Skeletal stem and progenitor cells maintain cranial suture patency and prevent craniosynostosis

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Cranial sutures are major growth centers for the calvarial vault, and their premature fusion leads to a pathologic condition called craniosynostosis. This study investigates whether skeletal stem/progenitor cells are resident in the cranial sutures. Prospective isolation by FACS identifies this population with a significant difference in spatio-temporal representation between fusing versus patent sutures. Transcriptomic analysis highlights a distinct signature in cells derived from the physiological closing PF suture, and scRNA sequencing identifies transcriptional heterogeneity among sutures. Wnt-signaling activation increases skeletal stem/progenitor cells in sutures, whereas its inhibition decreases. Crossing Axin2LacZ/+ mouse, endowing enhanced Wnt activation, to a Twist1+/− mouse model of coronal craniosynostosis enriches skeletal stem/progenitor cells in sutures restoring patency. Co-transplantation of these cells with Wnt3a prevents resynostosis following suturectomy in Twist1+/− mice. Our study reveals that decrease and/or imbalance of skeletal stem/progenitor cells representation within sutures may underlie craniosynostosis. These findings have translational implications toward therapeutic approaches for craniosynostosis.
Cranial sutures are fibrous joints that comprise two approaching osteogenic bone fronts separated by intervening proliferative mesenchymal tissue. They represent the major sites of cranial morphogenesis and grow in close coordination with the rapidly developing brain. Their anatomy is remarkably well conserved in both mammalian and non-mammalian species. Murine and human cranial sutures are similar: both have an anterior-frontal suture (AF), located between the paired frontal nasal bones; a posterior-frontal suture (PF) (metopic, in humans), located between the paired frontal bones; a coronal suture (COR), located between the frontal and parietal bones; and a lamboid suture (LAM), between the occipital and parietal bones. The PF suture fuses in early life, through endochondral ossification, while the SAG, COR, and LAM remain patent through adulthood.

A delicate balance between cell proliferation, differentiation, migration, and apoptosis regulates the osteogenic fronts at the cranial sutures, ensuring a steady equilibrium of growth and separation. Previous studies have indicated that cranial suture closure versus patency is governed by canonical Wnt (cWnt) signaling. The molecular machinery coordinating cell proliferation with osteoblast differentiation must be under strict control: prolonged proliferation of the suture mesenchyme or delayed osteoblast differentiation results in pathological suture expansion, whereas insufficient proliferation or accelerated differentiation results in premature cranial suture fusion, known as craniosynostosis.

Craniosynostosis affects approximately one in 2500 live births, resulting in significant clinical sequelae including major craniofacial deformities. Craniosynostosis can be associated with an underlying genetic abnormality (syndromic) or, more commonly, non-syndromic. Unveiling the factors that give rise to non-syndromic craniosynostosis is therefore of relevance in determining the etiology of premature suture fusion.

Recently, the suture mesenchyme has been postulated to act as a niche for stem cells critical in suture development, cranial growth, and injury repair. Similar to the growth plates of the long bone, cranial sutures are the sites of growth for the cranial vault. Fueled by our prior isolation of skeletal stem cells and progenitors from long bones and mandibles, we aimed to examine cranial suture biology through the lens of these cells.

We first confirm that skeletal stem/progenitor cells (referred to as CD51+CD200+ cells) are resident in cranial sutures and validated their multipotency and ability to self-renew. CD51+CD200+ cells are then prospectively isolated from physiologically fusing and patent sutures, in addition to cranial sutures harvested from animal models of syndromic and non-syndromic craniosynostosis. We identify that aberrancies in CD51+CD200+ cell equilibrium may underlie both physiologic suture closure and pathologic craniosynostosis. Furthermore, we demonstrate that Wnt activation increases CD51+CD200+ cell frequency, preventing suture fusion, and ultimately rescues the craniosynostosis phenotype. These findings suggest that CD51+CD200+ skeletal stem/progenitor cells and Wnt3a may provide a combined cellular/molecular intervention to treat or even prevent craniosynostosis.

Results
In vivo profiling of CD51+CD200+ cells in cranial sutures. We initiated our study by confirming the presence of skeletal stem/progenitor cells (referred to as CD51+CD200+ cells), defined by the following immunophenotype: CD51+, CD200+, CD45−,

![Fig. 1 Temporal FACS profiling of CD51+;CD200+ cells in patient and fusing cranial sutures.](image-url)

(a) Outline of calvarial suture harvesting and processing. (b) Time-course profiling of skeletal stem/progenitor cells isolated in vivo by flow cytometric analysis from freshly harvested posterior frontal (PF), sagittal (SAG), and coronal (COR) sutures. Tibial growth plate (GP) was used as reference. Values are given as a percentage of the P3 population, representing viable (propidium iodide−), non-hematopoietic (CD45−) cells isolated from the sutures. The fusing PF suture shows a dramatic decrease in the percentage of skeletal stem/progenitor cells between day pN7 and pN9, corresponding to the initiation of PF suture closure. Conversely, the patent SAG and COR sutures and GP maintain a sustained percentage of these cells over time. Data are represented as means ± SEM; PF vs SAG P = 0.002, PF vs COR P = 0.0147, PF vs GP P = 0.0179. *P ≤ 0.05 unpaired, two-tailed student t-test were performed. n = 60 animals/timepoint, experiments were repeated 6 independent times for pN3, 4 for pN17 and 2 independent times for pN5, 7, 9, 11, and 15.

(c) Diagram illustrating the timing of the endochondral differentiation through which the PF suture closes. Representative FACS plots of skeletal stem/progenitor (CD51+CD200+) cells isolated in vivo from the PF, SAG, and COR sutures and GP at pN3, pN9, and pN17. CD51+CD200+ cells decrease as a function of time in the physiologically fusing PF suture. Refer to Supplementary Fig. 1 for complete FACS gating and isolation strategy. Source data are provided as a Source data file.
Transcriptomic analysis revealed a distinct clustering of transcriptomic profiles in cranial sutures using FACS analysis (Fig. 1a–d). CD51+/CD200+ cells were analyzed in the PF, SAG, and COR sutures between pN3 and pN17 (n = 60 mice/time-point), a time frame spanning the period of physiological PF suture closure10 (Fig. 1c). Cell numbers were normalized to the cells defined by the following immunophenotype, [PF-CD45−], hereafter referred to as the P3 population, in our FACS gating scheme (Supplementary Fig. 1a–c). CD51+, CD200+ cells from long bone growth plates (GP) were used as a reference.15,32

The PF suture contained a higher percentage of CD51+/CD200+ cells at early time-points (pN3-5), when this suture is patent, followed by a sharp decrease in CD51+/CD200+ cells starting at pN7 [Fig. 1b]. The PF is prior to the onset of chondrogenesis which precedes suture closure (Fig. 1c).10 By pN17, when the PF suture is fused10, CD51+/CD200+ cells represented only a small percentage of the P3 population. Conversely, CD51+/CD200+ cell frequency from the patent COR and SAG sutures were maintained throughout the time-course whereas their frequency in GP was relatively higher (Fig. 1b, d). Our data reveal significant differences in CD51+/CD200+ cell representation between patent versus fusing cranial sutures—specifically, the number of these cells in the PF suture decreases as a function of time in advance of physiological suture fusion.

