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Targeting the G*ai*1/3–G*ab*1 Axis Enhances BMP2-Mediated Bone Regeneration

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Abstract

Background: Bone morphogenetic protein 2 (BMP2) is a pivotal cytokine for skeletal formation, homeostasis, and regeneration. While its signaling through serine/threonine kinase receptors (BMPRI/II) and downstream pathways (PI3K-Akt-mTOR, Erk, Smad) is known to drive osteogenesis, the full mechanistic picture remains incomplete. Our prior work identified G*ai*1 and G*ai*3, inhibitory G protein alpha subunits, as key mediators for several receptor tyrosine kinase (RTK) signaling pathways. Their potential role in BMP2 signaling and bone formation, however, was undefined.

Methods: We employed a multi-modal approach combining clinical observation, genetic mouse models, and molecular/cellular techniques. Bone marrow mesenchymal stem cells (BMSCs) from young and elderly subjects, as well as from wild-type (WT) and G*ai*1/3 double knockout (DKO) mice, were analyzed. *In vitro*, BMP2-induced signaling and osteogenic differentiation were assessed in BMSCs, MC3T3-E1 osteoblasts, and mouse embryonic fibroblasts (MEFs) following genetic manipulation

(knockout, knockdown, overexpression) of *Gai1/3*. *In vivo* effects were evaluated in *Gai1/3* DKO mice and a femoral fracture model using mice with BMSC-specific *Gai1/3* knockdown (*Gai1/3*-CKD), treated with or without BMP2. Mechanistic studies involved co-immunoprecipitation, molecular docking, and analysis of the adaptor protein Gab1.

Results: *Gai1/3* expression was reduced in bone tissue and BMSCs from aged/osteoporotic subjects and mice. *Gai1/3* DKO mice exhibited delayed skeletal development, reduced bone mass, and impaired bone formation. *In vitro*, genetic ablation of *Gai1/3*, but not *Gai2*, abolished BMP2-induced activation of downstream Akt, Erk, and Smad pathways in MEFs, MC3T3-E1 cells, and BMSCs, and severely blunted BMP2-stimulated osteogenic differentiation and mineralization. Conversely, *Gai1/3* overexpression enhanced these effects. In the fracture model, BMP2 failed to promote healing in *Gai1/3*-CKD mice. Mechanistically, *Gai1/3* were essential for BMP2-induced phosphorylation of the scaffolding protein Gab1. *Gai1/3* physically associated with BMP2 receptors (BMPR1A/B, BMPR2) and Gab1, facilitating this complex formation. Disrupting this interaction via dominant-negative *Gai1/3* mutants impaired downstream signaling and osteogenesis.

Conclusions: Our findings establish the *Gai1/3*-Gab1 signaling axis as a critical and novel component of BMP2 receptor (serine/threonine kinase) signal transduction, essential for BMP2-induced osteogenesis both *in vitro* and *in vivo*. This work expands the functional repertoire of *Gai* proteins beyond GPCRs and RTKs and identifies *Gai1/3* as potential therapeutic targets for enhancing bone formation and treating bone disorders like fractures and osteoporosis.

Key Word: *Gai1/3*; BMP2; Osteogenesis; Signaling

Introduction

Bone formation ability is a fundamental physiological process crucial for maintaining skeletal integrity and systemic homeostasis^[1]. First of all, it ensures the normal development of the skeletal system, supports the human body structure and protects

internal organs, while participating in the balance of calcium and phosphorus metabolism^[1]. Secondly, adequate osteogenic activity prevents the occurrence of senile osteoporosis, significantly improves the quality of life in the elderly, and is conducive to bone regeneration and repair after fractures^[2]. With advances in regenerative medicine and molecular biology, targeted therapies to enhance bone formation have emerged as a pivotal strategy for managing bone injury repair and degenerative bone diseases^[3, 4]. This approach holds significant clinical and public health value by addressing unmet needs in conditions such as osteoporosis, fractures. Bone formation is mainly mediated by BMSCs and goes through stages such as osteogenic differentiation of BMSCs, osteoid secretion and mineralization^[1, 2]. The above processes are regulated by multiple cytokines. BMPs induce the differentiation of MSCs into osteoblasts^[5]. The Wnt/ β -catenin pathway promotes osteogenic proliferation and inhibits adipogenesis^[6]. TGF- β regulates bone matrix synthesis^[7]. These cytokines work synergistically to promote bone formation, which is of great significance for bone development, repair and the treatment of metabolic diseases. Among them, the BMPs family plays a key role in promoting bone formation ^[5].

BMP2, a key signaling molecule in the TGF- β superfamily, regulates multiple biological processes including osteogenesis and tissue repair ^[8]. Because of the vital role of BMP2 in bone development, maintenance, and regeneration, BMP2 signaling has become one of the focuses of bone regeneration research^[9]. BMP2 promotes osteogenesis by stimulating the proliferation, differentiation of bone marrow mesenchymal cells (BMSCs) and osteogenic precursor cell^[10]. In clinical practice, rhBMPs serve as effective bone-inducing agents for the treatment of skeletal defects ^[11]. At the most empirical level, BMP2 signaling relies on activates intracellular serine/threonine kinases of type I and type II BMP receptors^[12]. Ligand-binding events induce the transcription of multiple Osteogenesis-associated genes through the activation of a complex array of downstream intracellular mediators that includes PI3K-Akt-mTORC1, Erk-MAPK, and Smads pathways ^[13, 14]. Yet, the underlying signaling mechanisms for BMP2-induced signaling remain not been fully elucidated.

Among inhibitory G protein alpha subunits (G α i), three primary subtypes exist: G α i1, G α i2, and G α i3 ^[15]. When bound to GPCRs, G α i proteins exert an inhibitory effect on AC, leading to decreased cAMP generation ^[15]. Our prior studies demonstrated that G α i1 and G α i3 critically regulate non-canonical RTKs (Receptor Tyrosine Kinases) signaling by modulating both PI3K-Akt-mTOR and Erk pathways. This mechanism applies to multiple RTKs, including EGFR^[16], BDNF receptor TrkB ^[17], and VEGF receptor VEGFR2 ^[18]. After the stimulation of RTKs through the binding of growth factors and RTKs, G α i1 and G α i3 are required for the activation of downstream PI3K-Akt-mTOR and Erk signal transduction ^[16, 17]. Among transmembrane receptors in human cells, BMP type I/II receptors constitute a rare class that possesses intrinsic serine/threonine kinase activity ^[14]. Whether G α i mediates the signal transduction of receptor serine/threonine kinases remain unknown.

We previously established that G α i1/3 plays a critical role in activating multiple tyrosine kinase receptor signaling pathways. In this study, we observed that G α i1/3 is downregulated in the bone tissue of elderly osteoporosis patients, with its expression level correlating with bone formation capacity. Mechanistically, we demonstrated that G α i1/3 is essential for BMP2 signal transduction, primarily by interacting with BMP2 receptors (BMPRII, BMPRI) and Gab1 to regulate downstream signaling. Inhibition of G α i1/3 expression or function significantly impairs BMP2-mediated bone formation and enhances fracture healing. These findings underscore G α i1/3 breaks through its traditional biological functions and mediates the activation of tyrosine kinase receptors. It is also a key protein mediating the activation of downstream signals of BMP2 (receptor serine/threonine kinases). As a promising therapeutic target for enhancing bone formation and treating fractures, it offers a foundation for novel treatment strategies.

