

# Wnt/ $\beta$ -catenin signaling is differentially regulated by $G\alpha$ proteins and contributes to fibrous dysplasia

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**Skeletal dysplasias are common disabling disorders characterized by aberrant growth of bone and cartilage leading to abnormal skeletal structures and functions, often attributable to defects in skeletal progenitor cells. The underlying molecular and cellular mechanisms of most skeletal dysplasias remain elusive. Although the Wnt/ $\beta$ -catenin signaling pathway is required for skeletal progenitor cells to differentiate along the osteoblastic lineage, inappropriately elevated levels of signaling can also inhibit bone formation by suppressing osteoblast maturation. Here, we investigate interactions of the four major  $G\alpha$  protein families ( $G\alpha_s$ ,  $G\alpha_{i/o}$ ,  $G\alpha_{q/11}$ , and  $G\alpha_{12/13}$ ) with the Wnt/ $\beta$ -catenin signaling pathway and identify a causative role of Wnt/ $\beta$ -catenin signaling in fibrous dysplasia (FD) of bone, a disease that exhibits abnormal differentiation of skeletal progenitor cells. The activating  $G\alpha_s$  mutations that cause FD potentiated Wnt/ $\beta$ -catenin signaling, and removal of  $G\alpha_s$  led to reduced Wnt/ $\beta$ -catenin signaling and decreased bone formation. We further show that activation of Wnt/ $\beta$ -catenin signaling in osteoblast progenitors results in an FD-like phenotype and reduction of  $\beta$ -catenin levels rescued differentiation defects of FD patient-derived stromal cells.  $G\alpha$  proteins may act at the level of  $\beta$ -catenin destruction complex assembly by binding Axin. Our results indicate that activated  $G\alpha$  proteins differentially regulate Wnt/ $\beta$ -catenin signaling but, importantly, are not required core components of Wnt/ $\beta$ -catenin signaling. Our data suggest that activated  $G\alpha$  proteins are playing physiologically significant roles during both skeletal development and disease by modulating Wnt/ $\beta$ -catenin signaling strength.**

**F**ibrous dysplasia (FD) of bone [Online Mendelian Inheritance in Man (OMIM) 174800] is a skeletal dysplasia caused by mosaic gain-of-function mutations in *GNAS*, which results in constitutive activation of  $G\alpha_s$  (1). FD occurs when these mutations arise in skeletal progenitor cells (2); the result is a disorder that presents during childhood and is characterized by bone marrow fibrosis, the presence of intramedullary and immature woven bone, failure of mature lamellar bone to form, and high bone turnover (2). In particular, the bone marrow fibrosis phenotype is characterized by persistent proliferation and lack of differentiation of osteoblastic precursor cells (3). When FD occurs in combination with multiple endocrinopathies and skin pigmentation defects, it is classically called McCune–Albright syndrome (MAS). Although the genetic mutations that cause FD are well characterized, the molecular mechanism by which activated  $G\alpha_s$  leads to the FD phenotype is unclear.

During normal tissue development and homeostasis, tightly regulated progenitor cell proliferation/renewal and differentiation are required to ensure proper organ function. The Wnt/ $\beta$ -catenin (or “canonical” Wnt) signaling pathway plays critical roles regulating the formation and homeostasis of most tissues, and the importance of this pathway in the skeleton is underscored by the number of disorders caused by mutations in Wnt/ $\beta$ -catenin signaling components (4–7). Proper modulation of Wnt/ $\beta$ -catenin signaling levels is critical, and both decreases and increases in signaling activities are associated with abnormal bone formation. During skeletal development, conditional inactivation of  $\beta$ -catenin

in skeletal progenitor cells leads to reduced osteoblast differentiation and decreased bone formation (8–11). Considerable evidence also indicates that the effect of Wnt/ $\beta$ -catenin signaling depends on the stage of osteoblast differentiation and that pathway activation in uncommitted progenitor cells can also suppress osteoblast differentiation (9, 12–14) and block the terminal differentiation of committed osteoblasts (10, 15, 16).

During Wnt/ $\beta$ -catenin signaling, Wnt ligands bind to Frizzled (Fzd) and low-density lipoprotein-related protein 5 or 6 (Lrp5/6), initiating a pathway leading to disruption of the Axin-containing  $\beta$ -catenin “destruction complex.” Fzd receptors contain seven transmembrane domains and are classified as a distinct family of G protein-coupled receptors (GPCRs) (17), although the precise roles of G proteins in transducing Wnt/ $\beta$ -catenin signaling remain controversial. GPCRs signal through four relatively small families of  $G\alpha$  proteins ( $G\alpha_s$ ,  $G\alpha_{i/o}$ ,  $G\alpha_q$ , and  $G\alpha_{12/13}$ ), and if Fzd receptors are classic GPCRs, they should signal through one of these four  $G\alpha$  families. Indeed, several groups have suggested that Fzd receptors require G proteins for signaling (18–21). Despite these findings, genetic screens in *Drosophila* and targeted screens in mammalian cells failed to identify G proteins as core pathway components of Wnt/ $\beta$ -catenin signaling (22, 23). Here, we investigate the interaction of these pathways in the context of a disease of skeletal progenitor cells and explore mechanisms by which  $G\alpha$  proteins regulate Wnt/ $\beta$ -catenin signaling.

