

## DEVELOPMENTAL BIOLOGY

# GATA3 mediates nonclassical $\beta$ -catenin signaling in skeletal cell fate determination and ectopic chondrogenesis

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Skeletal precursors are mesenchymal in origin and can give rise to distinct sublineages. Their lineage commitment is modulated by various signaling pathways. The importance of Wnt signaling in skeletal lineage commitment has been implicated by the study of  $\beta$ -catenin-deficient mouse models. Ectopic chondrogenesis caused by the loss of  $\beta$ -catenin leads to a long-standing belief in canonical Wnt signaling that determines skeletal cell fate. As  $\beta$ -catenin has other functions, it remains unclear whether skeletogenic lineage commitment is solely orchestrated by canonical Wnt signaling. The study of the Wnt secretion regulator *Gpr177/Wntless* also raises concerns about current knowledge. Here, we show that skeletal cell fate is determined by  $\beta$ -catenin but independent of LEF/TCF transcription. Genomic and bioinformatic analyses further identify GATA3 as a mediator for the alternative signaling effects. GATA3 alone is sufficient to promote ectopic cartilage formation, demonstrating its essential role in mediating nonclassical  $\beta$ -catenin signaling in skeletogenic lineage specification.

## INTRODUCTION

Lineage specification is pertinent to the creation of an organism. In mammalian embryos, the first two distinct lineages to form are the outer trophoblast and the inner cell mass of the blastocyst (1). Subsequently, three germ layers are formed followed by the development of the fourth germ layer—the neural crest (2, 3). Their dynamic interactions via molecular signals in the form of proteins, RNAs, surface contacts, and mechanics modulate the commitment of each cell and its neighboring cells to form diverse lineages and specified cell types during organogenesis (4). Three distinct lineages—somites, lateral plate mesoderm, and cranial neural crest—give rise to the axial skeleton, limb skeleton, and craniofacial bone and cartilage, respectively (5). Studies of the origin of cells that generate these tissues have led to the isolation and characterization of skeletogenic/skeletal stem cells (6–14). Recent advancements in stem cell research further offer next-generation therapeutics for large craniofacial defects caused by various conditions, including trauma, infection, tumors, congenital disorders, and progressive deforming diseases (12, 15). Proper cell fate determination can further facilitate the efficacy of stem cell–based therapy.

Cell fate switching has been linked to the pathogenesis of human diseases. Activation of canonical Wnt signaling plays a crucial role in muscle stem cell conversion from a myogenic to a fibrogenic lineage in aging mice (16). Later evidence suggests that the Wnt/transforming

growth factor  $\beta$  (TGF $\beta$ )–mediated lineage conversion promotes muscle stem cells to acquire fibroblast phenotypes, leading to muscular dystrophy (17). Heterotopic ossification is another example of cell fate switching as a pathogenic cause (18). The transformation of primitive cells in mesenchymal origin into osteogenic cells results in bone formation within the soft connective tissue. Cell fate switching is most commonly triggered by traumatic injury—the acquired form (19). However, there is also a rare congenital form—fibrodysplasia ossificans progressiva (FOP) linked to the autosomal dominant mutation in bone morphogenetic protein (BMP) type I receptor *ACVRI* (20). Furthermore, the interplay of BMP and fibroblast growth factor (FGF) signaling is modulated by Wnt in stem cell–mediated intramembranous ossification during calvarial morphogenesis (21). Disrupting the balance of this signaling cross-talk can alter the stem cell from osteogenic to chondrogenic fate, leading to aberrant endochondral ossification and craniosynostosis (21).

The requirement of canonical Wnt signaling in skeletal lineage commitment is based on the disruption of  $\beta$ -catenin causing ectopic chondrogenesis in mice (22, 23). The mouse genetic study of *Lrp5* and *Lrp6* further supports the role of canonical Wnt signaling in the promotion of osteoblast fate (24). However, multiple functions of  $\beta$ -catenin and no cell fate alteration, detected by the loss of *Gpr177*/mouse *Wntless*, raise concerns about this theory (25). Therefore, we have created several mouse models to examine details of the skeletal cell fate decision mediated by  $\beta$ -catenin. Our findings provide evidence supporting that an alternative mechanism mediated by  $\beta$ -catenin independent of the transcriptional output of canonical Wnt signaling is necessary for inhibiting ectopic chondrogenesis. The whole genomics study further examines downstream effectors, leading to the identification of GATA3 as the key modulator associated with these alternative signaling effects of  $\beta$ -catenin. Functional analyses further demonstrate that the GATA3 transcription factor is sufficient to promote the commitment of skeletogenic mesenchyme to chondrocyte lineage. GATA3 mediates the downstream effects of  $\beta$ -catenin on switching the fate of skeletal precursor cells.

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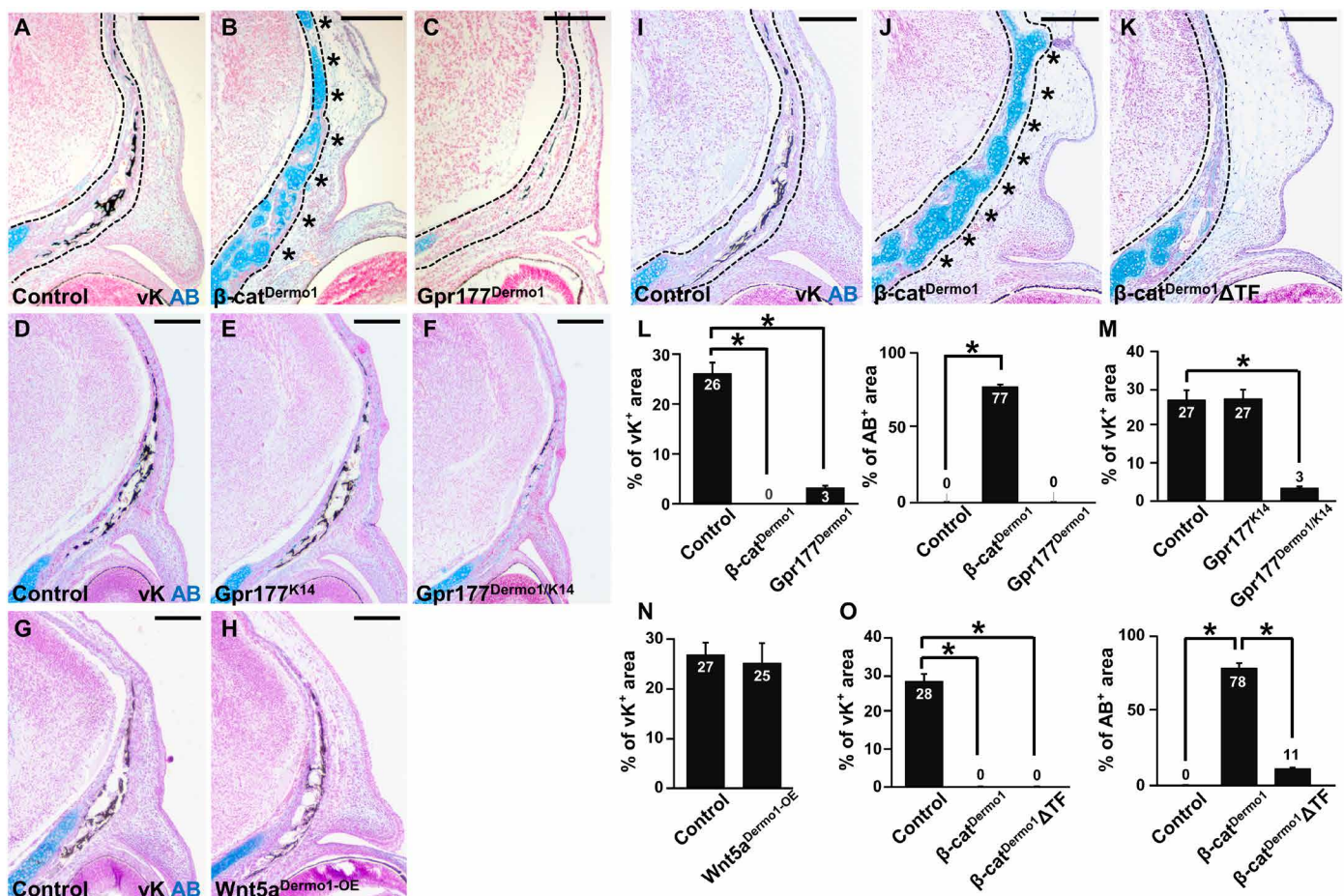
## RESULTS