Methods

Study approval

The human studies were approved by the Ethics Committee of the Second Affiliated

Hospital of Soochow University (Approval ID:JD-LK2022151-IR06). All participants provided informed consent. All animal studies were reviewed and approved by the Animal Ethics Committee of Soochow University (Approval ID: SUDA20181015A05) and conducted in compliance with institutional guidelines. The mice were housed under specific pathogen-free conditions at a controlled temperature of 22–24°C, under a 12-hour light/dark cycle, with free access to water and standard laboratory chow to ensure optimal health. All procedures were performed in accordance with international ethical guidelines and relevant national regulations ^[19].

Reagents and Antibodies

Recombinant human BMP2 was sourced from Calbiochem, while puromycin was acquired from Sigma-Aldrich. Cell culture media and supplements were obtained from Gibco BRL. The following primary antibodies were purchased from Santa Cruz Biotechnology: Tubulin, Erk1/2, Akt1/2, GSK3 β , Shp2, Shc, Gab1, Grb2, Gai1, Gai2, Gai3, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP. The β -actin monoclonal antibody (mouse) was supplied by Sigma. Additional phospho-specific and signaling antibodies-including p-Akt (Ser-473/Thr-308), p-Smad1/5, p-Gab1 (Tyr-627), p-S6K (Thr-389), p-S6 (Ser-235/236), p-GSK3 α/β (Ser21/9), p-Erk1/2 (Thr-202/Tyr-204), p-MEK1/2, Runx2, BMPR1A and BMPR1B were procured from Cell Signaling Technology.

Murine bone marrow mesenchymal stem cell isolation and culture

BMSCs were harvested from 6–8-week-old C57BL6/J mice following established protocols^[20]. Tissue Preparation: Euthanized mice were sterilized with 70% ethanol, and femurs were aseptically dissected into DMEM (Biological Industries) supplemented with 100 U/ml penicillin/streptomycin (P/S). Marrow Extraction: After removing soft tissues and trimming femur ends, marrow was flushed using a 27-gauge needle with growth medium (α -MEM + 10% FBS + P/S). The cell suspension was filtered through a 70- μ m strainer (BD Falcon) and plated in 100-mm dishes. Adherence Selection: Non-adherent cells were removed after 3 h. Medium was refreshed every 12

h for 72 h, then every 48 h until 70% confluence. Passaging: Cells were washed with PBS, detached with 0.25% trypsin/0.02% EDTA (5 min, RT), and expanded for subsequent experiments.

Cell culture

BMSCs from young (Average age is 27.7) or older (Average age is 71.3) patients with fracture were extracted and induced osteogenic differentiation. BMSCs were maintained in DMEM (1×) supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/L sodium pyruvate, and 1% penicillin/streptomycin, and cultured at 37°C with 5% CO₂. MC3T3-E1 osteoblastic cells were grown in high-glucose DMEM with 10% FBS and 1% penicillin/streptomycin under identical incubation conditions. For both cell types, the medium was replaced every 24 h during the initial 48 h to remove non-adherent cells, followed by changes every 2–3 days. Osteogenic differentiation was induced by culturing cells in growth medium supplemented with 100 mg/L sodium pyruvate, 50 μM ascorbic acid, and 10 mM β-glycerophosphate for 14 days^[20].

Mouse embryonic fibroblasts (MEFs)—including wild-type (WT), *Gai1/Gai3* double knockout (DKO), *Gai1/Gai2/Gai3* single knockout (SKO), and *Gab1* KO MEFs—were propagated in DMEM with 10% FBS. Prior to experiments, MEFs were serum-starved (0.5% FBS, overnight) and equilibrated in warm PBS for 30 min ^[21].

Alkaline phosphatase (ALP) and Alizarin Red S staining

BMSCs cultured in 6-well plates were processed as follows: Fixation: Cells were rinsed 3× with PBS and fixed with 4% paraformaldehyde (15–30 min). Alkaline Phosphatase (ALP) Activity: After PBS washes (2×), fixed cells were incubated with p-nitrophenyl phosphate substrate (Alkaline Phosphatase Assay Kit, Beyotime #C3206) for 2 h (RT, dark) per manufacturer's protocol. Mineralization Detection: Calcium deposits were stained using Alizarin Red S (Solarbio #G3280) following kit instructions.

Co-immunoprecipitation

Specific plasmids were transfected into cells using the SuperKine™ Lipo3.0 Efficient Transfection Reagent (Abbkine Scientific Co., Ltd.). Following transfection, proteins were extracted from the cells, and the cell lysates were subjected to immunoprecipitation with designated antibodies conjugated to magnetic beads, utilizing the Pierce™ Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific). The immunoprecipitates were collected and washed three times with lysis buffer, after which the immunoprecipitated proteins were employed for western blotting.

qPCR, Western blotting and others

Total RNA was isolated from BMMs using TRIzol reagent (ThermoFisher Scientific) according to the manufacturer's protocol. Synthesis of cDNA was performed using the PrimeScript RT Master Mix (Takara). RT-qPCR amplification was performed with TB Green Premix Ex Taq (Tli RNaseH Plus) on a Roche LightCycler 480 II system.

Western blotting, co-immunoprecipitation (IP) assay, and confocal microscopy were conducted as previously detailed [22]. For Western blotting, each SDS-PAGE Gel lane was loaded with 40µg of quantified protein lysates. Identical lysate samples were run in parallel on separate "sister" gels to test different proteins (same procedure for all figures).

Micro-computed tomography (Micro-CT) analysis

Femurs were dissected free of soft tissues and scanned using a SkyScan 1172 micro-CT system (Bruker, Belgium) at 9 µm isotropic voxel size (50 kV, 500 µA, 0.5° rotation step). Analyze the regions of interest (ROI) of femoral fractures in mice, such as the central area of callus (500µm at each end of the fracture line). Quantitative analysis of the fracture callus tissue images was performed using CTAn software (version 1.9, SkyScan). The following parameters were tested: callus bone mineral density(BMD, g/cm³), callus volume/total volume (CV/TV, %), callus number (1/mm), callus volume (mm³). CV, TV, CV/TV, BMD of each specimen were recorded for analysis^[23].

Animal surgery

A classic mice femoral fracture model was applied in this study^[23]. Six-week-old male

mice were divided into different groups. First, the mice were anesthetized with isoflurane. Cut the skin from the outside of the right knee to the greater trochanter side. Insert a 23G needle into the bone marrow cavity of the mice femur and break the middle part of the femur. X-rays were taken following fracture to exclude any mice with unsatisfactory fractures. According to the method used in previous studies^[24], 100 μ L of free BMP2 (500 ng/per mice) or PBS were injected into the fracture site. Analgesia was achieved by a single subcutaneous injection of buprenorphine sustained-release preparation (1 mg/kg). At the conclusion of the experiment, mice were euthanized via rapid anesthesia and death induced by hypoxia in a sealed container filled with CO₂.