## Results

**Wnt/ $\beta$ -Catenin Signaling Is Up-Regulated in FD/MAS Tissues.** Because Wnt/ $\beta$ -catenin signaling has been implicated to interact with  $G\alpha_s$  signaling (24–26) and accumulation of  $\beta$ -catenin protein is a hallmark of Wnt/ $\beta$ -catenin pathway activation, we first examined  $\beta$ -catenin protein levels in tissues isolated from patients with FD/MAS. We found that  $\beta$ -catenin levels were elevated in lesional bone tissue from three patients with FD relative to three control bones by immunohistochemistry (Fig. 1 *A* and *B*). In thyroid tissues from two controls, three patients with MAS [1 with thyroid carcinoma (27)], and one patient with Graves disease (OMIM 275000, increased activation of  $G\alpha_s$  through antibody-dependent activation of the thyroid-stimulating hormone receptor),  $\beta$ -catenin levels in patient tissues were also elevated compared with those in the control tissues by immunoblotting (Fig. 1*C*). These results suggest that Wnt/ $\beta$ -catenin signaling is

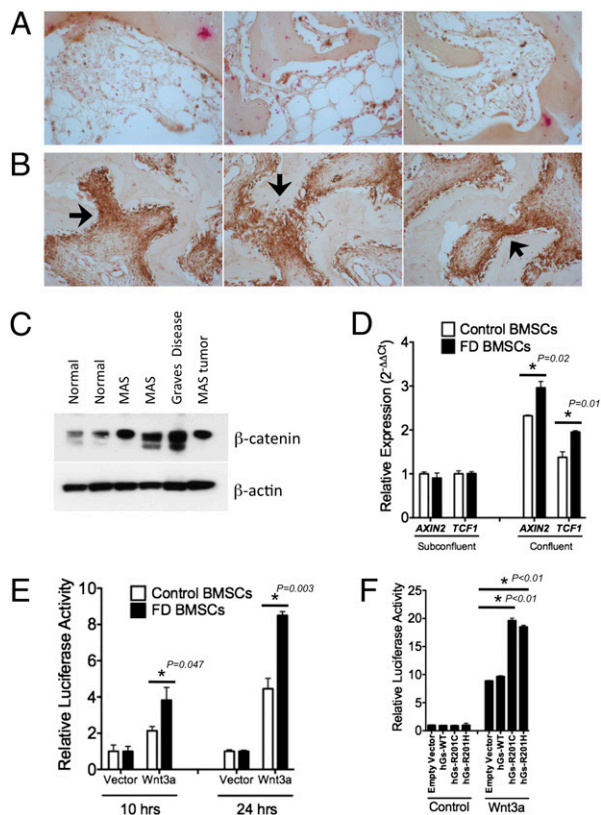
Author contributions: J.B.R., M.T.C., P.G.R., and Y.Y. designed research; J.B.R., N.C., and S.A.K. performed research; J.B.R., N.C., D.P., S.A.K., F.S.C., J.-M.G., M.C., N.B., J.W., S.R.C., L.S.W., M.T.C., and P.G.R. contributed new reagents/analytic tools; J.B.R., N.C., S.A.K., M.T.C., P.G.R., and Y.Y. analyzed data; and J.B.R. and Y.Y. wrote the paper.

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**Fig. 1.** Wnt/ $\beta$ -catenin signaling is up-regulated in FD and MAS tissues. (A) Immunohistochemistry for  $\beta$ -catenin in control adult human bone sections counterstained with nuclear fast red. (Magnification: 10 $\times$ .) (B) Immunohistochemistry for  $\beta$ -catenin on lesional bone sections from three patients with FD demonstrates elevated levels of expression. Arrows point to bone marrow stromal cells. (C) Immunoblotting for  $\beta$ -catenin and  $\beta$ -actin in thyroid gland lysates from two control individuals, three patients with MAS, and one patient with Graves disease.  $\beta$ -catenin levels were elevated in the patient samples relative to control. (D) qPCR data showing elevated expression of Wnt/ $\beta$ -catenin signaling target genes in BMSCs derived from FD bone when cultured under osteogenic conditions for 3 d ( $n = 3$ , average  $\pm$  SD;  $*P = 0.02$  and  $*P = 0.01$  for *AXIN2* and *TCF1*, respectively). (E) Transcription controlled by Wnt/ $\beta$ -catenin signaling as assayed by Supertopflash luciferase activities was up-regulated in FD BMSCs relative to control when cotransfected with a Wnt3a expression construct ( $*P = 0.047$  and  $*P = 0.003$  for 10 and 24 h, respectively). (F) When expressed in MEF cells, the human R201C/H mutant  $G\alpha_s$  proteins do not affect basal activation of Wnt/ $\beta$ -catenin signaling but potentiate signaling induced by exogenous Wnt3a ( $*P < 0.01$ ). human Gs, hGs.

more highly activated in affected tissues from the patients with FD/MAS that we have examined.

