (56), suggesting that Hh signaling plays critical roles in early rather than late stages of osteogenic differentiation. Interestingly, a recent study has suggested that Runx2 is required for intramembranous ossification from the initial phase when the mesenchymal cells express Prox1 and Sca1 until the time when osteoblast precursors express Osterix (57). Our data showing the failed expression of Runx2 and Osterix in the initial stage of the osteogenic mesenchyme suggest that a Sufu-regulated level of Hh signaling activity is required for the initiation of the osteoblast progenitor.

In the absence of Hh ligands or interference with the Hedgehog signaling transduction, transcriptional repression of Hh activity and targets would result in a variety of craniofacial abnormalities (44, 55, 58–61). Gli3 null mice with excessive proliferation and differentiation of osteoblast have craniosynostosis (25). In contrast, local application of recombinant FGF2 protein can rescue loss of Gli3 as it stabilizes the increased osteoblastic proliferation observed in Gli3 mutant mice (23). Interestingly, our current study provides genetic evidence that Sufu, a crucial negative regulator of Hh signaling, acts via repressive regulation of Hedgehog activity in the preosteogenic mesenchyme to ensure osteogenesis in calvarial bone formation. Our data suggest that the function of Gli2 and Gli3 in the calvarial mesenchyme requires the presence of Sufu (Fig. 8). Sufu stabilizes full-length Gli2 and Gli3 and promotes the generation of their repressor forms that are consistent with previous studies (62, 63). We demonstrate that calvarial bone development arrests at the initial mesenchyme condensation associated with excessive activation of Gli2 and Gli3 activity by deletion of Sufu in CNC-derived mesenchyme, which leads to suppression of both osteoprogenitor proliferation and expression of osteoblastic markers. The complete restoration of calvarial bone formation in simultaneous inactivation of Sufu and Gli2 suggests that the Gli2 activator primarily contributes to maximal activation of Hh activity in CNC-derived mesenchyme of the Sufu mutant. These findings therefore provide novel evidence that a relatively low level of Hh signaling activity is required for successful progression of osteogenesis beyond the initial condensation phase in intramembranous ossification.

inhibits the specification and proliferation of mesenchymal progenitor cells by suppressing BMP signaling during calvarial bone development.

Previous studies have shown that Jagged1, the molecule in the Notch signaling pathway, is one of the downstream genes of Hh signaling (48–50). Notch signaling is involved in regulation of osteogenesis by inhibition of endochondral bone formation and osteoblastic differentiation. Notch signaling in the bone marrow maintains mesenchymal progenitors by suppressing osteoblast differentiation through direct interaction of Hes/
Experimental procedures

Animals
All mice used in this study were raised in a standard specific pathogen-free mouse facility, and the animal experiments were approved by the Committee of Laboratory Animals, Hangzhou Normal University. Sufu-floxed mice containing loxP sites flanking exon 7 were generated as we described previously (38). Wnt1-Cre (64), Dermo1-Cre (65), R26R-LacZ (66), and Gli2 (67) mice were purchased from the Jackson Laboratory, and maintained on the C57BL/6 background. The morning of vaginal plug appearance was determined as embryonic day 0.

Scanning electron microscopy analysis
Embryos were fixed overnight with 1.25% glutaraldehyde in PBS. Samples were dehydrated through a series of increasing ethanol concentration from 30 to 100%. After a 15-min treatment with isoamyl acetate, samples were critically point-dried using CO2 for 2 h. The dried samples were mounted on conductive paper and sputter-coated with gold. Images were recorded with a scanning electron microscope (Hitachi S-3000N) with a 15-kV accelerating voltage.

Skeletal preparation
Mice were fixed in 95% ethanol for 2 days after the removal of skin and viscera. They were incubated in Alcian blue staining solution (0.03% Alcian blue in 80% ethanol and 20% glacial acetic acid) for 2–3 days at room temperature. Skeletons were rehydrated and cleared in 1% KOH overnight. Samples were stained with Alizarin red solution (0.03% Alizarin red in 1% KOH) for 1–2 days followed by further clearing with 1% KOH in 20% glycerol. The stained skeletons were finally stored in 100% glycerol.

Histological analysis and von Kossa staining
Samples were fixed in 4% paraformaldehyde (PFA) overnight and embedded in paraffin. Tissue paraffin sections were cut at 7 μm for histological analysis and von Kossa staining. H&E staining was performed using a standard protocol. For von Kossa staining, the sections were flooded with 5% silver nitrate and exposed under a UV lamp for 20 min. The staining was stopped with 2% sodium thiosulfate. The stained sections were counterstained with 1% neutral red.