The sample size was determined based on preliminary data from the evaluation of bone volume fraction (BV/TV), the primary outcome measure. Before conducting the formal experiment, we performed a preliminary exploratory study and observed a relatively consistent trend in treatment effects with low intra-group variability. In the preliminary experiments, we observed a significant effect size (Cohen's $d = 1.8$) and a within-group standard deviation ($SD = 0.1278$). Based on these findings, we performed an a priori sample size estimation using G*Power 3.1 software. Test type: Two-sample independent t-test (two-tailed); α error probability: 0.05; statistical power ($1-\beta$): 0.80; effect size d : 1.8. The calculation results showed that a minimum sample size of 5 animals per group is required to achieve 80% statistical power. In addition, in high-quality publications in the field of osteoporosis research using OVX-induced osteoporotic mouse models, the typical sample size ranges from 5 to 7 mice per group^[25-27]. In accordance with the "3R" principles of animal research (Reduction, Replacement, Refinement), the number of animals used should be minimized while still satisfying statistical requirements.

Bioinformatics studies

Publicly available scRNA-seq data (GSE150703) from 1.5 and 16-month-old mice were analyzed using Seurat (v3.0). Key steps included: Data Processing: 1) Quality-controlled raw counts. 2) Identified top 5,000 variable genes (FindVariableFeatures). 3)

Reduced dimensions using PCA (top 30 PCs). Cluster Analysis: 1) Unsupervised clustering (FindClusters). 2) UMAP visualization. 3) Cluster annotation using retinal cell markers from GeneCards. Downstream Analysis: 1) Density plots for *Gai1*/*Gai3* expression. 2) Osteoblast-specific DEG analysis (up-/down-regulated genes). 3) Top 10 enriched pathways (BP & KEGG).

Molecular docking prediction

Structure Acquisition: 1) Experimental structures: Obtained from Protein Data Bank. 2) Predicted structures: Generated using AlphaFold. Structure Preparation: 1) Processed with AutoDockTools-1.5.7. 2) Removal of water molecules. 3) Addition of polar hydrogens. Docking Analysis: 1) Performed using GRAMM web server (protein-protein docking). 2) Complex optimization. 3) Manual refinement of docking results. 4) Repeat water removal and hydrogen addition. Interaction Analysis: 1) Protein-protein interaction prediction. 2) Visualization of complexes using PyMOL^[28].

Alcian blue/Alizarin red double staining

Postnatal day 3 mouse specimens were fixed in 4% paraformaldehyde (PFA, Solarbio, P1110) at 4°C for 24 h, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Sagittal sections (5 µm) were cut using a Leica RM2016 microtome. For staining, sections were deparaffinized, rehydrated, and incubated with 0.1% Alcian Blue 8GX (#C0155, Beyotime) in 3% glacial acetic acid for 24 h at room temperature. After washing with acidic ethanol (1% HCl in 70% ethanol), sections were counterstained with 0.5% Alizarin Red (Sigma-Aldrich, #A5533) in 0.1% ammonium hydroxide for 10 min to visualize calcium deposits, followed by brief differentiation in 0.1% KOH (15 s). GAG specificity was verified by hyaluronidase pretreatment (10 mg/mL, 37°C, 2 h). Imaging was performed with an OlyVIA slide scanner using brightfield and spectral unmixing.

Histology and IHC

Following 48-hour fixation in 10% formalin and 2-week decalcification in 10% EDTA,

femurs and tibiae were paraffin-embedded and sectioned at 5 μ m using an HM 355S rotary microtome (Thermo Fisher). For morphological assessment, longitudinal coronal sections (femurs) and transverse sections (tibiae) were prepared, while fracture analysis utilized femoral coronal sections. Deparaffinized sections underwent histochemical staining (H&E and Masson's) and immunohistochemical analysis according to established protocols [32]. Primary antibodies targeting Runx2 (CST #12556S), Osterix (Abcam #ab209484), and OPN (Proteintech #22952-1-AP) were applied overnight at 4°C, with subsequent detection using an HRP-streptavidin system (Dako) and hematoxylin counterstaining. Stained sections were imaged by light microscopy (Leica) and quantitatively analyzed using ImageJ software .

Generation of *Gai1/3* DKO and *Colla1*-Cre; *Gai1/3*^{fl/fl} mice

Gai1/3 double knockout (DKO) mice were created using CRISPR-Cas9 technology as we previously reported [18]. The *Colla1*-Cre mice were purchased from Jiangsu Jicui Yaokang Biotechnology Co., LTD. *Gai1/3*^{fl/fl} mice were obtained from Jackson Laboratory (Sacramento, CA, USA,). We crossed *Gai1/3*^{fl/fl} mice with *Colla1*-Cre transgenic mice to generate *Colla1*-Cre; *Gai1/3*^{fl/+} progeny, which were used for subsequent mating to produce homozygous *Colla1*-Cre; *Gai1/3*^{fl/fl} mice.

Femoral metaphysis injection of AAV

The adult C57BL/6 mice, (23.5-25.2 g, 6-8-week-old) were anesthetized, and femoral injection of the virus was performed as reported previously. *Gai1* or *Gai3* shRNA sequences were inserted into an adeno-associated virus 9 (AAV9)-Runx2 construct containing the sequence of the osteoblasts promoter, Runx2. AAV injection (0.1 μ L virus per mouse) was performed following a previously reported protocol [29].

***Gai1/3* shRNA.**

Following seeding at 1×10^5 cells/well in 6-well plates, MEFs/MC3T3-E1/BMSCs were transduced with lentiviral particles encoding *Gai1*- or *Gai3*-specific shRNAs [17, 18]. After 48h, medium was replaced with puromycin-containing medium (changed every 48h) for 10-12 days. Knockdown efficiency exceeding 90% was validated at

both protein (Western blot) and mRNA (qPCR) levels.

CRISPR/Cas9 knockout of *Gai1* and *Gai3*.

CRISPR/Cas9 lentiviral constructs for *Gai1* and *Gai3* knockout were acquired from Genechem (Shanghai, China) ^[18], and the constructs were selected with puromycin after being transfected into cells. Control groups received non-targeting sgRNA (Santa Cruz Biotechnology). Successful *Gai1/3* knockout was validated through Western blot and quantitative PCR analysis.

***Gai1/3* overexpression.**

Cells were cultured in 6-well plates at a density of 100,000 cells per well prior to adenoviral transduction (Ad-*Gai1*, Ad-*Gai3* or empty vector control). After puromycin selection, transgene expression was validated through Western blotting for protein detection and qPCR for mRNA quantification ^[17].

Statistical analysis.