Transcriptomic signature of suture-CD51+/CD200+ cells. Next, we compared the transcriptomes of CD51+/CD200+ cells resident in fusing and patent sutures. We performed bulk RNA-seq profiling of CD51+/CD200+ cells harvested from the PF, SAG, and COR sutures (hereafter referred to as PF-CD51+, CD200+ cells, SAG-CD51+, CD200+ cells and COR-CD51+, CD200+ cells respectively) of pN3 mice, again using GP-isolated CD51+/CD200+ cells (GP-CD51+, CD200+ cells) as controls (n = 60 mice/time point). pN3 was chosen based on two features shared at this time point by all three sutures: first, their potency; second, their comparable degree of endogenous active cWnt signaling (Supplementary Fig. 3a)10,13.

Our analysis revealed significant transcriptomic differences among skeletal stem/progenitor cells from the different sutures. We identified 171 (out of 24,015 mouse mm9) genes whose expression levels were significantly different with a fold change greater than 3 between the different sutures (Fig. 2a). Hierarchical transcriptional analysis revealed a distinct clustering of transcripts that were up- or downregulated in CD51+/CD200+ cells from each suture. We identified a uniquely downregulated cluster of genes in PF-CD51+/CD200+ cells which are associated with craniosynostosis. Interestingly, the PF suture is the only suture that physiologically fuses during early life. Among these genes were Cdc45, Huw1, Efnb, Abhd1, Dusp6, Jag1, and Ezh237–42, while Ptpn11, a gene associated with gain-of-function mutations resulting in craniosynostosis43, was upregulated in PF-CD51+/CD200+ cells exclusively (Fig. 2b) and Supplementary Fig. 3b, c. The expression profile of these genes was validated by RT-PCR (Supplementary Fig. 3b).

To further characterize these differences in transcriptional programming, we performed single-cell RNA-seq analysis on PF-CD51+/CD200+ cells, SAG-CD51+/CD200+ cells, and COR-CD51+/CD200+ cells isolated at pN3 (Fig. 2c). Single cell data for each of the sutures were pooled, and blinded partitional analysis identified five transcriptionally-defined clusters (Fig. 2d).

Interestingly, PF-CD51+/CD200+ cells and COR-CD51+/CD200+ cells exhibited similar distributions and together comprised the majority of clusters 0, 2, and 3, while the SAG-CD51+/CD200+ cells transcriptional profiles appeared more distinct (cluster 1 and 4) (Fig. 2e, f). Downstream analyses of cluster characteristics provided further insights into what may represent transcriptional meta-states among suture-derived CD51+/CD200+ cells, such as those with comparatively pro-osteogenic versus chondrogenic expression patterns. (Fig. 2f, Supplementary Fig. 4a–c, and Supplementary Fig. 5a–d). Moreover, these transcriptional programs appeared distinct from those of the periosteal stem cell (PSC) population previously identified44 (Supplementary Fig. 6a) and comparatively pure with our earlier CD51+/CD200+ cell population (Supplementary Fig. 6b)15,46.

Imbalance of CD51+/CD200+ cells marks craniosynostosis. The different trends of resident CD51+/CD200+ cells between physiologically fusing versus patent sutures set the stage for investigating these cells in syndromic and non-syndromic craniosynostosis. Saethre-Chotzen syndrome, defined by TWIST1 loss-of-function mutation, is the second most prevalent form of syndromic COR craniosynostosis31,47. TWIST1+/− mice represent an established model to study this genetic syndrome48,49, displaying premature COR fusion (Fig. 3a). We FACS-profiled CD51+/CD200+ cells to determine their representation in TWIST1+/− COR sutures relative to COR sutures from wild-type mice. This analysis revealed a significant decrease of these cells in craniosynostotic TWIST1+/− as compared to wild-type COR sutures (Fig. 3b).

The majority of human craniosynostoses are non-syndromic (85%); of these, synostoses of the SAG are the most common (40–55%). Mutations in the inhibitory SMAD6 gene have been reported to lead to SAG craniosynostosis30. Inhibition of endogenous TGF-β has been shown to trigger downregulation of SMAD6, leading to enhanced activation of BMP signaling and induction of osteogenesis31,32. We thus hypothesized that SAG craniosynostosis could be phenocopied in wild-type SAG suture by inhibiting TGF-β signaling with the small molecule SB431542, SB431542. To test this approach, SAG sutures explanted from pN3 wild-type mice were treated with SB431542. After 8 days, treated sutures fused, whereas untreated SAG sutures remained patent (Fig. 3c). In treated SAG sutures, the suture mesenchyme was replaced by bony tissue bridging the osteogenic fronts, while in untreated sutures, mesenchyme remained present within the approaching osteogenic fronts. Consistent with previous reports31,32, PCR analysis confirmed significant downregulation of Smad6 gene expression in SB431542-treated sutures (Fig. 3d). SB431542 treatment also triggered upregulated expression of Id2 (Fig. 3e), a specific target of activated BMP signaling53, and osteocalcin (Bglap), a marker of terminal osteoblast differentiation (Fig. 3f).

Having phenocopied a SAG model of non-syndromic craniosynostosis, we next performed FACS profiling of CD51+/CD200+ cells. We observed a significant decrease of these cells in SB431542-treated SAG sutures compared to untreated sutures (Fig. 3g). Collectively, these results demonstrate that a significant change in the ratio of skeletal stem/progenitor cell populations is a shared signature in our models of both syndromic and non-syndromic craniosynostosis.