**Wnt signaling mediated by  $\beta$ -catenin-dependent transcription is not associated with skeletal lineage specification**

Ectopic chondrogenesis caused by the loss of  $\beta$ -catenin (22, 23) prompted us to elucidate the mechanism underlying the skeletal cell fate switching. In the *Gpr177* (mouse ortholog of *Drosophila Wntless*)-deficient model, we found impaired Wnt secretion in the signal-producing mesenchymal cells causing defects in calvarial and skeletal bone formation, similar to the loss of  $\beta$ -catenin (22, 23, 25). In the skeletogenic mesenchyme, the loss of *Gpr177*-mediated Wnt secretion disrupted bone ossification but did not cause ectopic chondrogenesis, which is evident in the  $\beta$ -catenin-deficient calvaria (Fig. 1, A to C and L; \* $P < 0.001$ , two-sided Student's *t* test;  $n \geq 3$ ; means  $\pm$  SD). Although osteogenesis was similarly defective in both mutants, ectopic chondrogenesis only occurred in the  $\beta$ -cat<sup>Der<sup>mo</sup>1</sup> but

not *Gpr177*<sup>Der<sup>mo</sup>1</sup> mice when gene deletion is activated by *Der<sup>mo</sup>1-Cre* (also known as *Twist2-Cre*) in the mesenchyme (Fig. 1, A to C and L; \* $P < 0.001$ , two-sided Student's *t* test;  $n \geq 3$ ; means  $\pm$  SD). We hypothesized that this discrepancy may be attributed to (i) the presence of Wnt secreted by nearby epidermal tissue, (ii) the effect of noncanonical Wnt, or (iii) the alternative function, e.g., cell adhesion or lymphoid enhancer factor/T cell factor (LEF/TCF)-independent transcription of  $\beta$ -catenin.

First, epidermal secretion of Wnt may be able to maintain skeletal cell fate via paracrine signaling effects upon removal of mesenchymal Wnt in the *Gpr177*<sup>Der<sup>mo</sup>1</sup> mutant. To test this possibility, we examined whether epidermal Wnt contributes to the maintenance of skeletal cell fate by creating mice with epidermal loss of *Gpr177* (*Gpr177*<sup>K14</sup>;  $n = 6$ ). At embryonic day 15.5 (E15.5), the epidermal deletion of *Gpr177* to eliminate its supply of Wnt did not induce ectopic chondrogenesis (Fig. 1, D and E). Next, we generated mice



**Fig. 1. Examination of Wnt signaling and  $\beta$ -catenin-dependent transcription in skeletal cell fate determination.** Coronal sections of control (A and I; genotype:  $\beta$ -catenin<sup>Fx/Fx</sup> and *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>;  $\beta$ -catenin<sup>Fx/+</sup>; D: genotype: *Gpr177Fx/Fx*; G: genotype: *R26StopWnt5aFx/+*),  $\beta$ -cat<sup>Der<sup>mo</sup>1</sup> (B and J; genotype:  $\beta$ -catenin<sup>Fx/Fx</sup>; *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>), *Gpr177*<sup>Der<sup>mo</sup>1</sup> (C: genotype: *Gpr177Fx/Fx*; *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>), *Gpr177*<sup>K14</sup> (E: genotype: *Gpr177Fx/Fx*; *K14-Cre*<sup>+</sup>), *Gpr177*<sup>Der<sup>mo</sup>1/K14</sup> (F: genotype: *Gpr177Fx/Fx*; *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>; *K14-Cre*<sup>+</sup>), *Wnt5a*<sup>Der<sup>mo</sup>1-OE</sup> (H: genotype: *R26StopWnt5aFx/+*; *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>), and  $\beta$ -cat<sup>Der<sup>mo</sup>1</sup>  $\Delta$ TF (K: genotype:  $\beta$ -catenin<sup>dm/Fx</sup>; *Der<sup>mo</sup>1-Cre*<sup>+/-</sup>) skulls are examined by double labeling of von Kossa (vK) and alcian blue (AB) at E15.5 (A to K). All sections are counterstained by nuclear fast red. Note that bone ossification is stained by vK, and ectopic chondrogenesis is only detected in the  $\beta$ -cat<sup>Der<sup>mo</sup>1</sup> mutants (B and J). Asterisks indicate the site of skeletal stem cells switching from osteoblast to chondrocyte fate. Broken lines define the skeletogenic mesenchyme. Images are representatives of at least three independent experiments. (L to O) Graphs show quantitation of the average percentage of the positively stained area (mm<sup>2</sup>) over the calvarial mesenchymal area (mm<sup>2</sup>) from three mice per group and are presented as  $P < 0.001$ , two-sided Student's *t* test (means  $\pm$  SD). (N) No significant difference between the vK<sup>+</sup> area in control and *Wnt5a*<sup>Der<sup>mo</sup>1-OE</sup>. Scale bars, 200  $\mu$ m (A to K).

with the loss of Gpr177 in both skeletogenic mesenchymal cells and epidermal cells (Gpr177<sup>Dermo1/K14</sup>;  $n = 8$ ). These double mutants still did not exhibit ectopic chondrogenesis (Fig. 1F). Although bone ossification was disrupted, no ectopic chondrogenesis was detected in the Gpr177<sup>Dermo1/K14</sup> mutant calvaria (Fig. 1M and fig. S1A;  $*P < 0.001$ , two-sided Student's  $t$  test;  $n \geq 3$ ; means  $\pm$  SD). Immunostaining analysis supported the efficiency of Cre-mediated disruption of Gpr177 in these models (fig. S2A). We also did not detect ectopic chondrogenesis in the limb, although a clear delay in endochondral ossification was associated with mesenchymal deletion of Gpr177 (fig. S3, A and B). The results suggested that the cell fate switching was not caused by the loss of mesenchymal and epidermal Wnts.

Second,  $\beta$ -catenin deficiency affects canonical Wnt signaling, while the secretion of all Wnt requires Gpr177, whose disruption impairs canonical and noncanonical Wnts (26). The balance of these two pathways may be critical for skeletal lineage commitments. Noncanonical signaling is known to counterbalance the canonical signaling of Wnt (27). Therefore, another possibility is that the loss of  $\beta$ -catenin/canonical Wnt signaling led to an elevation of non-canonical Wnt signaling responsible for the alteration of skeletal cell fate. Therefore, we generated a mouse model with transgenic expression of Wnt5a in the skeletogenic mesenchyme (fig. S2B;  $n = 5$ ). However, overexpression of Wnt5a failed to detect any ectopic chondrogenesis in the calvaria and limbs (Fig. 1, G to H and N, and fig. S3C). These results suggested that the balance of canonical and noncanonical Wnt signaling may not be associated with skeletal cell fate switching.

Third, as  $\beta$ -catenin has additional functions, e.g., cell adhesion, skeletal lineage specification may be independent of canonical Wnt signaling. To rigorously examine the requirement of canonical Wnt signaling for skeletal fate determination, we created mice deficient for the transcriptional output of  $\beta$ -catenin in the endogenous locus. This mutant, containing one amino acid substitution in the first armadillo repeat (D164A) and deletion of the C terminus ( $\Delta$ C), affected the transcription function but not the cell adhesion function of  $\beta$ -catenin (28). Cell-cell interaction mediated by  $\beta$ -catenin remains intact in the  $\beta$ -catenin<sup>dm</sup> allele (29). Using this allele, we created  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutants in which only  $\beta$ -catenin-dependent transcription is deficient. Both  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF and  $\beta$ -cat<sup>Dermo1</sup> mutants exhibited bone ossification defects (Fig. 1, I to K and O;  $*P < 0.001$ , two-sided Student's  $t$  test;  $n \geq 3$ ; means  $\pm$  SD). However, ectopic chondrogenesis was not evident in the calvarial and mandible regions of  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF, highly reminiscent of the Gpr177-deficient mutant (Fig. 1, I to K and O, and fig. S1B;  $*P < 0.001$ , two-sided Student's  $t$  test;  $n \geq 3$ ; means  $\pm$  SD). Molecular characterizations with various markers were carried out to examine the cell types affected by the mutations. The ectopic chondrocytes expressing type 2 collagen (Col2) were present in the  $\beta$ -cat<sup>Dermo1</sup> but not in control and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mice (Fig. 2A). This skeletogenic region normally formed calvarial bones via intramembranous ossification with the presence of osteoblast cells positive for osterix (Osx), Col1, and osteocalcin (OC) in the E15.5 control (Fig. 2, B to D). The disruption of  $\beta$ -catenin-dependent transcription had no apparent effects on the Osx<sup>+</sup> progenitor but significantly impaired the differentiation of Col1<sup>+</sup> and OC<sup>+</sup> osteoblast cells similar to the  $\beta$ -cat<sup>Dermo1</sup> mutant (Fig. 2, C and D;  $*P < 0.001$ , two-sided Student's  $t$  test;  $n \geq 3$ ; means  $\pm$  SD). Our findings implied that the skeletal lineage commitment is orchestrated by  $\beta$ -catenin but independent of transcriptional activation.