Statistical analysis

Data are presented as mean $\pm$ SD. The sample size for each experiment was determined based on preliminary data or prior studies in the field to ensure adequate statistical power (typically aiming for >80% power and $\alpha = 0.05$). Statistical analyses were performed using GraphPad Prism 8 software. Prior to parametric testing, the normality of data distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances was assessed using Brown-Forsythe test or F test, as appropriate. For comparisons between two groups, unpaired two-tailed Student's *t*-test was used when data met the assumptions of normality and equal variance; otherwise, the nonparametric Mann-Whitney U test was applied. For comparisons among three or more groups, one-way ANOVA followed by Tukey's post hoc test was used when data met the assumptions of normality and homogeneity of variances. If the assumptions for ANOVA were violated, the Kruskal-Wallis test followed by Dunn's post hoc test was employed. All experiments were repeated independently at least three times. A *P* value

of < 0.05 was considered statistically significant. Randomization and blinding were adopted to reduce bias.

Results

1. *Gai1* and *Gai3* are low expressed in aging osteoblasts or BMSCs, and participates in osteogenic function and signaling.

Loss of *Gai1/3* affects the formation of bone and cartilage in mice^[30]. *Gai1/3* is involved in bone metabolism and may be related to osteogenic function. To confirm our hypothesis, We first conducted an analysis of publicly accessible single-cell RNA (scRNA) sequencing data (GEO: #GSE150703) comparing 1.5 and 16 month-old mice bone marrow cells. We categorized individual cells from bone marrow into 16 distinct clusters (Fig. 1A). We found osteoblasts and chondrocytes in bone marrow of 16 month-old mice were significantly reduced compared with 1.5 month-old mice, while adipocytes were significantly increased (Fig. 1B). Analysis of differentially expressed genes showed that the expression of *Gai1/3* was decreased in 16 month-old mice osteoblasts (Fig. 1C). Weakened osteogenic ability in older male^[31]. To confirm the correlation between *Gai1/3* and osteogenic function, the expression of *Gai1/3* in BMSCs in young and old mice was examined. The results showed that the expression of *Gai1/3* was decreased in elderly BMSCs (Fig. 1D).

Next, we analyzed the differentially expressed genes to uncover co-expressed genes with *Gai3* in osteoblasts of 1.5 and 16 month-old mice. Functional enrichment analysis identified the top 10 biological processes (BPs) associated with *Gai3*, revealing significant involvement in angiogenesis-related pathways, including “Ossification”, “Osteoblast differentiation”, “Cell migration” and others (Fig. 1E-F). The KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment results for *Gai3* CEGs (Co-Expressing Genes) highlighted 10 key pathways (Fig. 1G), including “ TGF-beta signaling pathway”, “PI3K-Akt signaling pathway”, with a subset being functionally linked to osteogenic regulation.

Furthermore, BMSCs from senile osteoporosis were extracted and induced osteogenic differentiation. ALP and Alizarin red staining showed that the osteogenic ability of

BMSCs in elderly patients was significantly lower than BMSCs in young patients (Fig. 1H-I). The expression of *Gai1/3* in BMSCs of senile osteoporosis is significantly lower than that in BMSCs of young people (Fig. 1J). These results suggest that *Gai1/3* is related to the osteogenic ability of osteoblasts or BMSCs.

2. Loss of *Gai1/3* reduces bone formation ability and bone mass

Gai1/3 is involved in mice skeleton development^[32]. To test whether *Gai1* and *Gai3* deficiency could impair bone formation in vivo, we constructed mice with deletion of *Gai1/3* gene (*Gai1/3* DKO). We next performed the whole-mount skeletal staining of mice 3 days after birth with alcian blue and alizarin red^[33]. Newborn mutant pups (*Gai1/3*-DKO) showed small size and growth retardation compared with the wild-type littermates (Fig. 2A). In particular, the membranous ossification of the skull was impaired and clavicle more shorter in *Gai1/3*-DKO newborns when compared with the control littermates (Fig. 2B-C). The above results suggest that *Gai1/3* plays an important role in skeleton development and bone formation.

We performed microcomputed tomography (μ -CT) scans and three-dimensional reconstructions on femur metaphysis of 6-weeks and 40-weeks-old mice. The bone mass of *Gai1/3*-DKO mice was lower than that of wild type mice (Fig. D-E). The μ CT analysis revealed there were decreases in the trabecular bone mineral density (BMD), trabecular bone volume (BV), trabecular bone volume/tissue volume (BV/TV), trabecular numbers (Tb.N.), and trabecular thickness (Tb.Th.), while trabecular separation (Tb. Sp.) increased significantly in *Gai1/3* knockout (Fig. 3F). However, the reduction of cortical bone mineral density (BMD), bone volume (BV) and cortical bone volume/tissue volume (BV/TV) were not very obvious in *Gai1/3*-DKO mice (Fig. 3G). Taken together, these data suggest that bone mass was reduced in *Gai1/3* knockout mice.

In agreement with the above results, HE staining of femurs showed that the trabecular bone area and the number of osteoblasts on the bone surface (N.OB/B.S) were decreased in *Gai1/3*-DKO mice compared to WT mice (Fig. 2H-I). Immunofluorescence staining showed that the number of Sp7 positive cells in the bone marrow of *Gai1/3*-DKO mice was significantly lower than that of WT mice (Fig. 2J-

K). The dual-labeled xylenol orange and calcein double labeling revealed that *Gai1/3*-DKO mice had significantly lower cortical bone mineral apposition rate (MAR) compared with those of their littermate controls (Fig. 2L-M). The above results reflect that *Gai1/3*-DKO mice have insufficient bone formation ability and low bone mass.

3. Knockout *Gai1/3* blocked BMP2-induced activation of Akt-mTOR and Erk and Smads in MEFs

BMP2 is important to bone development, maintenance, and regeneration [9, 34]. BMP2 promotes osteogenesis by activating downstream signals such as Akt, Erk, Smads [35, 36]. Conversely, blocking Akt, Erk, Smads would inhibit osteogenesis [37, 38]. Our previous studies have shown that *Gai1/3* bind to multiple receptors to mediate downstream signal transduction [16, 17, 39]. Here, we tested whether *Gai1/3* would also associate with BMP2 receptor in mouse embryonic fibroblasts (MEFs).

BMP2-mediated phosphorylation events were compared between WT MEFs and *Gai1/3*-deficient cells [16, 17, 39]. BMP2 treatment (5-100 ng/mL) significantly enhanced phosphorylation of Akt, S6K, Erk1/2 and Smad1/5 in WT MEFs, demonstrating activation of Akt-mTOR, Erk and Smad pathways (Fig. 3A-B). Strikingly, this activation was nearly abolished in *Gai1/3* DKO MEFs, despite comparable total protein levels (Fig. 3A-B). Time-course analysis using 20 ng/mL BMP2 (the optimal activating concentration) revealed progressive phosphorylation of Akt, S6K, S6, Smad1/5 and Erk1/2 in WT MEFs, with complete impairment in DKO cells (Fig. 3C-D).