Modulation of cWnt signaling alters CD51+/CD200+ frequency. We previously showed that physiologic closure of the PF suture is dependent on inactivation of endogenous cWnt signaling at the onset of endochondral ossification53. Conversely, patency of the SAG and COR sutures is maintained by sustained activation of
cWnt signaling over time. \textsuperscript{13} Furthermore, manipulation of endogenous cWnt signaling is sufficient to reverse the fate of PF and SAG sutures. \textsuperscript{13} These findings, along with the observation that CD51\textsuperscript{+}; CD200\textsuperscript{+} cells sharply decrease prior to PF suture fusion (Fig. 1b), prompted us to investigate whether continuous activation of cWnt signaling (leading to PF suture patency) would affect the representation of these cells in the suture. PF sutures were treated with Wnt3a protein (Fig. 4a). Effective activation of cWnt signaling was confirmed by treating PF sutures of Axin2\textsuperscript{LacZ/LacZ} reporter mice (enabling detection of cWnt signaling through the X-gal reporter) with Wnt3a and performing X-gal staining as a readout of cWnt activity. \textsuperscript{13,54} Yellow: upregulation; purple: downregulation. cWnt signaling over time. \textsuperscript{13} Furthermore, manipulation of endogenous cWnt signaling is sufficient to reverse the fate of PF and SAG sutures. \textsuperscript{13} These findings, along with the observation that CD51\textsuperscript{+}; CD200\textsuperscript{+} cells sharply decrease prior to PF suture fusion (Fig. 1b), prompted us to investigate whether continuous activation of cWnt signaling (leading to PF suture patency) would affect the representation of these cells in the suture. PF sutures were treated with Wnt3a protein (Fig. 4a). Effective activation of cWnt signaling was confirmed by treating PF sutures of Axin2\textsuperscript{LacZ/LacZ} reporter mice (enabling detection of cWnt signaling through the X-gal reporter) with Wnt3a and performing X-gal staining as a readout of cWnt activity. \textsuperscript{13,54} Yellow: upregulation; purple: downregulation. cWnt signaling over time. \textsuperscript{13} Furthermore, manipulation of endogenous cWnt signaling is sufficient to reverse the fate of PF and SAG sutures. \textsuperscript{13} These findings, along with the observation that CD51\textsuperscript{+}; CD200\textsuperscript{+} cells sharply decrease prior to PF suture fusion (Fig. 1b), prompted us to investigate whether continuous activation of cWnt signaling (leading to PF suture patency) would affect the representation of these cells in the suture. PF sutures were treated with Wnt3a protein (Fig. 4a). Effective activation of cWnt signaling was confirmed by treating PF sutures of Axin2\textsuperscript{LacZ/LacZ} reporter mice (enabling detection of cWnt signaling through the X-gal reporter) with Wnt3a and performing X-gal staining as a readout of cWnt activity. \textsuperscript{13,54} Yellow: upregulation; purple: downregulation.
Conversely, inhibition of cWnt signaling in patent SAG sutures with sFrp-1 and Dkk-1 inhibitors markedly decreased CD51⁺; CD200⁺ cell frequency (Fig. 4d) and resulted in a nearly complete closure of the SAG suture (Fig. 4e). Clonal analysis of Wnt inhibitor-treated ActinCreERT2:Rainbow⁺/− sutures revealed a decrease in polyclonality relative to untreated and PBS-treated control groups (Supplementary Fig. 7f). These findings demonstrate that activation of cWnt signaling in the PF suture during the window of physiologic fusion enriches skeletal stem/progenitor cells representation, whereas inhibition of cWnt signaling in the patent SAG suture decreases them and promotes pathologic fusion.

Activation of cWnt signaling rescues craniosynostosis. We next questioned whether there would be decreased polyclonal proliferation in Twist1⁺/−:ActinCreERT2:Rainbow⁺/− COR sutures mirroring the
observed reduction of CD51^+;CD200^+ cells in Twist1^+/−:COR sutures (Fig. 3b). We observed decreased polyclonality in double transgenic Twist1^+/−:Axin2LacZ^+;ActinCreERT2 Rainbow^+/+ mice relative to control. By contrast, increased polyclonality was observed in COR sutures in Axin2^−/−;ActinCreERT2 Rainbow^+/+ mice, which displayed enhanced activation of cWnt signaling (Supplementary Fig. 8a–d). Given these findings, we next investigated whether sustained activation of cWnt signaling in a craniosynostotic background could restore the required balance of CD51^+;CD200^+ cells and thereby prevent/rescue the craniosynostosis phenotype.

We generated a double transgenic Twist1^+/−:Axin2^−/− mouse by crossing Twist1^+/− mice (bearing coronal craniosynostosis) with Axin2^−/− mice (Supplementary Fig. 9a–d). Pentachrome staining of COR sutures revealed suture patency and a large undifferentiated suture mesenchyme in both wild-type and Twist1^+/−:Axin2^−/− COR sutures as compared to synostotic Twist1^+/− sutures (Fig. 5a).

Remarkably, FACS analysis revealed that in Twist1^+/−:Axin2^−/− COR sutures, CD51^+;CD200^+ cells were restored to wild-type levels (Fig. 5b). Ensuing screening of the Twist1^+/−:Axin2^−/− double transgenic COR sutures for complete unilateral or bilateral craniosynostosis revealed a frequency of 0% in wild-type mice, as expected; 83% in Twist1^+/− mice; and 0% in Twist1^+/−:Axin2^−/− double transgenic mice. (Fig. 5c). Notably, a robust contribution of Axin2^+ (LacZ^+) cells was observed within the mesenchyme of the Twist1^+/−:Axin2^−/− COR suture at day pN3, day pN18, and 6 months (Fig. 5d and Supplementary Fig. 10). Moreover, immunostaining of activated β-catenin revealed levels of endogenous active cWnt signaling within the COR suture mesenchyme of Twist1^+/−:Axin2^−/− double transgenic similar to that of wild-type and Axin2^−/− COR sutures (Supplementary Fig. 11a–d). Conversely, a faint immunostaining was detected in Twist1^+/− COR sutures. Microcomputed tomography (μCT) confirmed COR suture patency.
Fig. 5 Sustained activation of cWnt signaling rescues COR suture craniosynostosis in Twist1\textsuperscript{+/−} mice. 

a Pentachrome staining and histological analysis of pN15 COR sutures from wild-type (top panel), Twist1\textsuperscript{+/−} (middle panel) and Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} (bottom panel) mice. Similar to the wild-type suture, the Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} COR suture is patent with a clear undifferentiated tissue mesenchyme between the osteogenic fronts, thus demonstrating a rescue of the COR suture craniosynostosis. Conversely, the Twist1\textsuperscript{+/−} COR suture is fused, showing a bony bridge and lack of a suture mesenchyme. Scale bars, 100 μm. Magnification at ×20. n = 3 animals/group, experiments were repeated 3 independent times.

b FACS analysis of CD51\textsuperscript{+}; CD200\textsuperscript{+} cells isolated from COR sutures of wild-type, Axin2\textsuperscript{LacZ+/}, Twist1\textsuperscript{+/−}, and Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} double transgenic. Values represent mean ± SEM; Axin2\textsuperscript{LacZ+/} vs. Twist1\textsuperscript{+/−}, P = 0.025; Twist1\textsuperscript{+/−} vs. Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/}, P = 0.0172; *P ≤ 0.05, unpaired, two-tailed student t-test were performed. n = 10 animals/group, experiments were repeated 3 independent times.

c Screening for coronal suture fusion in Twist1\textsuperscript{+/−} versus Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} mice reveals a frequency of COR craniosynostosis in Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} mice restored to that of wild-type mice (i.e., patent COR suture phenotype), compared to the widespread fusion seen in Twist1\textsuperscript{+/−}. Screening was performed on mice ranging from day pN6 up to 6 months. n = 282 animals screened. 