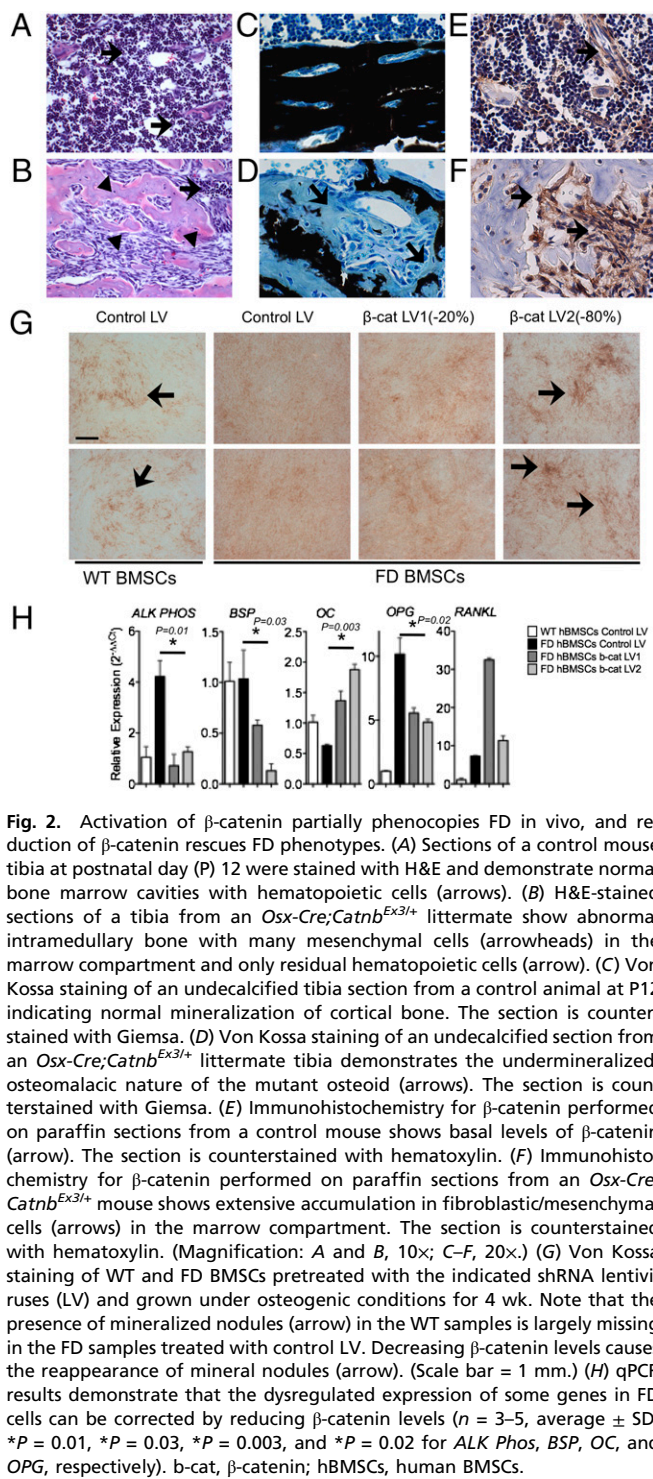
Because bone marrow stromal cells (BMSCs) from patients with FD demonstrate enhanced proliferation and decreased osteoblastic differentiation (3), we next asked if these cells also showed elevation of Wnt/ $\beta$ -catenin signaling. BMSCs isolated from healthy controls or patients with FD were cultured in vitro, and expression of transcriptional targets of Wnt/ $\beta$ -catenin signaling activity was examined. Under subconfluent conditions, *AXIN2* and *TCF1* expression was similar in the FD and control BMSCs (Fig. 1D). However, when allowed to reach confluence and placed in osteogenic media to promote differentiation, FD BMSCs showed elevated expression of both *AXIN2* and *TCF1* relative to control cells (Fig. 1D). This might be caused by more abundant Wnt ligands produced by the confluent cells. BMSCs were then transfected with a luciferase reporter construct of Wnt/ $\beta$ -catenin activity [Supertopflash (28)]. In the absence of exogenous Wnt ligands, control and FD BMSCs showed similar basal