Further studies in MEFs demonstrated that *Gai1* or *Gai3* single knockouts (SKO) resulted in partial inhibition of Akt, S6K, Erk1/2, Smad1/5 phosphorylation in response to BMP2 (Fig. 3E-F), while *Gai1/3* DKO provided almost complete blockage (Fig. 3E-F). In MEFs, *Gai3* SKO is more potent than *Gai1* SKO in suppressing BMP2-induced activation of AKT-mTOR, Erk, Smad1/5 (Fig. 3E-F). Contrarily, BMP2-induced Akt, S6K, Erk1/2, Smad1/5 phosphorylation were equivalent between WT and *Gai2* SKO MEFs (Fig. 3G-H). Together, these results show that *Gai1/3* DKO blocks BMP2-induced AKT-mTOR, Smads and Erk activation in MEFs.

To further demonstrate that *Gai1/3* are required for BMP2-induced signaling, a lenti CRISPR/Cas9-*Gai1* KO construct and a lenti-CRISPR/Cas9-*Gai3* KO construct were co-transfected into WT MEFs, and BMP2-induced phosphorylation Akt, Erk1/2, Smad1/2 were blocked as well (Fig. 3I-L). Next, we exogenously expressed adenovirus *Gai1* construct (“Ad-*Gai1*”^[18]) or adenovirus *Gai3* construct (“Ad-*Gai3*”^[18]) in *Gai1/3* DKO MEFs, and we found that BMP2-induced phosphorylation of Akt-S6K, Erk1/2, Smad1/5 were partially restored in DKO MEFs following ectopic re-expression of *Gai1* or *Gai3* (Fig. 3I-L).

4. *Gai1/3* are required for BMP2-induced signaling activation in MC3T3-E1 and BMSCs

In order to verify the key role of *Gai1/3* in BMP2 signal transduction in osteoblasts, we utilized the small interfering RNA (siRNA) strategy. As described^[17, 18], *Gai1* siRNA and *Gai3* siRNA were co-transduced into MC3T3-E1. *Gai1* plus *Gai3* double knockdown blocked BMP2-induced phosphorylation of Akt, S6K, Erk1/2, Smad1/5 (Fig. 4A-B). Significantly, single knockdown of *Gai1* or *Gai3* with siRNA oligos specific for *Gai1* and *Gai3* inhibited BMP2-induced Akt-mTOR, Erk, Smads activations in MC3T3-E1 (Fig. 4A-B). To silence *Gai1/3*, *Gai1* and *Gai3* shRNA lentiviral particles [22, 29] were co-added to MC3T3-E1, and following selection stable cells formed: “sh*Gai1/3*” MC3T3-E1. BMP2-induced Akt, S6K, Smad1/5 and Erk1/2 phosphorylation were almost completely blocked (Fig. 4C-D). Total Akt, S6K and Erk1/2 expression were intact (Fig. 4C-D). *Gai1/3* are required for BMP2-induced signaling activation in MC3T3-E1

Subsequently, the above conclusions were further verified in BMSCs. To elucidate the functional significance of *Gai1/3* in BMP2 signaling, we performed genetic manipulation in BMSCs through both knockout and overexpression approaches. CRISPR/Cas9-mediated depletion of *Gai1/3* (“Cas9-*Gai1/3*” BMSCs) significantly attenuated BMP2 (20 ng/mL)-induced phosphorylation of Akt (Ser-473), S6K, Smad1/5 and Erk1/2 (Fig. 4E-F). Conversely, lentiviral co-transduction of *Gai1/3* expression vectors (“OE-*Gai1/3*” BMSCs) markedly enhanced these phosphorylation

events (Fig. 4G-H). Consistent with these findings, shRNA-mediated knockdown of *Gai1/3* nearly abolished BMP2-triggered activation of these signaling molecules (Fig. 4I-J). These genetic interventions collectively demonstrate that *Gai1/3* are essential positive regulators of BMP2 signaling in BMSCs. The above results further confirm that *Gai1/3* is a regulatory protein necessary for BMP2 signal transduction.

5. *Gai1* and *Gai3* are required for osteogenic function mediated by BMP2

Previous studies have shown that BMP2 activates Akt, MAPK, Smad to promote osteogenesis of MC3T3-E1 and BMSCs [35, 40]. Above results show that *Gai1/3* are required for BMP2-induced Akt-mTOR, Erk, Smad1/5 activation in BMSCs and MC3T3-E1. Subsequently, we focused on exploring the effect of *Gai1/3* on osteogenic function mediated by BMP2.

Knockdown *Gai1/3* in BMSCs (*Gai1/3*-shRNA) by lentivirus transfection. The EdU staining results suggest that knockdown of *Gai1/3* inhibits the activity of BMSCs and blocks the promoting effect of BMP2 therein (Fig. 5A). Furthermore, the expression of Runx2, Sp7, OPN proteins was significantly downregulated when *Gai1* and *Gai3* absence (Fig. 5B). As expected, qPCR test results were consistent with the above results, BMP2 couldn't effectively induce the expression of *Runx2*, *OPN*, *Sp7* mRNA in *Gai1/3*-shRNA BMSCs (Fig. 5C). In Fig.1-2, we confirmed that *Gai1/3* affects BMP2 signal transduction, and signals such as Akt-mTOR, Erk, and Smad1/5 can promote the expression of osteogenesis-related proteins such as Runx2, Sp7, and OPN. We believe that the decreased expression of Runx2, Sp7 and OPN in *Gai1/3* deficient BMSCs is mainly caused by the inhibition of osteogenesis-related signals.

Extract BMSCs from WT and *Gai1/3*-DKO mice. Mineralization of BMSCs was measured by ALP and Alizarin Red staining. Compared to WT, results showed the mineralization of BMSCs from *Gai1/3*-DKO mice decreased. BMP2 was unable to promote mineralization capacity of BMSCs with *Gai1/3* defect (Fig. 5D-E). These results indicate that *Gai1* and *Gai3* are required for BMP2-induced osteogenesis. Knockout of *Gai1/3* inhibits BMP2-induced osteogenesis.

To further verify the above conclusion, different lentiviruses were introduced into BMSCs to achieve knockdown and overexpression of *Gai1/3*. On the basis of the above, osteogenic differentiation of BMSCs was induced, and the mineralization ability was detected by ALP and Alizarin Red staining. The results showed that after knocking down *Gai1/3* (sh-*Gai1/3*), the osteogenic ability of BMSCs decreased significantly. Conversely, overexpression of *Gai1/3* (OE-*Gai1/3*) increased significantly enhanced the osteogenic ability of BMSCs. More importantly, after knocking down *Gai1/3*, the osteogenic effect of BMP2 basically disappeared, and overexpression of *Gai1/3* significantly enhanced the osteogenic effect of BMP2 (Fig. 5F-G). These results further confirm that *Gai1* and *Gai3* are required for osteogenic function mediated by BMP2.