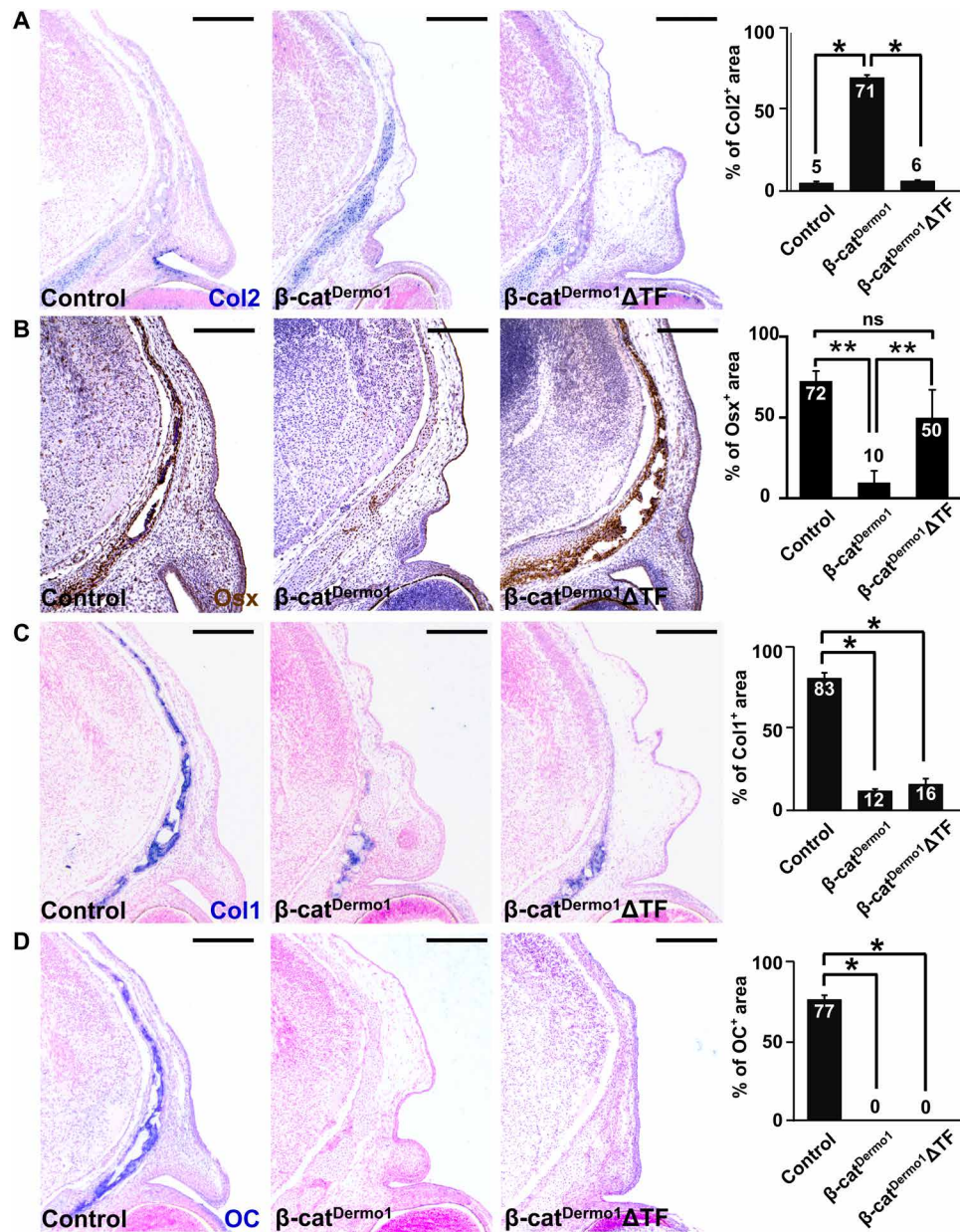
### Nonclassical $\beta$ -catenin signaling in skeletal cell fate determination

To determine the nature of mutation affecting the function in the  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mice, we performed immunostaining analyses. Using antibodies recognizing different domains of  $\beta$ -catenin, we showed that the N-terminal region remains intact in the skeletogenic mesenchyme of  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF (Fig. 3A). However, the C-terminal region is deleted in both mutants (Fig. 3B). The results demonstrated that the C-terminal transcriptional activation domain is abolished, while other regions critical for cell adhesion are not affected in the  $\beta$ -catenin<sup>dm</sup> allele. The mutation also impaired the activation of canonical Wnt targets whose expression is dependent on  $\beta$ -catenin-mediated transcription. Immunostaining of LEF1, TCF7, and DKK1 was reduced in both  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF skeletogenic mesenchyme (Fig. 3, D to F). The skeletogenic mesenchyme expresses OB-cadherin/cadherin-11 known to interact with  $\beta$ -catenin upon epithelial-mesenchymal transition to mediate cell adhesion and migration during bone metastasis (30, 31). The staining of OB-cadherin suggested that cell-cell interaction is disrupted in the  $\beta$ -cat<sup>Dermo1</sup> mutants but unaffected in the  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutants (Fig. 3C).

To test the alteration of skeletal cell fate, we examined their regenerative characteristics using transplantation assays. We previously showed the ability of a single SuSC (suture stem cells—skeletal stem cells within calvarial suture mesenchyme) to generate bone, thereby examining their stemness using in vivo clonal expansion analysis in the kidney capsule (12, 15). Therefore, we performed kidney capsule transplantation to examine the stem cell characteristics affected by the  $\beta$ -catenin mutations. Cells were isolated from the postnatal day 5 (P5)  $\beta$ -catenin<sup>Fx/Fx</sup> and  $\beta$ -catenin<sup>dm</sup>/<sup>Fx</sup> calvarial sutures. The  $\beta$ -catenin<sup>Fx/Fx</sup> and  $\beta$ -catenin<sup>dm</sup>/<sup>Fx</sup> suture cells were infected by lentivirus-Cre to generate  $\beta$ -cat-null and  $\beta$ -cat $\Delta$ TF cells, followed by immediately implanting into the kidney capsule (Fig. 4;  $n = 3$ , 100% transplantation success rate). At posttransplantation of 2 weeks, the implanted site was evaluated by histological and immunological staining to examine the stem cell-generated tissue structure and cell types. First, the efficacy of Cre-mediated deletion by lentiviral infection was analyzed by fluorescent images of the transplanted kidney in the whole mount and section (fig. S4, A to D). As expected, the expression of the red fluorescent protein (RFP) reporter associated with lentivirus-Cre was detected in the  $\beta$ -cat-null and  $\beta$ -cat $\Delta$ TF but not in control transplants (fig. S4, A and C). The  $\beta$ -catenin staining was positive in the control but lost in the  $\beta$ -cat-null and  $\beta$ -cat $\Delta$ TF (fig. S4D). Next, consistent with previous findings (12, 15), transplantation of  $5 \times 10^4$  control cells generated tissues resembling the calvarial bone (Fig. 4A) containing cells positive for Osx (Fig. 4B) but negative for alcian blue, and chondrocyte markers, Acan (aggrecan) and Col2 (Fig. 4, C to E). The implanted  $\beta$ -cat-null cells generated cartilages containing cells positive for alcian blue, Acan, and Col2, but not Osx (Fig. 4, A to E). However, the tissue generated by  $\beta$ -cat $\Delta$ TF cells was negative for Osx, Acan, Col2, or alcian blue (Fig. 4, A to E). The results from the transplantation assays supported mouse genetic studies in the calvaria, suggesting that  $\beta$ -catenin-dependent transcription is essential for osteogenesis. However, skeletal cell fate determination is independent of the transcriptional output of  $\beta$ -catenin.