(d) X-gal staining identifies a of cWnt-activated cells within the mesenchyme of the patent COR suture in Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} double transgenic mice at day pN3, pN18, and 6 months postnatal. (bottom panel) Movat’s pentachrome staining performed on adjacent slide sections as for top panels. Scale bars: 100 μm. Magnification at ×20. n = 3 animals/group, experiments were repeated 3 independent times. pN post-natal.

e Micro-CT analysis of wild-type, Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/}, Twist1\textsuperscript{+/−}, and Axin2\textsuperscript{LacZ+/} skulls at 6 months postnatal reveals the presence of a wide patent COR suture in the Twist1\textsuperscript{+/−}: Axin2\textsuperscript{LacZ+/} mouse, whereas a unilateral fused COR suture is observed in the Twist1\textsuperscript{+/−} mouse. n = 3 animals/group, experiments were repeated 3 independent times. COR coronal. Source data are provided as a Source data file.
in Twist1+/−;Axin2lacZ/+ mice (Fig. 5e). Collectively, these data support that cWnt activation in the suture mesenchyme of the Twist1+/−;Axin2lacZ/+ COR suture may suppress COR suture craniosynostosis observed in Twist1+/− mice, possibly through expansion of the CD51+/CD200+ cell population.

Preventing re-synostosis following suturectomy. Conventional methods of treatment for craniosynostosis require complex surgical intervention to correct the fused suture, frequently resulting in re-fusion of the suture. Thus, there is a strong unmet need for cellular or molecular therapies to treat craniosynostosis and prevent resynostosis. Our data suggest that skeletal stem/progenitor cell equilibrium within the suture mesenchyme may maintain suture patency. Moreover, active cWnt signaling may promote cell expansion within the suture mesenchyme to levels sufficient for sustaining patency. Based on these findings we hypothesized that by transplanting skeletal stem/progenitor cells and Wnt3a protein following ablation of a synostotic suture, we could model a physiological suture mesenchyme, thereby preventing suture re-fusion. To test this hypothesis, we surgically ablated the synostotic COR suture of Twist1+/− mice via suturectomy (Fig. 6a), reflecting the surgical management of human craniosynostosis. CD51+/CD200+ cells co-seeded with Wnt3a in a collagen sponge carrier were transplanted in the suturectomy site, with untreated suturectomies acting as controls (Fig. 6a). The extent of re-fusion was assessed by microcomputed tomography (μCT) and histology over a 14-week period (Fig. 6b–d). After 14 weeks the control suturectomy sites re-fused completely, while suturectomies treated with CD51+/CD200+ cells and Wnt3a protein remained patent with the appearance of a restored mesenchyme (Fig. 6d). Additionally, transplanted GFP+ CD51+/CD200+ cells were used to confirm their contribution to the suture mesenchyme (Fig. 6e). Interestingly, neighboring cells, in addition to the transplanted GFP+ CD51+/CD200+ cells, appeared to participate in recreating the suture mesenchyme, thus reestablishing a "suture niche/microenvironment" (Fig. 6f). Conversely, mice treated with Wnt3a or CD51+/CD200+ cells alone, developed recurrent synostosis (Supplementary Fig. 12a–d).

Discussion
This study examined cranial suture biology through the lens of a skeletal stem/progenitor cell population (namely CD51+;CD200+), demonstrating a link between CD51+;CD200+ cell equilibrium and cranial suture patency, thus highlighting the potential significance of these cells in craniosynostosis. Following isolation of CD51+;CD200+ population and validation of their multi-potency and self-renewal capacity, we performed an in vivo time course profiling of these cells derived from mouse cranial sutures using FACS analysis. Our data revealed significant differences in skeletal stem/progenitor cell representation between patent versus fusing cranial sutures—specifically, the number of these cell in the PF suture decreases as a function of time in advance of physiological suture fusion. Furthermore, we identified that a significant decrease in skeletal stem/progenitor cell is a shared feature in our models of both syndromic and non-syndromic craniosynostosis.

As a dynamic representation of CD51+/CD200+ cells is implicated in patent versus fusing cranial sutures, we evaluated transcriptional profiling of the cells derived from the patent (COR, SAG) versus the fusing (PF) sutures. Interestingly, genes in which loss-of-function mutations are known to lead to craniosynostosis were almost exclusively downregulated in PF-derived skeletal stem/progenitor cells. The PF is the only suture physiologically fusing during early life, and we found an array of genes to be uniquely downregulated in PF-derived cells relative to COR- and SAG-derived cells, suggesting a potential transcriptional signature of skeletal stem/progenitor cells fated for suture fusion. Furthermore, scRNA sequencing of cranial suture derived skeletal stem/progenitor cells identified multiple transcriptionally-defined clusters with gene expression profiles appearing to favor osteogenic versus chondrogenic cell fates. This would suggest that transcriptionally-defined clusters may represent transcriptional/functional primed cells. However, prospective isolation and evaluation of these putative subgroups would be required to determine whether they represent functionally distinct cell subpopulations. These findings highlight the transcriptional heterogeneity among suture-derived skeletal stem/progenitor cells, which may be reflective, in part, of differences in the niches wherein these cells reside and the complex tissue architectures within the cranial suture.

The importance of cWnt signaling in maintaining the undifferentiated state of stem cells by preventing them from progressing towards more differentiated lineages is well known5. Building on prior work by Behr et al.13, we interrogated if cWnt signaling rescued craniosynostosis by acting directly on these skeletal stem/progenitor cells. Our data indicated that cWnt signaling not only induces the expansion of this population, both in vitro and in vivo, but can also prevent craniosynostosis. The observation that Wnt3a induces proliferation of CD51+/CD200+ cells in vitro provides direct evidence for the role of cWnt on cell expansion independent of other potential paracrine signaling transmitted by neighboring cells within the suture.

The role of cWnt signaling on skeletal stem/progenitor cell expansion and craniosynostosis was further investigated by cross-breeding a Twist1+/− mouse harboring COR craniosynostosis with an Axin2lacZ/+ mouse, endowed with endogenous activation of cWnt signaling. The resulting double transgenic displayed suppressed expression of craniosynostosis phenotype and restoration of suture patency, possibly through the expanded skeletal stem/progenitor cell population observed. Furthermore, the preserved COR suture mesenchyme was largely composed of Xgal+ (cWnt active) cells in a “niche” harboring an active cWnt signaling environment.

Having illustrated that activation of cWnt signaling together with the expansion of skeletal stem/progenitor cells is associated with maintenance of suture patency, we explored whether transplantation of these cells combined with Wnt3a protein would have a synergistic effect in preventing re-synostosis following suturectomy in Twist1+/− coronal craniosynostosis. To functionally test the ability of these skeletal stem/progenitor cells to maintain patency post-suturectomy, we developed an "engineered" suture mesenchyme by transplanting CD51+/CD200+ cells combined with Wnt3a protein into an ablated COR suture site of Twist1+/− mice affected by unilateral COR suture synostosis. Transplantation of skeletal stem/progenitor cells with Wnt3a led to patency of the craniosynostotic suture following its ablation, which may be due to suture mesenchyme reconstitution or indeed arise as a result of failure of healing of the bone defect. Our findings are supported by a recent study39, showing that Gli-1+/MSCs transplanted into a surgically ablated Twist1+/− craniosynostotic suture prevented resynostosis, however, cWnt stimulation was not required. This difference could reflect ongoing autocrine cWnt signaling in Gli-1+MSCs, however, this remains a hypothesis. Based on GSE analysis of the cWnt pathway, our cells do not produce Wnt ligands, therefore require exogenous Wnt3a stimulation in order to activate the canonical signaling.