6. Loss of *Gai1/3* inhibits the osteogenesis induced by BMP2 in vivo.

Following a previously described protocol ^[20, 21], AAV9-mRunx2-*Gai1* shRNA and AAV9-mRunx2-*Gai3* shRNA were injected to femoral of C57B/6 mice. This led to BMSCs knockdown of *Gai1/3* (*Gai1/3*-CKD) as the viral construct contained the binding sequence for the BMSCs-specific promoter, Runx2 (Fig. S1A-B). After confirming that *Gai1/3* affects BMP2 signal transduction and BMP2-mediated osteogenesis, an established mouse femoral fracture model was performed to compare the healing process of bone fracture between "*Gai1/3*-CKD" and "Vehicle" mice with or without BMP2 treat. At week 2, the 3D reconstructed images of micro-CT confirmed the gap in the fracture sites almost disappeared in the WT group treated with BMP2, whereas it remained visible in *Gai1/3*-CKD group with BMP2 treatment (Fig.6A), which indicated that BMP2 cannot effectively promote bone regeneration and fracture healing in *Gai1/3*-CKD mice. Microcomputed tomography (μ -CT) scans analysis of the femur revealed that the reduces of callus mineral bone density (BMD), callus volume (CV), and callus volume/tissue volume (CV/TV) in *Gai1/3*-CKD group after BMP2 treatment were very obvious compare to Vehicle group treated with BMP2 (Fig.6B).

The H&E and Masson staining were performed to evaluate the histological properties of newly mineralized bone tissues. As shown, the fracture site of the *Gai1/3*-

CKD+BMP2 group was covered by less calluses, and the bone remodeling was more slowly when compared with WT group treated with BMP2 at week 2 post-surgery (Fig.6C). Moreover, by immunohistochemistry (IHC) staining and semiquantitative scoring, the decreased Runx2, OPN, Sp7 expressions were observed in the *Gai1/3*-CKD group. BMP2 cannot promote Runx2, OPN, Sp7 expressions in vivo (Fig.6D-F), suggesting the beneficial effect of *Gai1/3* on bone formation and remodeling when mediated by BMP2.

To further investigate the physiological role of *Gai1/3* in the determination of BMSCs commitment to the osteoblast lineage, we generated *Col1a1*-cre; *Gai*^{fl/fl} mice (hereafter referred to as *Gai1/3*^{BMSC-} mice) by crossing *Gai*^{fl/fl} mice with *Col1a1*-Cre (*Gai*^{BMSC+}) mice (Fig. S1C-D). *Gai* was knocked out in BMSCs *Gai*^{BMSC-} mice. Newborn *Gai*^{BMSC-} mice exhibited a significantly smaller body size than their control littermates (Fig. 6G). Moreover, a delay in bone formation was observed in the skull, clavicles of newborn *Gai*^{BMSC-} mice (Fig. 6H-I). Unfortunately, *Gai*^{BMSC-} mice have difficulty surviving for more than one week. These results indicate that *Gai1/3* plays an important role in BMP2-mediated bone formation.

7. The adaptor protein Gab1, downstream of *Gai1/3*, mediates BMP2-induced signaling activation

Following RTK stimulation, Gab1 translocates to the plasma membrane where it undergoes phosphorylation, subsequently functioning as a scaffold protein to facilitate downstream signaling propagation^[41]. Our findings reveal that EGFR activation induces Gab1-*Gai* complex formation, facilitating signal transmission from EGFR-*Gai* to the p85 regulatory subunit^[16]. BMP receptors represent a unique class of human transmembrane proteins possessing intrinsic serine/threonine kinase activity^[14]. Inhibition of BMPRI/II endocytosis can inhibit BMP2-induced signaling and osteogenesis^[42]. Ligand-binding events induce the transcription of multiple osteogenesis-associated genes through the activation of a complex array of downstream intracellular mediators that includes PI3K-Akt-mTORC1, Erk-MAPK, and Smads pathway^[13, 14, 43]. Previously, our research group confirmed that *Gai1/3* and Gab1 are

coupled to mediate the activation of downstream signal pathways under the stimulation of BDNF and VEGF^[17, 18]. However, the role of Gab1 in serine/threonine kinase receptor signal transduction is not clear. We speculate that Gai1/3 may mediate BMP2 downstream signal transduction through coupling and activating Gab1.

In western blotting studies, we found that Gai1/3 DKO blocked BMP2-induced Gab1 activation (Tyr-627 phosphorylation, Fig. 7A-B), whereas Gai2 SKO was ineffective in Gab1 activation by BMP2 (Fig. 7C). In WT MEFs, Gab1 activation by BMP2 was significantly attenuated by the knockout of Gai1 and/or Gai3 (Fig. 7D). In Gai1/3 DKO MEFs, re-expression of Gai1 or Gai3 partially restored BMP2-induced Gab1 activation (Fig. 7E). In BMSCs, overexpression of Gai1/3 also promoted BMP2-induced Gab1 activation (Fig. 7F). Conversely, in Gai1/3-shRNA BMSCs, the ability of BMP2 to induce Gab1 phosphorylation was significantly reduced (Fig. 7G). These results show that Gai1/3 are essential for BMP2-induced Gab1 activation, a necessary step for downstream Akt, Erk1/2, Smad1/5 activation of serine/threonine kinase receptors.

In Gab1 KO BMSCs, BMP2-induced phosphorylation of Akt, S6K, Erk1/2 and Smad1/5 were significantly blocked (Fig. 7H-J). Thus, Gab1 plays an important role in the activation process of BMP2-induced signal a mediated by Gai1/3. Gab1 is an important adaptor protein for downstream signal activation of serine/threonine kinases.

Gai1/3 DKO BMSCs were transiently transfected with the adenovirus Gab1 construct ("Ad-Gab1"), the adenovirus Gai3 construct ("Ad-Gab1") or the empty vector ("Vecor"). Overexpression of Gab1 in Gai1/3 DKO BMSCs did not restore the transduction of downstream signals by BMP2. The Western blot results showed that the phosphorylation of Akt, S6K, Erk1/2 and Smad1/5 was significantly inhibited in Gai1/3 DKO BMSCs. Introducing Gab1(Ad-Gab1) into Gai1/3 DKO BMSCs had no significant effect on the phosphorylation of downstream proteins (Fig. S2A). Inducing osteogenic differentiation of the above-mentioned BMSCs, ALP and alizarin red staining showed that introducing Gab1 into Gai1/3 DKO BMSCs did not restore the osteogenic capacity of BMSCs (Fig. S2B-C). These results suggest that Gai1/3 affect downstream signal transduction and osteogenesis through Gab1.

8. Mutants of *Gai1* and *Gai3* disrupt BMPR1A/B binding of adaptor proteins and prevent activation of downstream signaling

Protein-protein interaction analysis (PyMOL) identified and categorized functional residues based on their binding patterns. Hydrogen bond formation was observed between *Gai3* and BMPR1A/B, as well as between *Gai3* and Gab1 (Fig. 8A-C). In addition, there are multiple groups of residues for *Gai1* and BMPR1A, BMPR1B, Gab1 (Fig. S3A-C). With these interaction forces, *Gai1/3* may mediate the binding of BMPR1A/B to Gab1, thereby activating downstream signals.