### $\beta$ -Catenin-dependent transcription in endochondral ossification and palatogenesis

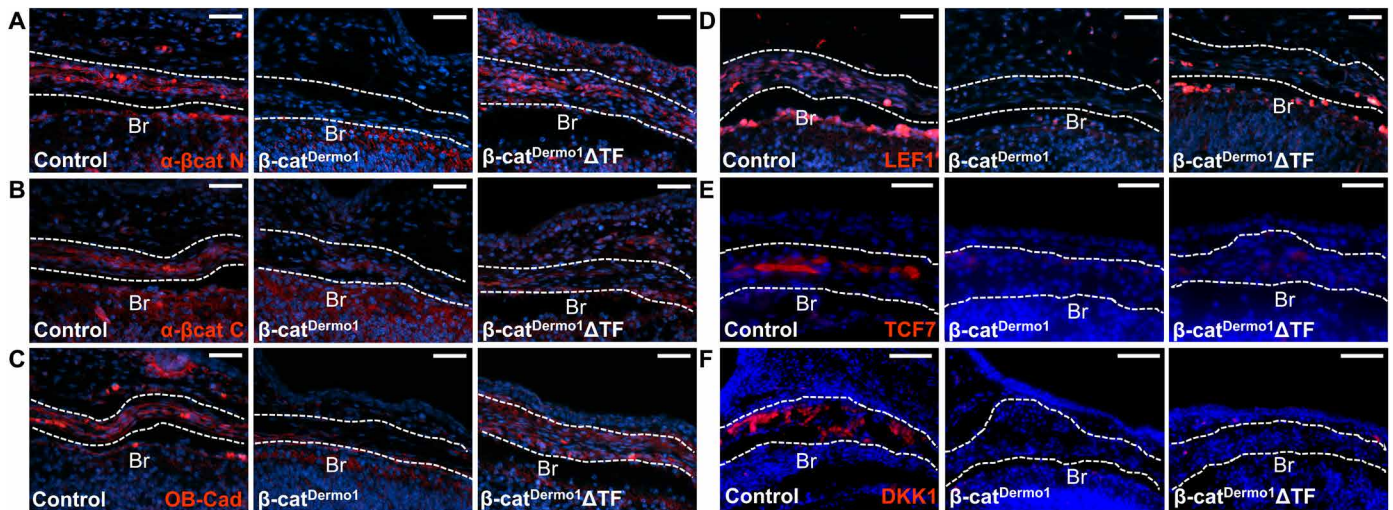
Canonical Wnt signaling mediated by  $\beta$ -catenin-dependent transcription is known to regulate the development of craniofacial and



**Fig. 2.  $\beta$ -Catenin-dependent transcription is essential for osteoblastogenesis but not skeletal lineage specification.** Coronal sections of E15.5 control (genotype:  $\beta\text{-catenin}^{\text{Fx/Fx}}$ ),  $\beta\text{-cat}^{\text{Dermo1}}$ , and  $\beta\text{-cat}^{\text{Dermo1}}\Delta\text{TF}$  are analyzed by in situ hybridization of Col2 (A), Col1 (C), and OC (D) and counterstained by nuclear fast red and immunostaining of osterix (Osx) and counterstained by hematoxylin (B). Graphs show quantitation of the average percentage of the positively stained area ( $\text{mm}^2$ ) over the calvarial mesenchymal area ( $\text{mm}^2$ ) from three mice per group ( $*P < 0.001$ ;  $**P < 0.02$ , two-sided Student's *t* test; means  $\pm$  SD). Images are representatives of three independent experiments. Scale bar, 200  $\mu\text{m}$  (A to D). ns, not significant.

body skeletons. To further dissect the requirement of  $\beta$ -catenin for endochondral ossification, we examined limb development at an early embryonic stage. In the humeri, alcian blue staining and expression of Col2 and Col10 indicated that chondrogenesis is severely delayed in both  $\beta\text{-cat}^{\text{Dermo1}}$  and  $\beta\text{-cat}^{\text{Dermo1}}\Delta\text{TF}$  mutants (fig. S5, A to C). The delay caused a cascade effect on subsequent osteoblast differentiation as evidenced by the staining of Osx and Col1 (fig. S5, D and E). No ectopic chondrogenesis was detectable in the  $\beta$ -catenin mutants. The results demonstrated that  $\beta$ -catenin-mediated transcription is

essential for chondrocyte maturation and subsequent ossification. The highly similar defects exhibited in these two mutants also suggested a complete disruption of canonical Wnt signaling in the  $\beta\text{-cat}^{\text{Dermo1}}\Delta\text{TF}$  limb. Similar conclusions are supported by the development of the cleft palate in both mutants (fig. S6). Consistent with previous reports (22, 23), the findings indicated canonical Wnt/ $\beta$ -catenin signaling essential for endochondral ossification and palatogenesis. Therefore, no cell fate switching detected in the calvaria of  $\beta\text{-cat}^{\text{Dermo1}}\Delta\text{TF}$  was not attributed to incomplete abrogation of Wnt/ $\beta$ -catenin signaling.



**Fig. 3. Disruption of transcription but not cell adhesion of  $\beta$ -catenin in the skeletogenic mesenchyme of  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF.** Coronal sections of the control (genotype:  $\beta$ -catenin<sup>Fx/Fx</sup>),  $\beta$ -cat<sup>Dermo1</sup>, and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF calvaria are examined by immunostaining using antibodies recognizing the N-terminal ( $\alpha$ - $\beta$ cat N; **A**) or C-terminal ( $\alpha$ - $\beta$ cat C; **B**) domain of  $\beta$ -catenin, OB-cadherin (OB-Cad; **C**), LEF1 (**D**), TCF7 (**E**), or DKK1 (**F**) at E15.5. Broken lines define the skeletogenic mesenchyme, and Br indicates the brain. Images are representatives of three independent experiments. Scale bars, 100  $\mu$ m (A to E) and 200  $\mu$ m (F).

### Gene expression profiling of $\beta$ -cat<sup>Dermo1</sup> and $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF

To further delineate the nonclassical signaling mechanism underlying skeletal lineage specification orchestrated by  $\beta$ -catenin-dependent but transcription-independent function, we performed RNA sequencing (RNA-seq) analysis comparing gene expression profiles of the E14.5 control,  $\beta$ -cat<sup>Dermo1</sup>, and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF calvarial mesenchymes. Our strategy was to reveal common differentially expressed genes (DEGs) affected by both  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutations compared to the control. Next, we identified unique DEGs specific to  $\beta$ -cat<sup>Dermo1</sup>, thereby differentiating nonclassical  $\beta$ -catenin effects on cell fate switching from the classical  $\beta$ -catenin signaling linked to osteoblastogenesis (Fig. 5A). The downstream effectors of nonclassical signaling were uncovered by the subtraction of the  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF from  $\beta$ -cat<sup>Dermo1</sup> DEGs to obtain a total of 431 DEGs potentially affected by the  $\beta$ -catenin-dependent but transcription-independent pathway (Fig. 5A).

To gain mechanistic insight from the identified 1093 common DEGs, we performed Gene Set Enrichment Analysis using MetaCore (32). The pathway analysis mapped 7 of the top 50 pathways related to Wnt signaling in tissue development and maintenance (fig. S7A). As  $\beta$ -catenin functions as a cofactor for TCF transcription factors to mediate canonical Wnt signaling, we next examined the effect of the mutations on the  $\beta$ -catenin/TCF-dependent downstream targets (33). Comparative gene expression analysis revealed that 20 genes known to be directly regulated by  $\beta$ -catenin-LEF/TCF-dependent transcription were substantially decreased in  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutants (fig. S7B), including TCF7 and DKK1, confirmed by immunostaining analysis (Fig. 3, E and F). The results showed a clear reduction of canonical Wnt signaling in the  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mesenchymes.