Taken together, the findings stemming from this study shift our focus towards viewing skeletal stem/progenitor cells through a lens magnifying these cells as an appealing tool suitable for preventing bone formation. Moreover, the findings from this study may lay the foundation for therapeutic options using...
skeletal stem/progenitor cells coached with effectors of cWnt signaling as an early intervention therapy for craniosynostosis.

Methods

Animals. All experiments using animals were performed in accordance with Stanford University Animal Care and Use Committee guidelines. This study complies with all relevant ethical regulations for animal testing and research under the guidance and approval of Stanford University’s Administrative Panel on Laboratory Animal Care IACUC/APLAC. Animals were bred and housed in light (12 h light cycle: 7 am on, 7 pm off), temperature (69–79 °F) and moisture-controlled (30–70%) Research Animal Facility and were given food and water ad libitum in accordance with Stanford University guidelines, APLAC Protocol 8397 and 21067. CD-1 and C57BL/6 mice were purchased from Charles Rivers Laboratories Inc. B6 Twist<sup>1/−</sup> (Stock# 2221) and B6 ACTb-EGFP (Stock# 3291) mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Axin2<sup>LoxP</sup>/LOx mice were as previously described<sup>13</sup>. ActinCre<sup>ERT2</sup>Rainbow
mice were a kind gift from the laboratory of Dr. Irv Weissman, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University. Twist1+/mice were crossed with Axin2mice to generate Twist1+/Axin2mice. Twist1+/mice were crossed with ActinCreERT2Rainbow mice to obtain ActinCreERT2Rainbow mice. Axin2mice to obtain ActinCreERT2Rainbow mice. Twist1+/Axin2mice, Twist1+/ACTB-EGFP, and ActinCreERT2Rainbow mice were genotyped as previously described14,55,59,60. Genotyping primer sequences and expected results can be found in Supplementary Table 1.

**In-vivo suture harvesting and cell dissociation.** Animals (n = 60) were sacrificed by CO2 asphyxiation for each postnatal (p) time-point, p3, p5, p7, p9, p11, p15, and p17 on the exact postnatal day determined by date of birth. The entire cranium was harvested, lightly washed in cold PBS and placed into cold FACS buffer composed of PBS (Gibco-Life Technologies, Grand Island, NY, USA), 2% fetal bovine serum (Gibco-Life Technologies, Grand Island, NY, USA), 1% penicillin–streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), 1% Penicillin–streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), 1% Phosphoric F-68 (Gibco-Life Technologies, Grand Island, NY, USA), the PPF, SAG and COR sutures were carefully dissected on ice with the aid of a dissecting stereo microscope (Zeiss, Oberkochen, Germany). Approximately 0.5 mm by 1–3 mm (depending on pN time-point) region was carefully removed including the osteogenic fronts of the cranial plates, suture mesenchyme, underlying dura mater and overlying periosteum (Suture Complex) using fine forceps and a small scalpel. Cell dissociation and digestion were performed as previously described31,32. Suture tissue was gently minced using fine tipped, dissecting scissor in a 2 ml microcentrifuge tube and chemically digested in 1.5 ml of serum-free Medium 199/EBs (HyClone, Logan, UT, USA) supplemented with 2.2 mg/ml Collagenase Type II (Sigma-Aldrich, St. Louis, MO, USA), 2 mg/ml Dispase II (Roche Diagnostics, Indianapolis, IN, USA), 1% Pluronic F-68 (Gibco-Life Technologies, Grand Island, NY, USA), the PPF, SAG and COR sutures were carefully dissected on ice with the aid of a dissecting stereo microscope (Zeiss, Oberkochen, Germany). Approximately 0.5 mm by 1–3 mm (depending on pN time-point) region was carefully removed including the osteogenic fronts of the cranial plates, suture mesenchyme, underlying dura mater and overlying periosteum (Suture Complex) using fine forceps and a small scalpel. Cell dissociation and digestion were performed as previously described31,32. Suture tissue was gently minced using fine tipped, dissecting scissor in a 2 ml microcentrifuge tube and chemically digested in 1.5 ml of serum-free Medium 199/EBs (HyClone, Logan, UT, USA) supplemented with 2.2 mg/ml Collagenase Type II (Sigma-Aldrich, St. Louis, MO, USA), 2 mg/ml Dispase II (Roche Diagnostics, Indianapolis, IN, USA), 1% Pluronic F-68 (Gibco-Life Technologies, Grand Island, NY, USA), 2% HEPES Buffer (Gibco-Life Technologies, Grand Island, NY, USA), 0.4% 2.5 M CaCl2 (Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml deoxyribonuclease I (Worthington Biochemistry, Lakewood, NJ, USA), incubated in a 37°C water bath for 10 min followed by 2 digestions, each of 25 min, carried out in an orbital shaker at 275 rpm at 37°C. Dissociated cells were filtered through a 70 μm filter and neutralized with 2x volume FACS buffer. The digestions were pooled together and total dissociated cells were pelleted at 1350 rpm at 4°C, resuspended in FACS buffer, blocked with rat IgG and stained with fluorochrome-conjugated antibodies (10 μg/ml) and directed against cell markers, followed by 15 min FACS staining. The resulting progeny will be marked with the same color as the parent cell, creating a fluorescent mosaic pattern upon analysis. For the induction, ActinCreERT2Rainbow Twist1+/−ActinCreERT2Rainbow and ActinCreERT2Rainbow mice were subcutaneously injected with 50 μl tamoxifen at 20 mg/ml at day pN3 and harvested at pN7 or pN8 depending on experimental design.

**FACS normalization methods.** All in-vivo FACS analysis were normalized by calculation of percentage of P3. Total number of CD51+CD200+ cells encompassing the skeletal stem/progenitor cell population (CD45+, Ter119+, Tie2+, Thy1.1+, Thy1.2+, 6C3+, CD105−, CD51+, CD200−) were normalized to the cranial population (pN16−Ter119+/CD45+) for comparison among the three cranial sutures and various treatment groups. For in-vitro FACS analysis (Fig. 3), CD51+CD200+ cells were normalized as total number of cells per 500,000 events. (Additional information can be found in Supplementary Fig. 1). Analysis was performed using FACS Diva (BD) v8.0.1 and FlowJo (TreeStar) 10.1r5 software packages.