Wnt/ $\beta$ -catenin signaling activities (Fig. 1E). When cotransfected with a Wnt3a expression construct, however, FD cells showed higher Wnt/ $\beta$ -catenin signaling activity relative to control BMSCs (Fig. 1E). These results suggest that mutant activated  $G\alpha_s$  in FD cells caused the elevated Wnt/ $\beta$ -catenin activities. To test this more definitively, either WT or mutant  $G\alpha_s$  carrying the common human FD/MAS-associated mutations (R201C and R201H) was transfected into mouse embryonic fibroblast (MEF) cells. Expression of both  $G\alpha_s$  mutants but not the WT  $G\alpha_s$  resulted in potentiated Wnt3a-dependent transcription (Fig. 1F). The relatively rapid kinetics of this interaction (3–4 h) suggest that the interaction of  $G\alpha_s$  and the Wnt/ $\beta$ -catenin signaling pathway may be direct, although we cannot exclude the possibility that secondary effects also exist. These observations indicate that activated  $G\alpha_s$  sensitizes BMSCs to Wnt signaling and activated  $G\alpha_s$  mutations in FD/MAS may exert their effects by up-regulating Wnt/ $\beta$ -catenin signaling.

**Loss of  $G\alpha_s$  Leads to Reduced Wnt/ $\beta$ -Catenin Signaling.** Because both Wnt/ $\beta$ -catenin and  $G\alpha_s$  signaling are important for bone formation, we tested whether  $G\alpha_s$  is required to regulate Wnt/ $\beta$ -catenin signaling in vivo during skeletal development. We conditionally removed *G $\alpha_s$*  (29) from the developing limb mesenchyme with the *Prx1-Cre* line (30) and used the *Topgal* reporter mice (31) to assess endogenous Wnt/ $\beta$ -catenin signaling. At embryonic day 16.5, the *Prx1-Cre;G $\alpha_s$ <sup>lox/-</sup>;Topgal* mutant mice demonstrated decreased X-gal staining in the bone collar and primary spongiosum (SI Appendix, Figs. S1 and S2), indicating that loss of  $G\alpha_s$  leads to decreased Wnt/ $\beta$ -catenin signaling in vivo in osteoblastic cells. In further support of this, expression of endogenous Wnt/ $\beta$ -catenin target genes in the developing limb of the mutant mice was decreased relative to littermate controls (SI Appendix, Fig. S3). Consistent with  $G\alpha_s$  promoting Wnt/ $\beta$ -catenin signaling during osteoblast differentiation, removal of  $G\alpha_s$  from mouse BMSCs cultured under osteoblastic conditions also led to reduced expression of Wnt/ $\beta$ -catenin target genes (SI Appendix, Fig. S4). Therefore,  $G\alpha_s$  signaling can regulate bone formation by enhancing Wnt/ $\beta$ -catenin signaling.

**Activation of  $\beta$ -Catenin in Osteoblast Precursors Phenocopies Key Aspects of FD in Vivo.** Because Wnt/ $\beta$ -catenin signaling is elevated in the FD BMSCs, we tested whether inappropriate activation of Wnt/ $\beta$ -catenin signaling in osteoprogenitor cells is sufficient to cause the FD phenotypes in vivo. This was accomplished by mating mice harboring a conditional allele of  $\beta$ -catenin (*Catnb<sup>Ex3</sup>*) (32) that produces an activated form of  $\beta$ -catenin following Cre-mediated recombination to *Osterix (Ox) - Cre* mice (10). FD is characterized by extensive marrow fibrosis associated with increased proliferation and reduced differentiation of osteoblastic cells and poorly mineralized bone (3). In the limbs of the *Ox-Cre;Catnb<sup>Ex3/+</sup>* mice, large portions of the marrow cavity were filled with osteoblastic cells at the expense of hematopoietic cells. In addition, intramedullary bone was immature and woven in the mutant (Fig. 2A and B). Von Kossa staining of the undecalcified tibia showed the mutant osteoid to be under-mineralized (Fig. 2C and D). Immunohistochemical staining of bone sections with an anti- $\beta$ -catenin antibody shows elevated expression of  $\beta$ -catenin in the osteoblastic cells in the marrow compartment of *Ox-Cre;Catnb<sup>Ex3/+</sup>* mutant mice (Fig. 2E and F). This staining is similar to  $\beta$ -catenin staining in human FD bone (Fig. 1B) and suggests that activation of Wnt/ $\beta$ -catenin signaling in osteoprogenitor cells can lead to their direct expansion in the marrow compartment. These analyses demonstrate that activation of Wnt/ $\beta$ -catenin signaling in early osteoblasts can recapitulate several important phenotypes associated with FD, including the presence of marrow fibrosis, inappropriate presence of intramedullary and woven bone, and failure of lamellar bone to form and mineralize correctly.