Next, the examination of osteoblast and chondrocyte markers showed that their expression coincides with skeletogenic abnormalities (Fig. 5, B and C). The expression of osteoblast genes was dramatically down-regulated in both  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutants (Fig. 5B). The canonical Wnt/ $\beta$ -catenin signaling regulates osteoblast differentiation at very early stages as evidenced by the alteration of

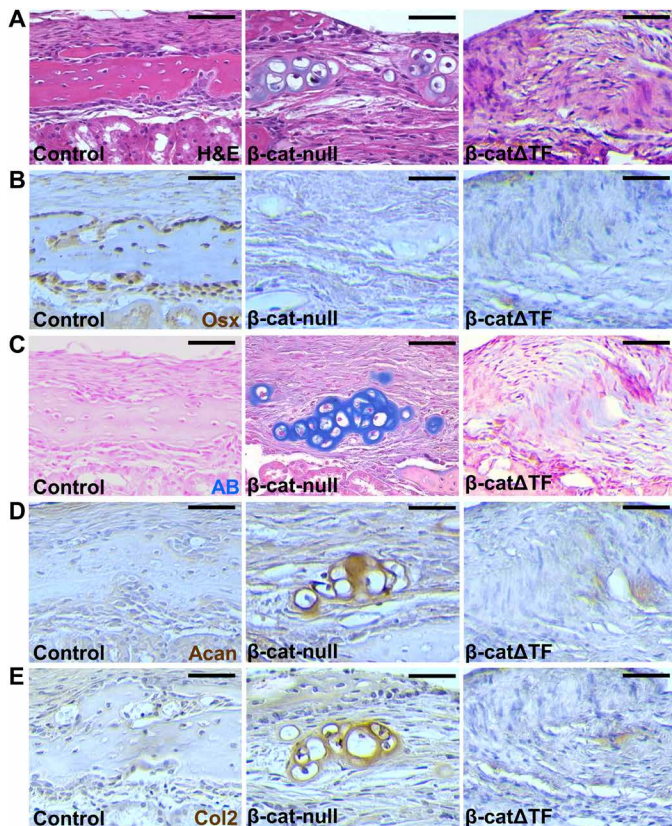
osteoprogenitor markers, Runx2 and Osx, and osteoblast markers, Col1a1, Ibsp (bone sialoprotein 2), OCN (osteocalcin) and OPN (osteopontin) (Fig. 5B). Consistent with the alteration of skeletal lineage commitment, the chondrocyte genes were strongly elevated only in the calvaria of  $\beta$ -cat<sup>Dermo1</sup> (Fig. 5C). The disruption of the canonical Wnt/ $\beta$ -catenin pathway did not alter skeletal cell fate. Our findings provide molecular profiling support for the skeletal characterizations and suggest the suitable use of these sequencing datasets to further decipher the regulatory process associated with nonclassical  $\beta$ -catenin (transcription-independent) signaling effects on cell fate switching.

### Identification of GATA3 associated with skeletal cell fate switching

To elucidate the mechanism underlying nonclassical  $\beta$ -catenin signaling, we first revealed its primary effect by upstream regulator assay using MetaCore Interactome analysis (34). By analyzing the downstream “effectors” (unique DEGs), our goal was to find upstream “regulators” with dynamic effects on stem cell fate switching (Fig. 6A). We were able to identify 11 key factors statistically over-connected within the downstream effectors (fig. S8). GATA3 was identified as the top candidate linked to 24 network objects in unique DEGs. GATA3 elevation was predicted to promote the dynamic changes of the downstream effectors that lead to the alteration of skeletal lineage commitment (Fig. 6B). This prediction was further validated by an immunostaining study showing an increased expression of GATA3 in the  $\beta$ -cat<sup>Dermo1</sup> but not  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF skeletogenic mesenchyme (Fig. 6C), as well as in the ectopic chondrocytes (Fig. 6D). The results suggest the link of GATA3 elevation to nonclassical  $\beta$ -catenin signaling in the cell fate alteration.

### GATA3 is sufficient to alter skeletal lineage commitment

To determine the role of GATA3 in skeletogenic lineage specification, we performed functional studies in the cells and mice. First, C3H10T1/2 mesenchymal cells were infected with lentivirus expressing green fluorescent protein (GFP) (control) or GATA3



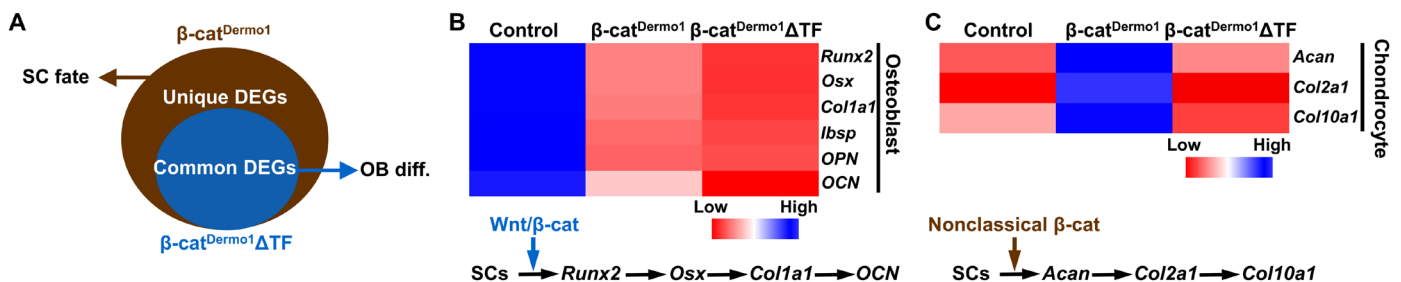
**Fig. 4. Assessment of skeletal lineage commitment by stem cell transplantation.** Kidney capsule transplantation of approximately  $5 \times 10^4$  control ( $\beta$ -catenin $Fx/Fx$ ),  $\beta$ -cat-null ( $\beta$ -catenin $Fx/Fx$  plus lentivirus-Cre), and  $\beta$ -cat $\Delta TF$  ( $\beta$ -catenin $^{dm}/Fx$  plus lentivirus-Cre) cells, isolated from the P5 calvarial suture mesenchyme, examines the stem cell property. The transplants are examined 2 weeks after transplantation. Ectopic bone generated by the transplanted stem cells is assessed by hematoxylin and eosin (H&E) staining (A) and immunostaining of Osx (B). The presence of chondrocytes is determined by AB staining (C) and immunostaining of Acan (D) and Col2 (E). Note that  $\beta$ -cat-null stem cells alter their fate and develop into chondrocytes instead of osteoblasts. No detection of osteoblasts or chondrocytes in the  $\beta$ -cat $\Delta TF$  transplant. Images are representative of three independent experiments (100% transplantation success rate). Scale bars, 100  $\mu$ m (A to E).

(Gata3<sup>OE</sup>), followed by in vitro differentiation into chondrocytes. Alcian blue staining revealed that the average of positively stained areas in the Gata3<sup>OE</sup> culture is two times more than the control (Fig. 7A). Next, the GATA3<sup>Dermo1-OE</sup> model was developed by crossing mice homozygous for the *R26StopGATA3* allele with *Dermo1-Cre* mice for mesenchymal expression of GATA3 (fig. S9A). The transgenic expression of GATA3 was detected in craniofacial skeletal mesenchyme (fig. S9B).