**Histology.** Whole cranium was harvested and fixed overnight at 4°C, either in 0.4% or 4% PFA (Electron Microscopy Sciences, Hatfield, PA, USA) and decalified in 19% EDTA at 4°C for 2 days to 2 months (depending on the age of the specimen). Following appropriate decalcification, specimens were prepared for cryo-embedding by soaking in 30% (mass/vol) sucrose in PBS at 4°C for 24 h and embedded in Tissue Tek O.C.T. (Sakura Finetek, Torrance, CA, USA). The entire PPF, SAG, and COR suture were cut in 10 μm sections. Sitter slides were then selected and stained for X-gal and Pentachrome staining or confocal imaging for direct comparison of the different sutures. X-gal (Roche Indianapolis, IN, USA) and Pentachrome staining was performed as previously described43. Images are representative of at least 2 independent samples or experiments. Details of reagents used are listed in Supplementary Table 4.

**Tamoxifen induction of rainbow reporter system.** Rainbow mice were employed for clonal analysis of the cranial sutures. Rainbow mice were crossed with the ubiquitous ActinCreERT2 driver to mark cells under the actin promoter after systemic tamoxifen injection. The Rainbow reporter (R26ERT2flucGFP) is a multicolorCre-dependent reporter system with a four-color reporter construct in the ROSA locus. Once recombination occurs, cells are randomly and genetically marked with one of ten possible color combination. The resulting progeny will be marked with the same color as the parent cell, creating a fluorescent mosaic pattern upon analysis. For the induction, ActinCreERT2Rainbow Twist1+/−ActinCreERT2Rainbow and ActinCreERT2Rainbow mice were subcutaneously injected with 50 μl tamoxifen at 20 mg/ml at day pN3 and harvested at pN7 or pN8 depending on experimental design.

**Imaging analysis.** Laser scanning confocal microscopy was performed with a LEICA TCS SP8 xci confocal microscope (LEICA Microsystems, Buffalo Grove, IL, USA) with an objective lens (×10 HC PL APO, air, N.A. 0.40; ×20 HC PL APO IMM CORR CS2, H2O/Glycerol/oil, N.A. 0.75), located in the Cell Sciences Imaging Facility (Stanford University, Stanford, CA). Raw image stacks were imported into ImageJ (NIH) for further analysis. All clones were individually examined to confirm that they reported a single color. For visualizing individually labeled cells expressing the Rainbow reporter, the brightness and contrast were...
adjacently determined by the green (eGFP), blue (mCerulean), orange (mOrange), and red (mCherry) channels and composite serial image sequences were assembled. Tiled images were produced following a grid/collection stitching plugin. Clones were determined by isolating the rendered composite image containing all four channels, using ImageJ (NIH) software. Cells that were visually determined to be clones were traced with a surface area measurement tool for analysis based on the pixel density of each clone (300 pixels per inch). Clones with a surface area greater than or equal to 3.2 mm$^2$ were included. Values are representative of three independent fields analyzed. Experiments were performed two times.

Transcriptomic analysis. All RNA sequencing, bulk and single cell, were conducted at the Stanford Functional Genomics Facility (SFGF) core at Stanford University.

Bulk RNA Sequencing. Skeletal stem/progenitor cells, freshly isolated from the PF, SAG, COR, and posterior plate of pN3 CD-1 mice (n = 60) were double sorted and directly into TRIzol Reagent (Ambion-Life Technologies, Carlsbad, CA, USA) using the FACS Aria II. RNA extraction was done using the Qiagen MiRNeasy Kit (Cat#217084, Qiagen, Hilden, Germany). The quality of extracted RNA was evaluated using pico bio-analyzer chip on an Agilent bio-analyzer instrument. cDNA was synthesized and amplified using CloneTech Ultra low input RNA kit v4 (Cat # 634888, CloneTech, Mountain View, CA, USA) and fragmenting using Covaris. Quality of the fragmented cDNA was assessed using high-sensitivity chip on an Agilent bio-analyzer. Then were prepped using CloneTech Low Input Library Rep Kit v2 (Cat # 634899, CloneTech, Mountain View, CA, USA). Equal nanomoles of each uniquely indexed library were pooled and sequenced at an Illumina HiSeq 4000 (purchased from NIH funds under award number S10OD018220). Details of reagents used for bulk RNA-sequencing are listed in Supplementary Table 4.

Single-cell RNA sequencing (scRNA-seq). Freshly isolated skeletal stem/progenitor cells from the PF, SAG, COR and growth plate of pN3 CD-1 mice (n = 60) were double sorted and single cells sorted into 96-well plates with 4 µl per well of lysis buffer consisting of 4 units of Recombinant RNase inhibitor (RRI) (Cat # 231B3, CloneTech, Mountain View, CA, USA), 0.1% Triton X-100 (CAT# 85111, Thermo Fisher Scientific, Rockford, IL, USA), 2.5 mM dNTP (CAT# 10297018, Thermo Fisher Scientific, Rockford, IL, USA), 2.5 µl oligodT30VN (5′AGCA GTGGGTAACCAAGCAGTAC3′ VN-3′, Integrated DNA Technologies, Skokie, IL, USA). Once sorted, cells were immediately spun down and frozen at −80 °C. Single-cell RNAseq was performed via the Picelli method. Lysis buffer plates were thawed on ice, then heated at 72 °C for 3 min in a Biorad C1000 Touch thermal cycler. First strand cDNA synthesis was performed in a 10 µl reaction with 100 ng of total RNA, CloneTech’s Smartscribe reverse transcriptase (Cat# 639038, CloneTech, Mountain View, CA, USA), 10 Units RRI, 1× First Strand Buffer (CloneTech, Mountain View, CA, USA), 1× T4 DNA ligase (Invitrogen, Carlsbad, CA, USA), 1× E. coli competent cell solution (Invitrogen, Carlsbad, CA, USA), 25 µM dNTPs. Reactions were cleaned with SPRI beads on a Biomek FX and eluted in 1× lysis buffer. cDNA quality was assessed on a Bioanalyzer chip and cDNA pooling was performed using a Bioanalyzer chip. cDNA libraries were sequenced on an Illumina HiSeq 4000 (purchased from NIH funds under award number S10OD018220). Details of reagents used for bulk RNA-sequencing are listed in Supplementary Table 4.

Bulk RNA-sequencing data analysis. Analysis were performed on the paired-end fastq files using FastQC v0.11.5 prior to proceeding with the read mapping. Fastq reads were mapped to the mouse mm9 reference genome using STAR v2.5.1b in paired-end mode to yield BAM files sorted by coordinates. To estimate transcript abundances of gene features in terms of FPKMs, Cuffdiff was run on the various datasets from Debnath et al., 2018 (GSE106237) and Chan et al., Cell 2015 (GSE64447).

RT-PCR validation of selected genes identified from bulk RNA-Seq analysis. Total RNA was isolated from PF, SAG and COR derived skeletal stem/progenitor cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Upon DNase I (Ambion; Austin, TX, USA) treatment to clear genomic DNA, RNA was reverse transcribed using a SuperScript III First-Strand Kit (Invitrogen, Carlsbad, CA, USA) as previously described. Gene expression profile was analyzed by RT-PCR. The relative mRNA level in each sample was normalized to its Gapdh content. Values are provided as relative to Gapdh primers. Primers sequence and annealing temperature are described in Supplementary Table 5.