The co-immunoprecipitation (Co-IP) results showed that following BMP2 stimulation, *Gai1/3* associated with BMP2 receptors (BMPR2, BMPR1A/B) and Gab1 in BMP2 (Fig. 8D and Fig. S3D). Consistently, in situ proximity ligation assay (PLA) confirmed that the direct interaction between endogenous BMPR1A/B and Gab1 was abolished in *Gai1/3*-DKO BMSCs (Fig. 8C). Introducing *Gai1/3* into *Gai1/3*-DKO BMSCs restored the mutual binding of BMPR1A/B and Gab1. Introducing *Gai1/3* into *Gai1/3*-DKO BMSCs restored the mutual binding of BMPR1A/B and Gab1 (Fig. 8D, Red). The above results confirm that *Gai1/3* binds to BMP2 receptors and Gab1, mediating downstream signal transduction.

To investigate *Gai1/3*-dependent BMP2 signaling mechanisms, we employed dominant-negative (DN) constructs [43], in which the conserved Gly (G) residue in the G3 box was substituted with Thr (T) to disrupt *Gai1/3*-protein interactions^[44]. The DN-*Gai1* and DN-*Gai3* constructs were co-transduced into WT BMSCs, and after selection stable BMSCs, “DN-*Gai1/3*”, formed. BMPR1A binds to BMP2 with high affinity and is the main receptor for BMP2 signaling^[45, 46]. Here, we focus on the impact of *Gai1/3* mutation on BMPR1A. Co-IP assay results shown in Figure 8A demonstrate that DN-*Gai1/3* disrupts BMP2 While BMP2 treatment induced BMPR1A-Gab1 binding (without affecting expression, Fig. 8E Input), this interaction was strictly dependent on functional *Gai1/3* (Fig. 8F). Western analysis verified DN mutant expression (Fig. 8E Input). Importantly, DN-*Gai1/3* expression markedly suppressed BMP2-triggered

Smad1/5, Akt and Erk1/2 phosphorylation (Fig. 8G). This implies Gαi1/3 dominant negative mutants may disrupt BMP2-induced BMP2 receptors associate with Gab1 association, BMP2 signaling activation. The results of ALP and Alizarin Red staining showed that the mutation of Gαi1/3 inhibited BMP2 and promoted osteogenesis of BMSCs (Fig. 8H-I). In conclusion, mutants of Gαi1 and Gαi3 disrupt BMPR1A/B binding of adaptor proteins and prevent activation of downstream signaling.

Discussion

Previous study has confirmed penetrance and expressivity of the rib fusion phenotype is altered in mice knockout Gαi^[30]. However, the molecular mechanism of Gαi1/3 in bone development and bone formation is not clear. Our research highlights the pivotal roles of Gαi1 and Gαi3, key members of the Gαi protein family, in the process of bone formation. We found that Gαi1/3 expression was abnormally decreased in senile osteoporosis. Our results corroborate the findings of Lutz Birnbaumer by demonstrating delayed skeletal development in newborn Gαi1/3-DKO mice^[30]. In addition bone mass and quality decreased in Gαi1/3-DKO mice. These results suggested that Gαi1/3 may be involved in bone formation and development. The molecular mechanisms through which Gαi mediates bone formation was investigated. Especially the role and mechanism of Gαi1/3 in BMP2 signal transduction.

Previous studies have found that Gα(i) subfamily of heterotrimeric G proteins regulate development of skeletons^[30]. Lutz Birnbaumer et al. believe that Gαi may affect signals such as BMPs, Wnt, Hedgehog, etc., which are closely related to bone development. However, it is not for studying the role of Gαi in the signal transduction of BMPs, Wnt, Hedgehog, etc^[30]. Besides, Gαi3 knock-down significantly decreased the odonto/osteogenic differentiation ability of stem cells of dental apical papilla (SCAPs)^[47]. Based on the above research, this study explored the expression of Gαi in BMSCs and osteoblasts. With aging, both in mice and humans, the osteogenic capacity of bone marrow mesenchymal stem cells (BMSCs) or osteoblasts declines, which is accompanied by reduced expression of Gαi1/3. In contrast, no significant change was observed in the expression of Gαi2.

Therefore, in the subsequent study, we focused on the role of *Gai1/3* in bone formation. In *Gai1/3*-DKO mice, the skeletal development of neonatal mice was delayed. The bone mass of adult mice was lower than that of control mice, and their bone formation ability was insufficient. Although *Gai2* shows no change in senile osteoporosis, its influence on bone metabolism cannot be ruled out. Perhaps *Gai2* only plays a specific role in skeleton development. The role of *Gai2* in bone metabolism is still worthy of further exploration.

In the physiological process of bone formation and regeneration, expression of BMP2 and its binding to the BMPRI/II receptor lead to activation of multiple downstream signal pathways including Erk, PI3K-Akt-mTOR, and Smads^[14, 48]. Activation of these cascades promote BMSCs and Osteogenic precursor cells proliferation, differentiation, and osteogenesis^[14]. Previous studies have demonstrated that impaired signal transduction in response to BMP-2 underlies the delayed fracture healing and osteoporosis^[49]. Recombinant human bone morphogenetic protein-2 (hrBMP-2) has been used in clinical to treat delayed fracture healing^[50]. However, the underlying mechanisms of BMP2-induced activation of Akt, Erk, Smads remain largely unknown.

The present study provides a novel mechanism of the role of *Gai1/3* in BMP2 signal transduction and osteogenesis. We show that *Gai1/3* are essential players in BMP2-induced Akt, Erk, Smads signaling activation. In BMSCs, MC3T3-E1 or MEFs, *Gai1/3* KO/shRNA, CRISPR-induced *Gai1/3* gene editing, or dominant negative *Gai1/3* mutations potentially inhibited BMP2 induced downstream activation of Akt, Erk, Smads. Contrarily, overexpression of *Gai1/3* facilitated Akt activation by BMP2. Functional studies demonstrated that MC3T3-E1/BMSCs with *Gai1/3* deficiency were refractory to osteogenesis. Thus, *Gai1/3* are required for BMP2-induced downstream activation of Akt, Erk and Smads, which then lead to osteogenesis.

Furthermore, we constructed *Gai1/3* knockdown mice (*Gai1/3*-CKD) in BMSCs. In *Gai1/3*-CKD mice, fracture healing is delayed, and the bone healing promoting effect of BMP2 disappears. Although we failed to construct a fracture model in *Gai1/3* conditional knockout mice (*Gai1/3*^{BMSC-} mice), we confirmed that bone development

was delayed in $G\alpha i1/3^{BMSC-}$ mice. The above results all indicate that $G\alpha i1/3$ plays an indispensable and crucial role in bone formation, and its deficiency will inhibit the osteogenic effect of BMP2.