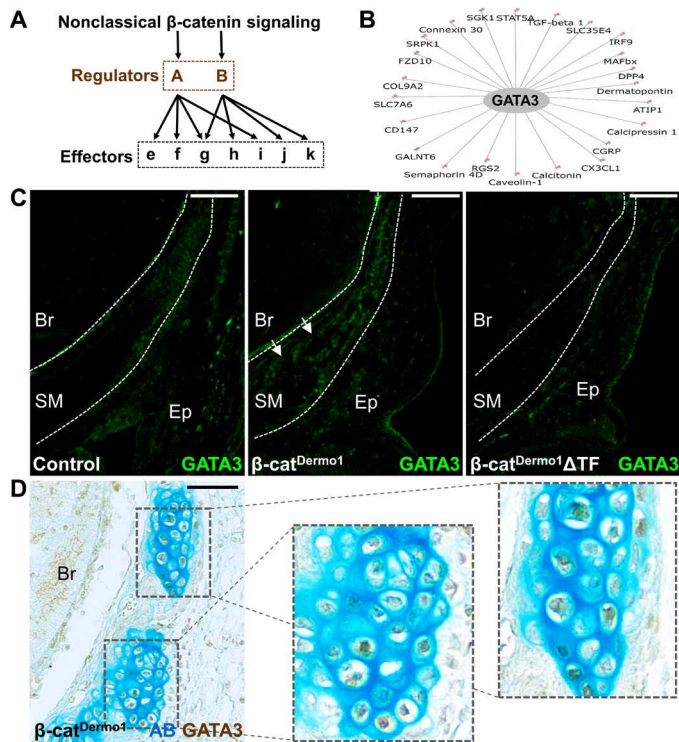
In GATA3<sup>Dermo1-OE</sup> mice, we detected ectopic cartilages in various regions of the skull (Fig. 7, B and C;  $n \geq 3$ , 100% penetrance). In the calvaria suture mesenchyme, several areas displayed severe abnormalities in chondrogenesis (Fig. 8, A to C, and fig. S10, A and B; E15.5 to 18.5,  $n = 24$ , 100% penetrance). Because calvarial bone formation was mediated by intramembranous ossification, only Osx<sup>+</sup> cells were present in the control mesenchyme (Fig. 8, A and B). In the GATA3<sup>Dermo1-OE</sup> mice, we identified cells expressing Acan, indicating a switch of osteogenic to chondrogenic fate in the mesenchymal regions (Fig. 8, A and B, and fig. S10, C and D). The anterior skull, containing nasal cartilage and bones, also exhibited a drastic expansion of the nasal cartilage in the Gata3<sup>Dermo1-OE</sup> mutant (Fig. 8C). These results provided definitive proof of GATA3 in skeletal cell fate switching. Next, we perform a functional test to determine the role of GATA3 in mediating nonclassical  $\beta$ -catenin signaling. The ex vivo culture of primary cells isolated from control and  $\beta$ -cat<sup>Dermo1</sup> showed that the loss of  $\beta$ -catenin enhances chondrogenesis (Fig. 9, A and B). However, the enhanced chondrogenesis of  $\beta$ -cat<sup>Dermo1</sup> was significantly alleviated by lentivirus-mediated knockdown of GATA3 (Fig. 9, A and B; means  $\pm$  SD;  $n = 3$  animals;  $P < 0.01$ , Student's *t* test). The quantitative reverse transcription polymerase chain reaction (RT-PCR) further revealed the elevated expression of chondrogenic markers significantly alleviated by the reduction of GATA3 (Fig. 9C; means  $\pm$  SD;  $n = 3$  animals;  $P < 0.01$ , Student's *t* test). The results thus suggested the dependence of GATA3 on the  $\beta$ -catenin-mediated commitment of skeletogenic lineage.

**DISCUSSION**

This study provides evidence that  $\beta$ -catenin signaling independent of its transcriptional function specifies skeletal cell fate. The loss of  $\beta$ -catenin-dependent transcription does not alter skeletal lineage commitment, arguing against the previous knowledge where canonical



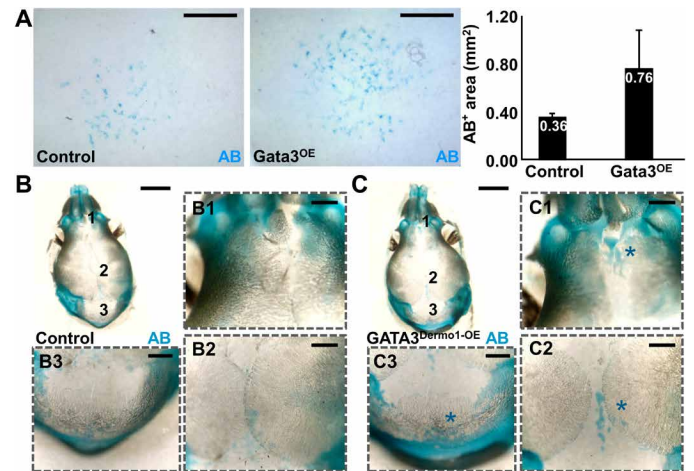
**Fig. 5. Gene expression profiling of classical and nonclassical  $\beta$ -catenin signaling.** (A) Diagrams illustrate our strategy to identify differentially expressed genes (DEGs) for skeletal fate determination and OB differentiation linked to nonclassical signaling effects of  $\beta$ -catenin (unique DEGs) and canonical Wnt/ $\beta$ -catenin signaling (common DEGs), respectively. SC, skeletal cell; OB, osteoblast. Heatmap showing the expression of osteoblast (B) and chondrocyte (C) genes associated with the  $\beta$ -catenin mutations identified by gene expression profiling of the calvarial mesenchyme using RNA-seq analysis. Acan, aggrecan; Col1a1, collagen type I  $\alpha$ 1; Col2a1, collagen type II  $\alpha$ 1; Col10a1, collagen type X  $\alpha$ 1; lbsp, bone sialoprotein 2; OPN, osteopontin; OCN, osteocalcin; Osx, osterix; Runx2, runt-related transcription factor 2.



**Fig. 6. Identification of GATA3 mediating the effect of nonclassical  $\beta$ -catenin signaling.** (A) Diagrams illustrate our strategy to identify upstream regulators in the alternative pathway mediating the primary effects based on the statistical overconnection in the DEGs. (B) GATA3 is the top candidate identified by an Interactome/Upstream Regulator analysis to mediate the effect of  $\beta$ -catenin but independent of canonical Wnt signaling using MetaCore. (C) Immunostaining of the E18.5 control (genotype:  $\beta$ -catenin $Fx/Fx$ ),  $\beta$ -cat $^{Dermo1}$ , and  $\beta$ -cat $^{Dermo1}\Delta TF$  calvarial sections using anti-Gata3 antibodies. Broken lines define the skeletogenic mesenchymal region. (D) Double-labeling indicates GATA3 expression in AB-positive chondrocytes. Arrows and insets show ectopic cartilages. Br, brain; Ep, epidermis; SM, skeletogenic mesenchyme. Scale bars, 200  $\mu$ m (C) and 100  $\mu$ m (D).

Wnt signaling is required for skeletal fate determination. On the basis of our genetic studies, we propose that nonclassical signaling mediated by  $\beta$ -catenin is essential for skeletal lineage commitment. Gene expression profiling and bioinformatics analyses further identify GATA3 to mediate the nonclassical signaling effect of  $\beta$ -catenin on skeletal cell fate determination. The importance of GATA3 in chondrogenic fate is further demonstrated by clear evidence from in vitro cell differentiation and in vivo transgenic animal studies. The programming of skeletal precursors is switched from an osteogenic to chondrogenic fate by the expression of GATA3 alone, suggesting that it acts as a master regulator in skeletal lineage commitment.