Immunofluorescence and in situ hybridization
Immunofluorescence analysis was carried out according to the standard protocol for paraffin section samples (68). Embryos were fixed in 4% PFA for 30 min, embedded in paraffin, and sectioned at 7 μm. Antigen epitopes were unmasked by heat-induced epitope retrieval. The sections were incubated with primary antibodies at 4 °C overnight. Secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:1000; Invitrogen) were applied for 30 min to detect the primary antibody. Antibody against Sufu was from LifeSpan BioSciences (LS-C482700; 1:150). Antibodies against Hes1 (ab712559; 1:100) and Osterix (ab22552; 1:200) were from Abcam. Antibody against Runx2 (1:300) was from MBL International (D130-3). The protocol for immunofluorescence analysis with cryostat sections was described previously (68). Images were acquired using a Nikon 80i fluorescence microscope.

In situ hybridizations using whole-mount and section of embryonic samples were performed as described previously (38, 69) using digoxigenin-labeled RNA probes.

Figure 8. Schematic of hypothesized model for Sufu-dependent balance of Gli repressors and activators in regulating calvarial bone development.

In wild-type calvarial mesenchymal cells, Sufu is required for maintaining the balance of Gli2 and Gli3 activity (A). Calvarial mesenchymal cells in Sufufx/fx;Wnt1-Cre mutant cannot undergo normal condensation and osteogenic differentiation due to the deletion of Sufu that leads to a decrease of Gli repressors and an increase of Hh signaling activity (B). This leads to activation of downstream Jag1/Notch signaling and inhibition of BMP signaling, respectively, resulting in dysregulation of osteogenic markers Runx2 and Osterix. Simultaneous removal of Sufu and Gli2 can compromise Hh activity by rebalancing the Gli repressors and full-length activators, which promotes the proliferation and differentiation of osteoprogenitor cells (C). The regulatory relationships shown in dashed lines are drawn according to previous studies from other researchers (41, 44, 45, 51–53).
**Sufu is required for calvarial bone development**

**Statistical analysis**

Student’s t test was used to compare the differentials between data sets. The threshold for statistical significance was \( p<0.05 \).

**Author contributions**—J. L. and Zunyi Zhang designed the research. X. Z. collected animal materials. J. L., Y. C., J. X., Q. W., X. Y., Y. L., and X. Z. performed the experiments. J. L. and Zunyi Zhang analyzed the data. M. Q. and Ze Zhang helped perform the analysis with constructive discussions. J. L. and Zunyi Zhang wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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**References**

1. Jiang, X., Iseki, S., Maxson, R. E., Sucov, H. M., and Morriss-Kay, G. M. (2002) Tissue origins and interactions in the mammalian skull vault. *Dev. Biol.* **241**, 106–116
2. Gross, J. B., and Hanken, J. (2008) Review of fate-mapping studies of osseous cranial neural crest in vertebrates. *Dev. Biol.* **317**, 389–400
3. Yoshida, T., Vivatbutsiri, P., Morriss-Kay, G., Saga, Y., and Iseki, S. (2008) Cell lineage in mammalian craniofacial mesenchyme. *Mech. Dev.* **125**, 797–808
4. Chai, Y., Jiang, X., Itô, Y., Bringas, P., Jr., Han, J., Rowitch, D. H., Soriano, P., McMahon, A. P., and Sucov, H. M. (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* **127**, 1617–1679
5. Pan, A., Chang, L., Nguyen, A., and James, A. W. (2013) A review of hedgehog signaling in cranial bone development. *Front. Physiol.* **4**, 61
6. Huycke, T. R., Eames, B. F., and Kimmel, C. B. (2012) Hedgehog-dependent proliferation drives modular growth during morphogenesis of a dental bone. *Development* **139**, 2371–2380
7. Brugmann, S. A., Allen, N. C., James, A. W., Mekonnen, Z., Madan, E., and Helms, J. A. (2010) A primary cilia-dependent etiology for midline facial disorders. *Hum. Mol. Genet.* **19**, 1577–1592
8. Lenton, K., James, A. W., Manu, A., Brugmann, S. A., Birker, D., Nelson, E. R., Leucht, P., Helms, J. A., and Longaker, M. T. (2011) Indian hedgehog positively regulates calvarial ossification and modulates bone morphogenic protein signaling. *Genesis* **49**, 784–796
9. Naruse, I., Ueta, E., Sumino, Y., Ogawa, M., and Ishikiriyama, S. (2010) Birth defects caused by mutations in human GLI3 and mouse GLI3 genes. *Conogenet. Anom.* **50**, 1–7
10. Dennis, J. F., Kurosaka, H., Julianella, A., Pace, J., Thomas, N., Beckham, S., Williams, T., and Trainer, P. A. (2012) Mutations in Hedgehog acyltransferase (Hhat) perturb Hedgehog signaling, resulting in severe acranioholoprosencephaly-agathyriani craniofacial disorders. *PLoS Genet.* **8**, e1002927
11. Abhazanov, A., Rodda, S. J., McMahon, A. P., and Tabin, C. J. (2007) Regulation of skeletogenic differentiation in cranial dermal bone. *Development* **134**, 3133–3144
12. Jacob, S., Wu, C., Freeman, T. A., Koyama, E., and Kirschner, R. E. (2007) Expression of Indian Hedgehog, BMP-4 and Noggin in craniosynostosis induced by fetal constraint. *Ann. Plast. Surg.* **58**, 215–221
13. Nott, R. L., Stelnicki, E. J., Mack, J. A., Ben, Y., Mitchell, R., and Mooney, M. P. (2002) Changes in the protein expression of hedgehog and patched-1 in perisutural tissues induced by cranial distraction. *Plast. Reconstr. Surg.* **110**, 523–532
14. Murakami, S., Nifuji, A., and Noda, M. (1997) Expression of Indian hedgehog in osteoblasts and its posttranscriptional regulation by transforming growth factor-β. *Endocrinology* **138**, 1972–1978
15. Hui, C. C., and Angers, S. (2011) GLI proteins in development and disease. *Annu. Rev. Cell Dev. Biol.* **27**, 513–537