**Fig. 2.** Activation of  $\beta$ -catenin partially phenocopies FD in vivo, and reduction of  $\beta$ -catenin rescues FD phenotypes. (A) Sections of a control mouse tibia at postnatal day (P) 12 were stained with H&E and demonstrate normal bone marrow cavities with hematopoietic cells (arrows). (B) H&E-stained sections of a tibia from an *Osx-Cre;Catnb<sup>Ex3/+</sup>* littermate show abnormal intramedullary bone with many mesenchymal cells (arrowheads) in the marrow compartment and only residual hematopoietic cells (arrow). (C) Von Kossa staining of an undecalcified tibia section from a control animal at P12 indicating normal mineralization of cortical bone. The section is counterstained with Giemsa. (D) Von Kossa staining of an undecalcified section from an *Osx-Cre;Catnb<sup>Ex3/+</sup>* littermate tibia demonstrates the undermineralized, osteomalacic nature of the mutant osteoid (arrows). The section is counterstained with Giemsa. (E) Immunohistochemistry for  $\beta$ -catenin performed on paraffin sections from a control mouse shows basal levels of  $\beta$ -catenin (arrow). The section is counterstained with hematoxylin. (F) Immunohistochemistry for  $\beta$ -catenin performed on paraffin sections from an *Osx-Cre;Catnb<sup>Ex3/+</sup>* mouse shows extensive accumulation in fibroblastic/mesenchymal cells (arrows) in the marrow compartment. The section is counterstained with hematoxylin. (Magnification: A and B, 10 $\times$ ; C–F, 20 $\times$ .) (G) Von Kossa staining of WT and FD BMSCs pretreated with the indicated shRNA lentiviruses (LV) and grown under osteogenic conditions for 4 wk. Note that the presence of mineralized nodules (arrow) in the WT samples is largely missing in the FD samples treated with control LV. Decreasing  $\beta$ -catenin levels causes the reappearance of mineral nodules (arrow). (Scale bar = 1 mm.) (H) qPCR results demonstrate that the dysregulated expression of some genes in FD cells can be corrected by reducing  $\beta$ -catenin levels ( $n = 3$ –5, average  $\pm$  SD; \* $P = 0.01$ , \* $P = 0.03$ , \* $P = 0.003$ , and \* $P = 0.02$  for *Alk Phos*, *BSP*, *OC*, and *OPG*, respectively). b-cat,  $\beta$ -catenin; hBMSCs, human BMSCs.

**Reduction of  $\beta$ -Catenin in Patient-Derived BMSCs Rescues FD In Vitro.**

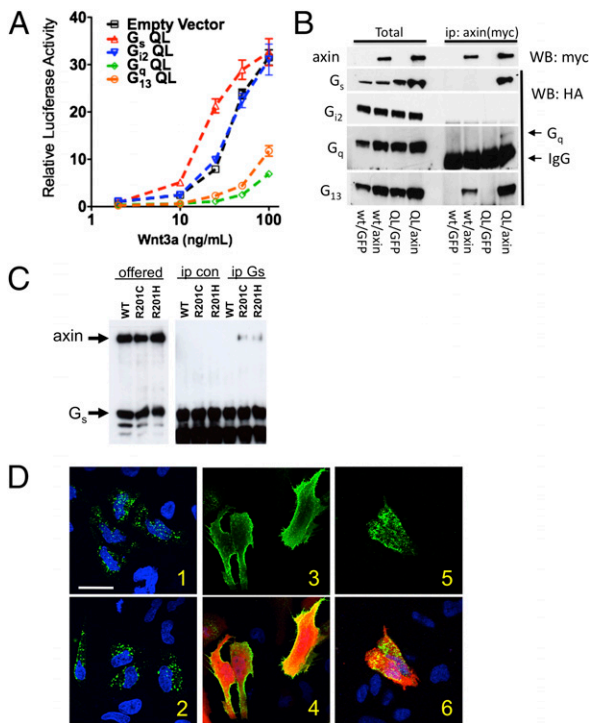
To assess whether the up-regulated Wnt/ $\beta$ -catenin signaling contributes to the pathology of FD in humans, we turned to a cell culture model of osteoblast maturation. Human BMSCs grown to confluence were switched to osteogenic media and cultured for 4 wk, allowing osteoprogenitor cells to differentiate into mature osteoblasts. Relative to control BMSCs, BMSCs isolated from patients with FD demonstrated a block in maturation as seen by the elevated expression of the osteoprogenitor marker *alkaline phosphatase (ALK PHOS)*, decreased mRNA expression

of the mature osteoblast marker *osteocalcin (OC)*, and decreased formation of calcified matrix nodules by Von Kossa staining (Fig. 2 G and H).