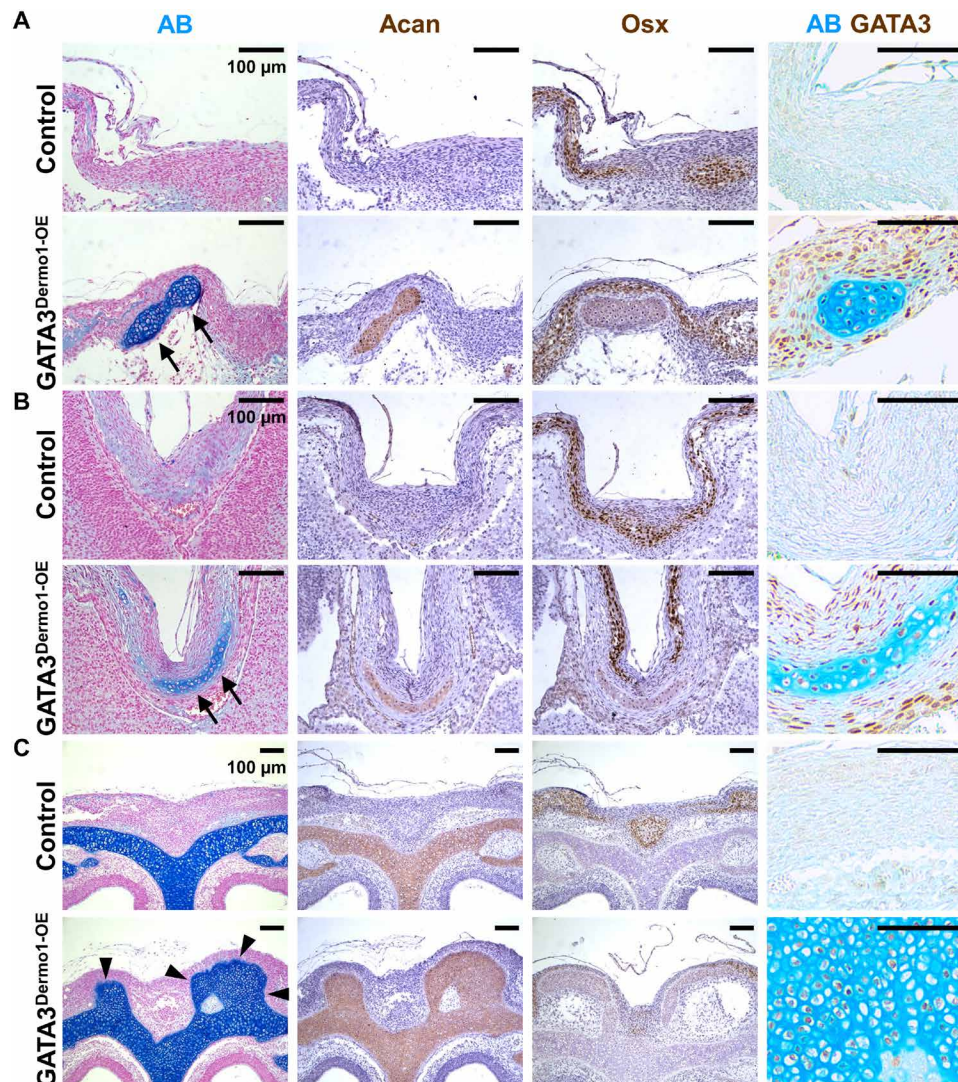
The  $\beta$ -catenin-dependent nonclassical effects may include those of cell-cell interaction and LEF/TCF-independent transcription. The  $\beta$ -cat  $\Delta TF$  mutant protein can associate with E-cadherin at the adherens junction and remains detectable in the nucleus upon Wnt stimulation, suggesting that the mutation does not affect the subcellular distribution of  $\beta$ -catenin (35). The loss of *Lrp5* and *Lrp6* in mice develops extra cartilage elements (24) that seem to favor the involvement of  $\beta$ -catenin-mediated transcription independent of LEF/TCF over the cell adhesion function. Wnt signals mediated through  $\beta$ -catenin may generate transcriptional outputs distinct



**Fig. 7. Chondrogenic lineage specification and chondrogenesis mediated by GATA3.** (A) In vitro culture of C3H10T1/2 cells is infected by lentiviruses expressing green fluorescent protein (GFP) (control) or mouse Gata3 ( $Gata3^{OE}$ ) in the chondrogenic condition, followed by AB staining and quantification of the AB-positive area (means  $\pm$  SD,  $n = 3$ ,  $P < 0.05$ ). (B and C) Whole-mount AB staining of E18.5 control (B; genotype:  $R26StopGATA3Fx^{+}$ ) and  $GATA3^{Dermo1-OE}$  (C; genotype:  $R26StopGATA3Fx^{+}$ ;  $Dermo1-Cre^{+}$ ) skulls reveals aberrant chondrogenesis caused by overexpression of GATA3 in skeletogenic mesenchyme. Asterisks indicate sites of aberrant chondrogenesis. Images are representative of at least three independent experiments. Scale bars, 200  $\mu$ m (A), 2 mm (B and C), and 500  $\mu$ m (B1 to B3 and C1 to C3).

from the LEF/TCF of canonical signaling (33). The interaction of  $\beta$ -catenin with other transcription factors, e.g., FOXO, hypoxia-inducible factor (HIF), and SOX17, via the armadillo repeats suggests such alternative downstream effects (36–38).  $\beta$ -Catenin, including  $\beta$ -cat  $\Delta TF$  mutant, may be a repressor directly or indirectly affecting GATA3. An interesting question is whether the  $\beta$ -catenin–GATA3 regulatory axis is modulated by Wnt. Mice with *Wnt9a* deficiency exhibit abnormal cartilage formation in the skull similar to those caused by overexpression of GATA3 (39). *Wnt9a* may exert nonclassical signaling effects through modulation of the  $\beta$ -catenin–GATA3 regulatory axis. However, it remains possible that the cell fate determination requires  $\beta$ -catenin-mediated cell adhesion in the craniofacial mesenchyme. Although the mechanism underlying nonclassical  $\beta$ -catenin signaling remains elusive, the role of GATA3 in skeletal cell fate determination is clear.

GATA3 belongs to the GATA family of transcription factors containing the zinc finger motif that recognizes G-A-T-A nucleotide sequences to activate or repress target genes (40). Germline deletion of *Gata3* in mice causes embryonic lethality and exhibits a variety of phenotypes in several tissues, including craniofacial bone and cartilage (41, 42). In humans, the haploinsufficiency of *GATA3* has been linked to HDR syndrome (hypoparathyroidism sensorineural deafness and renal disease) and craniofacial defects (43). Genome-wide association study has further revealed susceptibility loci for craniofacial microsomia, leading to the identification of mutations in *GATA3* (44). GATA3 is also known to regulate various stages of hematopoiesis both in the adult and during development (45). It has multiple functions with a role in developing the first hematopoietic stem cells in the embryo. Our findings support GATA3 in the control of key lineage-specific factors to drive the cell fate decision and tissue morphogenesis. This is likely attributed to GATA proteins belonging to a subclass of pioneer transcription factors capable of



**Fig. 8. GATA3 alters the commitment of skeletogenic mesenchyme in calvarial morphogenesis.** (A to C) Coronal sections of E18.5 control (genotype:  $R26^{Stop}GATA3^{Fx/+}$ ) and  $GATA3^{Dermo1-OE}$  (genotype:  $R26^{Stop}GATA3^{Fx/+}; Dermo1-Cre^{+/+}$ ) skulls are analyzed by AB staining, immunostaining of Acan and Osx, and double labeling of AB and GATA3. Arrows and arrowheads indicate ectopic chondrogenesis in the calvarial sutures and drastic expansion of nasal cartilage, respectively. Images are representative of three independent experiments.

promoting chromatin opening and recruitment of additional transcriptional regulators (46). In addition to their pioneer activity, GATA factors also have three-dimensional chromatin reorganization ability (40). Several GATA members act as primary regulators of various lineage decisions and cell fate determinations (40). Further deciphering its orchestral influence at the chromatin level promises important insights into the action of GATA3 as a master chondrogenic regulator.

## MATERIALS AND METHODS

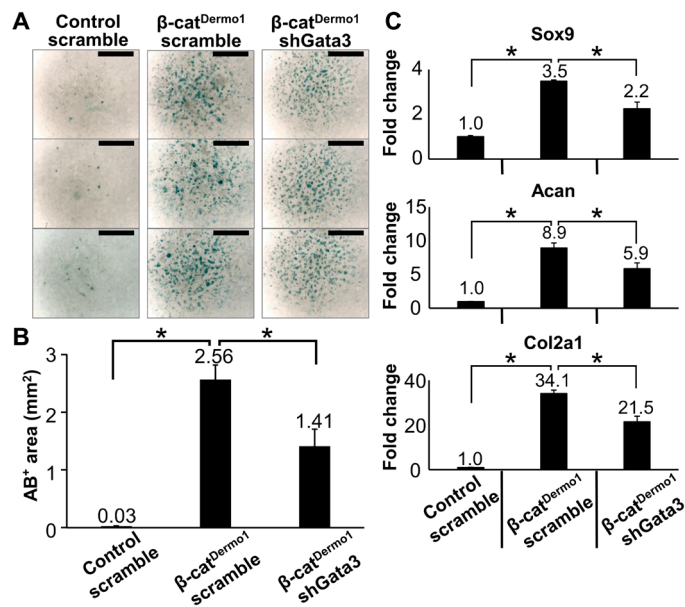
All experiments were performed according to the guidelines and Institutional Animal Use and Care Committees (IACUCs) of Forsyth Institute and the University of Rochester. This study is compliant with the ARRIVE (Animal Research Reporting of In Vivo Experiments) guidelines. Materials are freely distributed upon request to

the qualified academic investigator for noncommercial research, and mouse strains are available according to the National Institutes of Health (NIH) Grant Policy on Sharing of Model Organisms for Biomedical Research.

## Study design

This study was designed to elucidate the mechanism underlying skeletal cell fate determination. Using several mouse models with cell type-specific disruption of *Gpr177* and  $\beta$ -catenin and activation of *Wnt5a*, we found that Wnt signaling mediated by  $\beta$ -catenin-dependent transcription is not associated with skeletal lineage specification. The ectopic chondrogenesis caused by  $\beta$ -catenin deficiency is independent of its transcriptional output. Transplantation further revealed critical alteration in stem cell characteristics associated with the generation of cartilage and bone, suggesting that nonclassical  $\beta$ -catenin signaling is essential for skeletal cell fate determination.