Ex vivo suture explants. Sagittal sutures from day pN3 CD-1 mice and coronal sutures from day pN3 Twist1−/− mice were explanted and placed into 24-millwell plates and cultured for eight days in DMEM GlutaMax-supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin Gibco-Life Technologies, Grand Island, NY, USA), and 10 µM SB431542 (Selleckchem.com, Houston, TX) where needed. After eight days in culture, explants were washed twice with PBS digested with StemPro Accutase cell dissociation reagent (Gibco-Life Technologies, Grand Island, NY, USA) for 30 min at 37 °C and prepared for FACS analysis as described above. For information regarding reagents used for suture explants refer to Supplementary Table 3 and Supplementary Table 4. Primers sequence and annealing temperature are described in Supplementary Table 5.

cWnt activation and inhibition. cWnt signaling activation and inhibition animal surgical procedures were conducted as previously described. A skin incision was performed above the PF or SAG suture of anesthetized C57 Cd-1, Axin2LacZ−/−, or Axin2−/− mouse with 2.5 µl of a dissociation buffer consisting of 0.5% collagenase and 0.05% trypsin in 1× PBS as previously described. On day pN8 or pN17 animals were re-soaked with the corresponding factors. On day pN8 or pN17 animals were sacrificed by CO2 asphyxiation. The 50 most highly ranked genes from this analysis for each gene. However, in order to better account for the mutual information contained within highly correlated predictors genes, we also employed a characteristic direction analysis. The 50 most highly ranked genes from this analysis for each cluster were used to perform gene set enrichment analysis in a programmatic fashion using EnrichR v2.167.

Generation of the Twist1−/− Axin2LacZ−/− double transgenic mouse and screen generation. B6Twist1−/− (Stock# 2221) mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and Axin2LacZ−/− mice on B6Cd-1 mixed background were obtained as previously described. Twist1−/− male mice were crossed with Axin2LacZ−/− female mice to generate Twist1−/− Axin2LacZ−/− double transgenic mice. For screen of bilateral or unilateral COR craniosynostosis in wild-type, Axin2−/−, Axin2LacZ−/− and Axin2LacZ−/− double transgenic mice, animals were sacrificed by CO2 asphyxiation on the exact post-natal date determined by DOB. Entire cranial was dissected and a small portion of the tail harvested for genotyping as described above. Prior to genotyping, entire cranial were screened with the aid of a dissection microscope to determine the presence or absence of any craniosynostosis. For screen of bilateral or unilateral COR craniosynostosis in wild-type, Axin2−/−, Axin2LacZ−/− and Axin2LacZ−/− double transgenic mice, animals were sacrificed by CO2 asphyxiation on the exact post-natal date determined by DOB. Entire cranial was dissected and a small portion of the tail harvested for genotyping as described above. Prior to genotyping, entire cranial were screened with the aid of a dissection microscope to determine the presence or absence of any craniosynostosis.
Coronal sutectomy. pN16-18 Twist+/− mice (n = 2) were anesthetized by 2% isoflurane, shaved and disinfected prior to a longitudinal skin incision along the midline. The Cranial pericranium was harvested, coronal suture excised using a trephine drill with a 0.3 mm drill bit under constant irrigation and with meticulous care to avoid damaging the underlying dura mater. Preoperative microcomputed tomography (μCT) scans were used as a guide to identify the synostosed coronal suture for excision. Coronal suturoctomies were treated with 1.5 mm diameter Helastat collagen sponge (Integra LifeSciences, Plainsboro, NJ, USA) seeded with 3 × 10^5 skeletal stem/progenitor cells (see cell preparation for coronal sutectomy) resuspended in 2 μl of recombinant Wnt3a protein at a concentration of 100 ng/μl in PBS. Untreated and Wnt3a alone treated sutures were used as controls. The sutures was closed using 8-0 nylon suture and the animals were allowed to recover. The extent of re-fusion of the excised coronal suture was assessed by μCT over a 14-week period and by histology 14-weeks postoperatively. For information regarding reagents used refer to Supplementary Table 4.

Microcomputed tomography (μCT) scanning and analysis. Animals were anesthetized by 2% isoflurane prior to scanning. All scans were performed using a Bruker SkyScan 1276 at a resolution of 35 μm. μCT reconstructions were performed with NRecon software (Bruker, Billerica, MA, USA), and 3D solid volume images produced using CTVol software (Bruker, Billerica, MA, USA). To assess the extent of healing, standardized region of interest was applied to the suturoctomy area in μCT reconstructions from 1 biological replicates per condition and quantified using the software program (Adobe) as previously described36. Bone tissue was based upon tissue densities via an automatic thresholding process in CTAx (Bruker, Billerica, MA, USA). Mean values were compared across POD 1, 2wk, 4wk, 6wk, 8wk, 10wk, 12wk, and 14wk time-points.

Cell culture. Skeletal Stem Cells were cultured in alpha-MEM GlutaMax supplemented with 10% fetal bovine 1% penicillin–streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), and 0.1% ciprofloxacin HCl (bioWORLD, Dublin, OH, USA). Cells were incubated under low O2 conditions (2% atmospheric oxygen, 7.5% CO2) for 48 h and then moved to standard conditions (5% CO2). For information regarding reagents used refer to Supplementary Table 3.

Wnt3a proliferation assay. The Click-iT EdU Imaging Kit (Invitrogen, Eugene, OR, USA) was used to assess the proliferation of skeletal stem/progenitor cells with Wnt3a treatment. Skeletal stem/progenitor cells from the three cranial sutures were isolated, pooled (as described above) and seeded at the density of 3 × 10^5 cells/well in a 24-well plate. Skeletal stem/progenitor cells were cultured in alpha-MEM GlutaMax supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), 0.1% ciprofloxacin HCl (bioWORLD, Dublin, OH, USA) and Wnt3a (50 ng/ml) (R&D Systems). Cells were incubated under low O2 conditions (2% atmospheric oxygen, 7.5% CO2) for 48 h and then moved to standard conditions (5% CO2). Cells were incubated in EdU (10 μM) for 1.5 h under standard conditions. The Click-iT EdU Imaging Kit was used per the manufacturers protocol. The "Analyze Particle" tool on ImageJ imaging software was used for quantification of proliferating (GFP+) cells. The number of GFP+ proliferating cells, as well as Dapi− nuclei was measured, and proliferation presented as a percentage of GFP+ cells/Dapi− cells. For information regarding reagents used refer to Supplementary Tables 3 and 4.