To test whether elevated Wnt/ $\beta$ -catenin signaling contributes to the phenotypes of the human FD BMSCs, we reduced  $\beta$ -catenin levels by infecting cells with lentiviruses that express shRNAs targeting  $\beta$ -catenin (*SI Appendix, Fig. S5*). We found that reducing  $\beta$ -catenin levels rescued the ability of FD BMSCs to form calcified matrix nodules in vitro (Fig. 2G). This effect was more pronounced when  $\beta$ -catenin levels were decreased more efficiently (20% vs. 80% knockdown). Quantitative PCR (qPCR) analysis demonstrated that the expression of some genes dysregulated in FD was normalized by reducing  $\beta$ -catenin levels. Expression of *ALK PHOS* was decreased, and expression of *OC* was increased, suggesting that the blockage of osteoblast maturation of the FD osteoprogenitor cells had been partially corrected by reducing the  $\beta$ -catenin levels (Fig. 2H). *Osteoprotegerin (OPG)*, a target of Wnt/ $\beta$ -catenin signaling (33), was up-regulated in FD cells, and its expression was decreased by reducing  $\beta$ -catenin levels. Others have reported that *bone sialoprotein (BSP)* was up-regulated in FD-like cells (34). However, we found that *BSP* expression levels were similar in control and FD BMSCs, although reduction of  $\beta$ -catenin levels did decrease *BSP* expression. Receptor activator of NF- $\kappa$ B ligand (*RANKL*) was up-regulated in FD cells, and this elevation was not reversed by decreasing  $\beta$ -catenin levels but, instead, was further increased. This is consistent with the notion that *RANKL* expression is positively regulated by  $G_{\alpha_s}$  activation of protein kinase A (PKA) and cAMP response element-binding protein (CREB) (35) and inhibited by Wnt/ $\beta$ -catenin signaling (36). These data suggest that the fibrotic and osteolytic phenotypes in FD can be uncoupled. Taken together, our data indicate that enhanced Wnt/ $\beta$ -catenin signaling in osteoprogenitor cells contributes to the pathophysiology of FD and that inhibition of Wnt/ $\beta$ -catenin signaling may represent a novel therapeutic approach.

**$G_{\alpha}$  Proteins Differentially Regulated Wnt/ $\beta$ -Catenin Signaling.**

$G_{\alpha_s}$  can be activated by GPCRs, such as the parathyroid hormone receptor, and our results suggest that GPCR signaling may regulate bone formation by modulating Wnt/ $\beta$ -catenin signaling activities. Because GPCRs play significant roles during both embryonic development and adult homeostasis, and usually activate more than a single G protein family, we explored the mechanism by which  $G_{\alpha_s}$  and the other major G protein families ( $G_{\alpha_q/11}$ ,  $G_{\alpha_i/o}$ , and  $G_{\alpha_{12/13}}$ ) regulate Wnt/ $\beta$ -catenin signaling. We first tested whether all major  $G_{\alpha}$  protein families exhibit similar regulatory activities on Wnt/ $\beta$ -catenin signaling by generating dose–response curves using a wide range of Wnt ligand concentrations and the Super-topflash assay (Fig. 3A). MEF cells transfected with either an empty vector or a vector encoding constitutively active  $G_{\alpha_{12}}$  ( $G_{\alpha_{12}}\text{-QL}$ ) gave similar curves, demonstrating a lack of interaction between  $G_{\alpha_{12}}$  and the Wnt/ $\beta$ -catenin signaling pathway. Expression of  $G_{\alpha_s}\text{-QL}$  caused the dose–response curve to shift to the left, but it reached a similar maximum as the control curve. By contrast,  $G_{\alpha_q}\text{-QL}$  and  $G_{\alpha_{13}}\text{-QL}$  shifted the Wnt3a dose–response curve to the right, decreasing the cells’ responsiveness to Wnt3a. Our results demonstrate that  $G_{\alpha}$  proteins by themselves are not sufficient to stimulate Wnt/ $\beta$ -catenin signaling in MEF cells; however, they exhibit differential activities in modulating Wnt/ $\beta$ -catenin signaling in the presence of exogenous Wnt3a. Under the same experimental conditions,  $G_{\alpha_s}$  potentiates, whereas  $G_{\alpha_q}$  and  $G_{\alpha_{13}}$  inhibit, Wnt/ $\beta$ -catenin signaling and  $G_{\alpha_i}$  has no effect. Activation of different G protein pathways by distinct GPCRs yielded similar results (*SI Appendix, Figs. S6 and S7*). Expression of constitutively active forms of three distinct  $G_{\alpha_{i/o}}$  family members ( $G_{\alpha_{12}}\text{-QL}$ ,  $G_{\alpha_o}\text{-QL}$ , and  $G_{\alpha_z}\text{-QL}$ ) failed to activate basal or Wnt3a-induced  $\beta$ -catenin–dependent transcription (*SI Appendix, Fig. S8*). Because the stimulatory effects of  $G_{\alpha_s}$  were not mimicked by the PKA