**Fig. 9. GATA3 in mediating nonclassical  $\beta$ -catenin signaling for skeletal cell fate determination.** (A) Micromass culture of primary cells isolated from control (genotype:  $\beta$ -catenin<sup>Fx/Fx</sup>) and  $\beta$ -cat<sup>Dermo1</sup> infected by the lentivirus scramble or shGata3 (short hairpin RNA-mediated knockdown of Gata3) in the chondrogenic condition, followed by AB staining 7 days after culture. (B) Graphs show quantification of the AB-positive area (means  $\pm$  SD;  $n = 3$  animals;  $P < 0.01$ , two-sided Student's  $t$  test). (C) Quantitative reverse transcription polymerase chain reaction (RT-PCR) examines Sox9, Acan, and Col2a1 expression modulated by  $\beta$ -catenin and Gata3 (means  $\pm$  SD;  $n = 3$  animals;  $P < 0.01$ , two-sided Student's  $t$  test). Scale bars, 2 mm (A).

Using an unbiased genomic approach, we characterized gene expression profiling linked to the nonclassical effects of  $\beta$ -catenin and identified GATA3 associated with skeletal cell fate switching. A mouse model was generated to demonstrate that GATA3 is sufficient to alter skeletal lineage commitment. Micromass culture further indicated the dependence of GATA3 on promoting chondrogenesis caused by the loss of  $\beta$ -catenin. For scientific reproducibility, all studies were performed and repeated with proper controls, including mouse embryos carrying appropriate transgene(s). At least three independent experiments were performed for each study. No randomization, statistical method to predetermine the sample size, and inclusion/exclusion criteria defining criteria for samples were used.

### Animals and models

The *Twist2tm1.1(cre)Dor (Dermo1-Cre)*, *Tg(KRT14-cre)1Amc (K14-Cre)*, *Gpr177F<sub>x</sub>*, *R26StopWnt5a*, *R26StopGATA3*, *Ctrnb1tm2Kem* ( $\beta$ -catenin<sup>Fx</sup>),  $\beta$ -catenin<sup>dm</sup>, and *Prkdcscid (SCID)* mouse strains; the generation of *Gpr177<sup>Dermo1</sup>* and *Gpr177<sup>K14</sup>* models; and genotyping methods were reported previously (25, 26, 29, 47–54). The *Dermo1-Cre* mice were crossed with  $\beta$ -catenin<sup>Fx</sup> and  $\beta$ -catenin<sup>dm</sup> mice to create  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF models, respectively. To generate the *Gpr177<sup>Dermo1/K14</sup>* model, *K14-Cre* and *Dermo1-Cre* transgenes were bred into the *Gpr177F<sub>x</sub>/F<sub>x</sub>* background. The *Wnt5a<sup>Dermo1-OE</sup>* and *GATA3<sup>Dermo1-OE</sup>* models were generated by crossing mice heterozygous for the *Dermo1-Cre* allele with mice homozygous for the *R26StopWnt5a* or *R26StopGATA3* allele. Both male and female mice were used in this study. Care and use of experimental animals described in this work comply with the guidelines and policies of

IACUC at the Forsyth Institute and the University Committee on Animal Resources at the University of Rochester.

### Cell isolation and transplantation

Primary suture mesenchymal cells containing SuSCs were isolated from mouse calvaria as described (12, 15, 55). Briefly, an approximately 1.5-mm-wide tissue containing sagittal suture at P5 and its adjacent parietal bones were dissected, followed by separation of the parietal bone parts. Next, the suture parts were incubated with 0.2% collagenase in phosphate-buffered saline at 37°C for 1.5 hours. The dissociated cells were filtered and then resuspended in Dulbecco's Modified Eagle Medium for transplantation analysis. The transplantation of freshly isolated cells of control,  $\beta$ -cat<sup>Dermo1</sup>, and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF into the kidney capsule was performed as described (12, 15). The isolation of primary cells from the calvarial bone was performed as described (56). The isolated calvarial cells were cultured in the  $\alpha$  minimum essential medium containing 10% fetal bovine serum. The addition of ascorbic acid (50  $\mu$ g/ml) and 4 mM  $\beta$ -glycerophosphate promotes the differentiation of  $2.5 \times 10^5$  calvarial cells seeded in 24-well plates with infection of control or Gata3-expressing lentiviruses at multiplicity of infection (MOI) = 1, followed by alcian blue staining in 3 weeks (21, 57). For alcian blue staining, cells were fixed in a solution containing 30% ethanol, 0.4% paraformaldehyde, and 4% acetic acid for 15 min at room temperature, followed by incubation with 0.05% alcian blue staining solution in 75% ethanol:0.1 M hydrochloride (4:1) overnight at 37°C.

### Histology and staining

Sample preparation, fixation, and embedding for paraffin sections and histological analysis were performed as described (58, 59). Samples were subjected to hematoxylin and eosin staining for histology, alcian blue staining, von Kossa staining, or immunological staining with avidin:biotinylated enzyme complex (15, 60). The in situ hybridization analyses were performed as described (60). In brief, DNA plasmids containing *Col2a1*, *Col10a1*, *Col1a1*, and *OC* cDNAs were linearized for in vitro transcription using T3 or T7 RNA polymerase (Promega, Wisconsin, WI, USA) to generate digoxigenin-labeled RNA probes for in situ hybridization. Sections were then incubated with the RNA probes, followed by recognition with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN, USA). To visualize the bound signals, samples were incubated with BM-purple (Roche) for 4 to 5 hours. The immunological staining was visualized by enzymatic color reaction or fluorescence according to the manufacturer's specification (Vector Laboratories, Burlingame, CA). The sections underwent an antigen retrieval process by incubating with antigen unmasking solution (H3300, Vector Laboratories) in pressured cooking for 10 min. Mouse monoclonal antibodies  $\alpha$ - $\beta$ -catenin N (1:200; ALX-804-060, Enzo Life Sciences, Exeter, UK),  $\alpha$ - $\beta$ -catenin C (1:200; 610153, BD Transduction Laboratories, Franklin Lakes, NJ, USA), Acan (1:100; MABT84, Merck Millipore, Burlington, MA), Col2a1 (1:100; MA5-13026, Invitrogen, Waltham, MA), and OB-cadherin (1:200; 32-1700, Invitrogen); rabbit polyclonal antibodies Osx (1:500; ab22552, Abcam, Cambridge, MA, USA) and Lef1 (1:200; bs-1843R, Bioss, Beijing, China); and rabbit monoclonal antibody Gata3 (1:250; ab199428, Abcam) were used for immunological staining. Images were taken using a Zeiss Axio Observer microscope (Carl Zeiss, Thornwood, NY), Leica DM2500 microscope with a DFC7000T digital imaging system (Leica Biosystems Inc., Buffalo

Grove, IL), or a Nikon Eclipse Ts2 microscope with an Insight complementary metal-oxide semiconductor (CMOS) digital camera (Nikon, Melville, NY).

### RNA-seq and statistics

The calvarial mesenchymes isolated from E14.5 control,  $\beta$ -cat<sup>Dermo1</sup>, and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mice were subjected to RNA-seq analysis for the comparison of gene expression profiles using the ION Torrent PGM sequencer. The DEGs in the  $\beta$ -cat<sup>Dermo1</sup> and  $\beta$ -cat<sup>Dermo1</sup> $\Delta$ TF mutant mesenchymes were identified with the R package edgeR. The criteria to select DEGs were >2-fold changes and average gene counts > 10. The Interactome/Upstream Regulator analysis assay was performed using MetaCore software (34). R software version 3.2.1 or Microsoft Excel 2010 was used for statistical analysis. The significance was determined by two-sided Student's *t* tests. A *P* value of less than 0.05 was considered statistically significant. Before performing the *t* tests, the normality of the data distribution was first validated by the Shapiro-Wilk normality test. To quantify the AB<sup>+</sup> area, the RGB image was split into single channels. The resulting grayscale image with the red channel was used for quantification. The positive area is selected by threshold 0 to 62.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.adv6172>

[View/request a protocol for this paper from Bio-protocol.](#)

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