Cell preparation for coronal sutectomy. Skeletal stem/progenitor cells were isolated from entire cranial and tibia from pN3-5 wild type (C57BL/6) or Egfp+/− mice by flow cytometry. Skeletal stem/progenitor cells were pelleted at 1350 rpm at 4 °C for 20 min, resuspended in growth media (as described above) supplemented with 50 ng/ml Wnt3a protein (R&D Systems, Minneapolis, MN, USA) and seeded at the density of 3 × 10^5 cells/well in a 96-well plate. Cells were incubated under standard conditions (37 °C, 5% CO2) overnight (8–12 h). The following morning, cells were lifted using StemPro Accutase Cell Dissociation Reagent (Gibco-Life Technologies, Grand Island, NY, USA), pelleted at 1350 rpm for 20 min at 300 g using a tabletop centrifuge, and resuspended in 2 μl of recombinant Wnt3a protein at concentration of 100 ng/μl in PBS for transplantaion. For information regarding reagents used refer to Supplementary Tables 3 and 4.

Differentiation assays. Osteogenic and chondrogenic potential were evaluated using the Stem Pro osteogenesis or chondrogenesis differentiation kit (Gibco-Life Technologies, Grand Island, NY, USA). Freshly sorted skeletal stem/progenitor cells were seeded into a 96-well plate, pre-coated with 0.1% gelatin (EmbryoMax, Millipore, Billerica, MA, USA) and seeded with the density of 3 × 10^5 cells/well (pooled sutures and growth plate) or 1 × 10^5 cells/well (sutures individually) for osteogenic differentiation assays. For chondrogenic differentiation assays, 1 × 10^5 cells/well (whole skull and growth plate) or 1 × 10^5 cells/well (sutures individually) were seeded into a 96-well plate. "Pooled sutures" were comprised of equal number (1 × 10^5 cells) of skeletal stem/progenitor cells from the PF, SAG, and COR sutures. After reaching confluency growth media was replaced by Stem Pro osteogenic or chondrogenic media according to the manufactures protocol and media was changed every other day for 21 days (osteogenic) or 40 days (Chondrogenic). Osteogenic differentiation was assessed by alizarin red staining and chondrogenic by Alcian Blue staining as previously described36,39. For information regarding reagents used refer to Supplementary Table 3.

 Colony-forming units (CFUs) and self-renewal assay. Colonies were assessed as previously described36. 500 freshly sorted skeletal stem/progenitor cells from pN3, CD-1 mice PF, SAG, and COR sutures and the GP were seeded into a 10 cm² plate pre-coated with 0.1% gelatin (EmbryoMax, Millipore, Burlington, MA, USA) in alpha-MEM GlutaMax (supplemented with 10% fetal bovine serum 1% penicillin–streptomycin (Gibco-Life Technologies, Grand Island, NY, USA), and 0.1% ciprofloxacin HCl (bioWORLD, Dublin, OH, USA). Cells were incubated under low O2 conditions (2% atmospheric oxygen, 7.5% CO2) for two weeks. For further details see Supplementary Table 3. Colony formations of CFUs, colonies were seeded with 50 colonies or more and were counted. For evaluation for self-renewal ability, skeletal stem/progenitor cells were isolated by FACS and pooled from the PF, SAG, and COR sutures of pN3 CD-1 mice. 500 skeletal stem/progenitor cells were seeded into 10 cm² for two weeks as described above. After 2 weeks clones were lifted using Stem Pro Accutase (Gibco-Life Technologies, Grand Island, NY, USA) and skeletal stem/progenitor cells isolated by FACS and passaged onto 10 cm² plates as described above. For information regarding reagents used refer to Supplementary Table 3.

Immunofluorescent staining for activated β-catenin. Immunofluorescence was performed to evaluate activation of β-catenin signaling. Briefly, sagittal sections of COR sutures from wild-type, Twist1−/+ Axin2LacZ+/− and Twist1−/+Axin2LacZ−/− mice at day pN15 were allowed to equilibrate at room temp for 5 min followed by 2 × 5min washes in PBS and 20 min fixation in 4% PFA. After fixation, sections were washed for 3 × 5 min in PBST (PBS with 0.05% Tween-20) and blocked for 1 h at room temperature in 10% donkey serum in PBST. After blocking, sections were incubated overnight in a humid chamber at 4 °C with the primary antibody, Active-β-Catenin (EMD Millipore, Billerica, MA, USA, 05-665) at a 1:100 dilution with 1% donkey serum. Following incubation with the primary antibody, sections were washed for 5 × 5 min in PBST and incubated for 1 h in the secondary antibody, Goat anti-Mouse IgG (H + L), Alexa Fluor 488 (Thermo Fisher Scientific, Eugene, OR, USA, A-11001), at a 1:1000 dilution in 2% donkey serum. Following incubation with the secondary antibody, sections were washed 3 × 5min in PBST and 2 × 5 min in PBS. Sections were mounted with Fluoromount-G with DAPI and imaged using a LEICA TCS SPX X confocal microscope (n = 2). Experiments were performed 3 independent times. To determine the nuclear localization of β-Catenin, E26Localization39 plug-in for ImageJ was used to map the localization of β-Catenin (GFP) to nuclei (Dapi). Results are presented as a heatmap demonstrating cells with colocalization of β-Catenin to the nucleus. For information regarding reagents used refer to Supplementary Table 1.

Statistical analysis and reproducibility. Statistical significance was assigned for P ≤ 0.05 (P < 0.05 to ****P < 0.0001 representing a significant difference). Statistical analysis was performed using GraphPad Prism 8 (GraphPad) software package. No statistical method was used to determine sample size. Flow cytometry plots are representative of at least two individual experiments and one-way analysis of variance (ANOVA). All statistical calculations were performed using the Prism 8 (GraphPad) software package. No statistical method was used to determine sample size. Flow cytometry plots are representative of at least two individual experiments and up to ten. For all figures (n) indicate the number of animals used per each independent experiment, all experiments were performed at least three times unless otherwise indicated by the figure legend. For all figures data are presented as a percentage or absolute value. For all graphs data are presented as means or representative value with error bars representing ± the standard error.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Source data for the conclusions in this manuscript can be found in the figures. All source data plots for are available in the attached source data file and any other data can be requested from the corresponding authors. The mouse mm9 reference genome database was used for all RNA-seq experiments. Publicly available datasets were obtained from Gene Expression Omnibus under the following accession numbers; Debarth et al., Nature 2018 GSE106237 and Chan et al., Cell 2015 GSE64447. All RNA-seq and RNA-seq data generated from this study can be accessed from the Gene Expressions Omnibus (http://www.ncbi.nlm.nih.gov/geo/) using accession number GSE138882. Source data are provided with this paper.

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Author contributions

S.M. and N.Q. designed experiments. S.M., N.Q., A.S., S.S., B.B., C.K.F.C., R.C.R., M.J., and D.C.W. performed experiments. S.M. generated figure panels 1c, 2c, 4a, S1a, S2d, S9a, and S9b. M.J. performed the bioinformatics analysis; N.Q., S.M., R.T., M.J., D.C.W., and M.T.L. wrote the manuscript. N.Q. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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