**Fig. 3.** Activated  $G\alpha$  proteins differentially regulate Wnt/ $\beta$ -catenin signaling. (A) Dose-response curve obtained by treating MEF cells transiently transfected with activated (QL)  $G\alpha$  protein constructs with increasing concentrations of recombinant Wnt3a. Wnt3a stimulation with concomitant  $G$  protein activation demonstrates that  $G\alpha_s$ -QL leads to increased Wnt/ $\beta$ -catenin signaling, whereas both  $G\alpha_q$ -QL and  $G\alpha_{13}$ -QL cause decreased Wnt/ $\beta$ -catenin signaling. (B) HEK293T cells were cotransfected with WT or the activated QL form of rat  $G\alpha$  proteins and Axin or GFP. Activated forms of  $G\alpha_s$ ,  $G\alpha_q$ , and  $G\alpha_{13}$  showed enhanced coimmunoprecipitation with Axin relative to WT  $G\alpha$  forms. (C) Coimmunoprecipitation experiments performed with WT or mutant (R201C, R201H) forms of human  $G\alpha_s$  demonstrate enhanced binding of mutant forms to Axin. (D) Axin (green) subcellular localization is affected by the presence of  $G\alpha$  proteins. Under control conditions, transfected Axin is present in cytoplasmic puncta (1 and 2). When cotransfected with  $G\alpha_s$ -QL (red, 3 and 4), Axin distribution is more widespread and present at the plasma membrane. When cotransfected with  $G\alpha_{13}$ -QL (red, 5 and 6), Axin remains largely cytoplasmic and aligns parallel to f-actin (Magnification: 63 $\times$ ).

activators forskolin or 3-isobutyl-1-methylxanthine,  $G\alpha_s$  may act through a PKA-independent mechanism to regulate Wnt/ $\beta$ -catenin signaling (*SI Appendix, Fig. S8*). Forskolin treatment did, however, lead to phosphorylation of CREB and enhanced CREB transcriptional activities (*SI Appendix, Fig. S9*). The inhibitory effects of  $G\alpha_q$ -QL and  $G\alpha_{13}$ -QL were specific to Supertopflash, because these same  $G$  protein constructs promoted transcriptional activation of Ap1- and serum response factor-luciferase constructs, as expected (*SI Appendix, Fig. S10*).

To investigate the mechanism of Wnt/ $\beta$ -catenin signaling regulation by  $G\alpha$  proteins, we activated the pathway using lithium chloride (LiCl), a Gsk3 inhibitor. Because Gsk3 promotes  $\beta$ -catenin degradation, LiCl treatment abrogated all  $G\alpha$  effects, suggesting that the  $G\alpha$  proteins act upstream of Gsk3 (*SI Appendix, Fig. S8*). Indeed, activated  $G\alpha$  proteins differentially affected accumulation of cytoplasmic  $\beta$ -catenin, possibly by regulating the Gsk3-containing  $\beta$ -catenin destruction complex (*SI Appendix, Fig. S11*). In the absence of exogenous Wnt3a, cytoplasmic  $\beta$ -catenin levels were undetectable regardless of the activation status of  $G\alpha$  proteins. Addition of Wnt3a to MEF cells resulted in accumulation of cytoplasmic  $\beta$ -catenin. In the presence of  $G\alpha_s$ -QL, the amount of cytoplasmic  $\beta$ -catenin increased, whereas  $G\alpha_q$ -QL or

$G\alpha_{13}$ -QL expression resulted in decreased  $\beta$ -catenin levels (*SI Appendix, Fig. S11*). Addition of  $G\alpha_{12}$ -QL had no effect on cytoplasmic  $\beta$ -catenin accumulation. Taken together, these results demonstrate that activation of  $G\alpha$  proteins alone is not sufficient to activate the Wnt/ $\beta$ -catenin pathway de novo but can differentially regulate the activity of this pathway by acting upstream of Gsk3 to modulate  $\beta$ -catenin protein stability.

Because Axin contains a regulator of  $G$  protein signaling domain, a protein interaction module known to bind  $G\alpha$  subunits, and is required for the assembly of the  $\beta$ -catenin destruction complex, we explored the interaction between all four major  $G\alpha$  subunits and Axin. Consistent with previous observations (24), overexpressed  $G\alpha_s$ -QL could be coimmunoprecipitated with Axin with considerably higher efficiency than WT  $G\alpha_s$  (Fig. 3B). Similar to the constitutively activating Q227L  $G\alpha_s$  mutations, the common human FD/MAS-associated mutations (R201C and R201H) demonstrated increased affinity for Axin (Fig. 3C). Although robustly expressed, neither WT  $G\alpha_{12}$  nor  $G\alpha_{12}$ -QL could be coimmunoprecipitated with Axin. Interestingly, activated forms of both  $G\alpha_{13}$  and  $G\alpha_q$  could be coimmunoprecipitated with Axin, whereas WT forms either failed to coimmunoprecipitate or did so less robustly.  $G\alpha_q$ -QL tended to be coimmunoprecipitated less efficiently than either  $G\alpha_s$ -QL or  $G\alpha_{13}$ -QL. Because the three activated  $G\alpha$  subunits that interacted with Axin in this assay are the same that affected Wnt/ $\beta$ -catenin signaling in MEF cells (Fig. 3A), these  $G\alpha$  subunits may exert their effects by binding to Axin.