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**X-gal staining**

Whole-mount and section X-gal staining were performed according to the standard protocols (38, 70). Embryos were incubated in fixing solution (4% PFA with 5 mM EGTA and 2 mM MgCl2 in PBS) for 1 h at room temperature. Fixed embryos were rinsed three times in washing buffer (0.014% Nonidet P-40, 0.01% sodium deoxycholate, and 2 mM MgCl2 in PBS) and stained 2–4 h at 37 °C in the dark using standard staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM Tris (pH 7.3), 0.1% X-gal in washing buffer). The stained samples were rinsed twice in PBS and post-fixed in 4% PFA. The X-gal staining procedures for cryostat section samples were similar to that for whole-mount samples. The X-gal-stained sections were counterstained with Nuclear Fast Red.

**Cell proliferation and TUNEL assays**

The cell proliferation rate was evaluated by BrdU labeling and immunofluorescence staining using antibody against cyclin D1 (1:200; Abcam). BrdU labeling was performed by intraperitoneal injection of a pregnant mouse with BrdU solution (3 mg/100 g body weight) from a BrdU labeling and detection kit (Roche Applied Science) 30 min prior to harvesting embryos. The embryos were fixed in 10% neutral buffered formalin at 4 °C overnight and embedded in paraffin. Detection of BrdU-labeled cells was performed with an immunohistochemical staining method according to the manufacturer’s instructions. The cell proliferation rate was calculated by dividing the positively stained cells by the total number of cells within the zone of presumptive calvarial bone primordia. TUNEL analysis was performed on 5-μm paraffin sections using the In Situ Cell Death Detection kit (Roche Applied Science) by following the manufacturer’s protocol.

**Real-time quantitative PCR analysis**

Supraorbital mesenchyme that forms the presumptive frontal bone primordia was dissected out under a stereomicroscope and transferred to RNA stabilization reagent (Qiagen). RNA was extracted with an Ambion RNAqueous-4PCR kit and reverse-transcribed with an iScriptTM cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using the CFX96 real-time system (Bio-Rad) with SsoFast™ EvaGreen Supermix (Bio-Rad). The relative expression level of each target gene was calculated based on a standard curve of cycle thresholds, and 18S rRNA expression was used as an internal control for normalization. qRT-PCR analysis was performed in triplicate for each set of samples.

**Western blot analysis**

Western blot analysis was performed according to the standard protocol (70). The primary antibodies against Sufu (1:1000; LS-C482700, LifeSpan BioSciences), Gli2 (1:2500; AF3635, R&D Systems), and Gli3 (1:1000; AF3690, R&D Systems) were used for immunoblotting to detect the expression in calvarial bone primordia. Antibody against β-actin (1:1000; catalog no. 3700, Cell Signaling Technology) was used as an internal reference. Relative quantification of protein expression was analyzed with Image-Pro Plus software (version 6.0) based on the integrated optical density of the blotting bands.

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**J. Biol. Chem. (2017) 292(38) 15814–15825 15823**
Sufu is required for calvarial bone development

16. Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. (1999) Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* 274, 8143–8152.

17. Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999) Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 126, 3915–3924.

18. Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D., and Joyner, A. L. (2002) Sufu is required for calvarial bone development. *J. Biol. Chem.*

19. Rice, D. P., Connor, E. C., Veltmaat, J. M., Lana-Elola, E., Veistinen, L., Amano, K., Densmore, M., Nishimura, R., and Lanske, B. (2014) Indian hedgehog signaling regulates transcription and expression of collagen type X via Runx2/Smad interactions. *J. Biol. Chem.* 289, 24898–24910.

20. Amano, K., Denismore, M., Nishimura, R., and Lanske, B. (2014) Indian hedgehog signaling regulates transcription and expression of collagen type X via Runx2/Smad interactions. *J. Biol. Chem.* 289, 24898–24910.

21. Han, J., Ishii, M., Bringas, P., Jr, Maas, R. L., Maxson, R. E., Jr, and Chai, Y. (2010) Inactivation of Msx1 and Msx2 in neural crest reveals an unexpected role in suppressing heterotopic bone formation in the head. *Dev. Biol.* 334, 28–39.

22. Panman, L., Gilli, A., Lagarde, N., Michos, O., Soete, G., Zuniga, A., and Zeller, R. (2006) Differential regulation of gene expression in the digit forming area of the mouse limb bud by SHH and gremlin 1/FGF-mediated epithelial-mesenchymal signalling. *Development* 133, 3419–3428.

23. Xiao, G., Karaca, G., Swiderska-Syn, M., Michelotti, G. A., Krüger, L., Chen, Y., Premont, R. T., Choi, S. S., and Diehl, A. M. (2013) Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. *Hepatology* 58, 1801–1813.

24. Ishii, M., Merrill, A. E., Chan, Y. S., Gitelman, I., Rice, D. P., Sucov, H. M., and Maxson, R. E., Jr. (2003) Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. *Development* 130, 6131–6142.

25. Lee, M. H., Kwon, T. G., Park, H. S., Wozney, J. M., and Ryoo, H. M. (2003) BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem. Biophys. Res. Commun.* 309, 689–694.
53. Hilton, M. J., Tu, X., Wu, X., Bai, S., Zhao, H., Kobayashi, T., Kronenberg, H. M., Teitelbaum, S. L., Ross, F. P., Kopan, R., and Long, F. (2008) Notch signaling maintains bone marrow mesenchymal progenitors by suppress- ing osteoblast differentiation. Nat. Med. 14, 306–314

54. Rodda, S. J., and McMahon, A. P. (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133, 3231–3244

55. Long, F., Chung, U. I., Ohba, S., McMahon, J., Kronenberg, H. M., and McMahon, A. P. (2004) Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. Development 131, 1309–1318

56. Plaisant, M., Fontaine, C., Cousin, W., Rochet, N., Dani, C., and Peraldi, P. (2009) Activation of hedgehog signaling inhibits osteoblast differentiation of human mesenchymal stem cells. Stem Cells 27, 703–713

57. Takarada, T., Nakazato, R., Tsuchikane, A., Fujikawa, K., Iezaki, T., Yoneda, Y., and Hinoi, E. (2016) Genetic analysis of Runx2 function during intramembranous ossification. Development 143, 211–218

58. Sasaki, T., Ito, Y., Bringas, P., Jr, Chou, S., Urata, M. M., Slavkin, H., and Chai, Y. (2006) TGFβ-mediated FGF signaling is crucial for regulating cranial neural crest cell proliferation during frontal bone development. J. Biol. Chem. 281, 4975–4982

59. Iseki, S., Wilkie, A. O., and Morriss-Kay, G. M. (1999) Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. Development 126, 5611–5620

60. Wang, C., Pan, Y., and Wang, B. (2010) Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. Development 137, 2001–2009

61. Iwata, J., Hosokawa, R., Sanchez-Lara, P. A., Urata, M., Slavkin, H., and Chai, Y. (2010) Transforming growth factor-β regulates basal transcriptional regulatory machinery to control cell proliferation and differentiation in cranial neural crest-derived osteoprogenitor cells. J. Biol. Chem. 285, 15825

62. Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C., and Chen, Y. (2002) Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 129, 4135–4146

63. Zhu, X., Zhao, P., Liu, Y., Zhang, X., Fu, J., Ivy Yu, H. M., Qiu, M., Chen, Y., Hsu, W., and Zhang, Z. (2013) Intra-epithelial requirement of canonical Wnt signaling for tooth morphogenesis. J. Biol. Chem. 288, 12080–12089