Activation of  $G\alpha$  proteins leads to their subcellular redistribution (37, 38) and may modulate Wnt/ $\beta$ -catenin signaling by affecting Axin localization. To test this, we performed immunofluorescent staining to determine the effects of activated  $G\alpha$  proteins on Axin distribution. In the absence of  $G\alpha$  proteins, exogenously expressed Axin was present in cytoplasmic puncta (Fig. 3D, 1 and 2). Consistent with differential localization of distinct activated  $G\alpha$  proteins, coexpression of Axin with  $G\alpha_s$ -QL led to colocalization and redistribution of Axin to the plasma membrane and throughout the cytoplasm in a diffuse pattern (Fig. 3D, 3 and 4). Consistent with  $G\alpha_s$  potentiating Wnt/ $\beta$ -catenin signaling, others have shown that the membrane localization of Axin is associated with pathway activation (39). By contrast, coexpression with  $G\alpha_{13}$ -QL also colocalized and caused Axin puncta to aggregate between actin stress fibers (Fig. 3D, 5 and 6). These data suggest that the opposing effects of  $G\alpha_s$  and  $G\alpha_{13}$  on Wnt/ $\beta$ -catenin signaling may be attributable to altered subcellular localization of Axin. These results further suggest that different  $G\alpha$  proteins, when activated simultaneously, might exert their effects in modulating Wnt/ $\beta$ -catenin signaling by competing for binding to Axin. To test this, we coexpressed Axin along with either  $G\alpha_{13}$ -QL or  $G\alpha_s$ -QL, or Axin with both  $G\alpha_{13}$ -QL and  $G\alpha_s$ -QL. We found that when both  $G\alpha_{13}$ -QL and  $G\alpha_s$ -QL were coexpressed, they demonstrated decreased binding to Axin relative to when either  $G\alpha_{13}$ -QL or  $G\alpha_s$ -QL was expressed singly, suggesting that these  $G\alpha$  proteins are competing for common binding sites on Axin (*SI Appendix, Fig. S12*).

**$G\alpha$  Proteins Are Not Required for Wnt/ $\beta$ -Catenin Signaling.** Although it has been reported that Fzds may act as GPCRs and require  $G\alpha$  proteins to activate Wnt/ $\beta$ -catenin signaling (18, 40), the precise role of  $G$  proteins in Wnt signaling remains controversial. If  $G\alpha$  proteins are core components of Wnt/ $\beta$ -catenin signaling, they should be required for Wnt/ $\beta$ -catenin signaling in all cell types. To test this, we genetically removed either  $G\alpha_s$ ,  $G\alpha_{q11}$ , or  $G\alpha_{12/13}$  from MEF cells. Importantly, genetic removal of these  $G\alpha$  proteins failed to affect Wnt/ $\beta$ -catenin signaling as assayed by Supertopflash, accumulation of cytoplasmic  $\beta$ -catenin, or phosphorylation of Lrp6 (Fig. 4A–C and *SI Appendix, Figs. S13–15*). Inhibition of  $G\alpha_{i/o}$ , achieved by pertussis toxin pretreatment, alone or in a  $G\alpha_{q11}$ -null background, similarly did not affect Wnt/ $\beta$ -catenin signaling (Fig. 4B and *SI Appendix, Fig. S14*). Our data indicate





**Luciferase Assays.** Human BMSCs were transfected with Supertopflash/Renilla-null constructs and pRES-control or pRES-Wnt3a plasmids by nucleofection (Amaxa). Cells were allowed to recover overnight in  $\alpha$ -MEM/20% (vol/vol) FBS growth media and then placed in similar media containing 10% FBS for the indicated time points. MEF cells were transfected with Supertopflash/Renilla-null and other constructs and allowed to recover for 5–6 h. Cells were then placed in DMEM, 0.2% FBS, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mM glutamine overnight. On the following morning, cells were placed in 0.2% FBS media with recombinant or Wnt3a-conditioned media for 4–6 h. Luciferase activity was determined using the Dual Luciferase Reporter Assay (Promega). For

luciferase based assays,  $n = 3$ –4 and experiments were repeated a minimum of three times.

**Lentiviral Production.** Lentivirus was produced, and BMSCs were infected as described (52). Selection was performed for 48 h using puromycin (2  $\mu$ g/mL).

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