Although we have broken through the traditional view and confirmed that $G\alpha i1/3$ can mediate the signal transduction of serine/threonine kinases type receptors (BMPRI/II). However, how $G\alpha i1/3$ functions remains unclear. $Gab1$ functions as a critical scaffolding adaptor that mediates essential signaling transduction pathways [51]. Accumulated evidence indicate that $Gab1$ proteins can function to activate SHP2 phosphatase, which in turn activates MAPK signaling [52]. In addition, the $Gab1$ -p85 interaction is important in activating the PI3K/Akt pathway in mammalian cells [53, 54]. Activation of Akt and Erk and osteoblast function were impaired in $Gab1$ -deficient osteoblasts [51].

Prior work from our group has established that $G\alpha i1/3$ mediate $Gab1$ recruitment and activation downstream of multiple growth factors in RTK signaling pathways [16, 39]. In the present study, we also show that $G\alpha i1/3$ are essential for $Gab1$ activation in response to Although we have broken through the traditional view and confirmed that $G\alpha i1/3$ can mediate the signal transduction of serine/threonine kinases type receptors (BMPRI/II). However, how $G\alpha i1/3$ functions remains unclear. (RS/TK). In $Gab1$ KO BMSCs, BMP2-induced Akt, Erk, Smads activation were completely blocked. Therefore, $G\alpha i1/3$ are required for BMP2-induced recruitment and activation of $Gab1$, facilitation of PI3K-Akt, and activation and osteogenesis of MAPK. Without functional $G\alpha i1/3$, $Gab1$ would not be recruited and activated in response to BMP2, which then causes defective downstream signaling and osteogenesis of PI3K-Akt, MAPK, Smads. We speculate that $G\alpha i1/3$ may also be involved in BMPRI/II (the BMP2 receptor). Subsequent studies have also confirmed that $G\alpha i1/3$ can bind to BMPRI/II- $Gab1$. The detailed mechanisms may require further characterization.

In this study, we found that the expression of $G\alpha i1/3$ in the bone tissue of patients with osteoporosis is aberrant. In $G\alpha i1/3$ DKO mice, the bone mass and quality decreased. We confirmed BMP2 as an important cytokine to promote osteogenesis, while signal

transduction and osteogenesis are dependent on Gαi1/3. Loss of Gαi1/3 caused delayed fracture healing. In addition to BMP2 signaling, Hedgehog and Wnt signaling also participate in bone development, maintenance, and regeneration ^[55]. Wnt3a signaling to β-catenin is dependent on heterotrimeric G proteins ^[56]. As we found that Gαi1/3 mediates Wnt/β-catenin signal transduction. The Drosophila Hedgehog signaling cascade has been shown to engage G protein signaling through Smoothed-mediated mechanisms ^[57]. Suppression of Gnai2 and Gnai3 expression attenuates Shh-mediated proliferation in rat cerebellar granule cell precursors ^[58]. These evidences suggest that Gαi1/3 may regulate the signal pathways activated by multiple cytokines to affect osteogenesis.

In short, The results of this study suggest that Gαi1/3 could be novel and key mediators of BMP2 signal transduction osteogenesis. Targeting Gαi1/3 could provide a new therapeutic modality for fracture or osteoporosis.

Conclusions

This study establishes the Gαi1/3-Gab1 axis as a novel and essential signaling module for BMP2-induced osteogenesis. We demonstrate that Gαi1/3 are indispensable for BMP2 receptor (BMPRI/II) complex formation with the adaptor protein Gab1, which is required for the subsequent activation of downstream PI3K-Akt-mTOR, Erk, and Smad pathways both in vitro and in vivo. Genetic ablation of Gαi1/3 in mice leads to skeletal defects, reduced bone mass, and impairs BMP2-mediated fracture healing. Mechanistically, Gαi1/3 act as critical scaffolds bridging BMP2 receptors to Gab1, facilitating signal transduction. These findings expand the functional repertoire of Gαi proteins beyond GPCRs and RTKs to include receptor serine/threonine kinases. Consequently, targeting the Gαi1/3-Gab1 axis presents a promising therapeutic strategy for enhancing bone formation and treating conditions such as fractures and osteoporosis.

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Figure1. *Gai1* and *Gai3* are low expressed in aging osteoblasts or BMSCs, and participates in osteogenic function and signaling. The tSNE plot of visualization of the publicly available bone marrow cells scRNA-seq data (GEO: #GSE150703) of 1.5 and 16 month-old mice (A). Proportion of cell types in bone marrow of 1.5 and 16 month-old mice (B). Expression of *Gai1* and *Gai3* in osteoblasts of 1.5 and 16 month old mice (C). Expression of *Gai1* and *Gai3* in BMSCs of young and old mice (D). Biological Processes (BP, E-F) and KEGG pathways (G) of differentially expressed genes in osteoblasts of 1.5 and 16 month old mice. Expression of *Gai* subunits in BMSCs of young and old male. Representative images and quantitative analysis of Alp staining and Alizarin red staining of BMSCs from young and old male (H-J). ***p < 0.001, **p < 0.01, Data were presented as mean ± standard deviation (SD).

Figure 2. Loss of *Gai1/3* reduces bone formation ability and bone mass. Alcian blue and Alizarin red staining of the whole skeletons, calvarium and clavicle of the newborn WT and *Gai1/3* DKO mice, and statistical analysis (A-C). μ CT analysis of distal femurs from 6 and 40-week-old WT and *Gai1/3* DKO mice. Representative three-dimensional reconstruction of μ CT images (G, H). Cortical BMD, BV/TV and BV (I-K) of *Gai1/3*-DKO mice and control littermates. BMD, BV/TV, BV, Tb.Th, Tb.N, Tb.Sp of *Gai1/3* DKO mice of trabecular bone and control littermates (D-G). BMD, bone mineral density, BV, bone volume, BV/TV, bone volume/tissue volume Tb. N, trabecular numbers, Tb.Th, trabecular thickness, Tb. Sp, trabecular separation, Data were presented as mean ± standard deviation (SD). Representative images of H&E staining of femur sections from *Gai1/3*-DKO (H) and quantification of the number of osteoblasts in bone surface(I). Representative images of immunofluorescence (Sp7, Red) staining of femur sections from *Gai1/3*-DKO (J) and quantification of the number of Sp7 positive cells in bone tissue (K). Calcein labeling to visualize bone formation, with quantification of mineral apposition rate (MAR) (L-M). ***P < 0.001; **P < 0.01; *P < 0.05 versus WT. n=5 per group.

Figure 3. Knockout *Gai1/3* blocked BMP2-induced activation of Akt-mTOR and Erk and Smads in MEFs. Wild-type (WT), *Gai1* and *Gai3* double knockout (*Gai1/3*-DKO) (A-D). *Gai1*, *Gai2* or *Gai3* single knockout (SKO) (E-H) mouse embryonic fibroblasts (MEFs) were treated with BMP2 (20ng/mL) for applied time, tested by Western blotting of listed protein. For all the Western blotting assay, each lane was loaded with exact same amount of quantified protein lysates (20 μ g in each treatment), the same set of lysate samples were run in parallel (“sister”) gels to test different proteins (Same for all Figures). For the quantification of Western blotting, the indicated proteins were quantified using the ImageJ software (B,D,F,H). *Gai1/3* DKO MEFs were transiently transfected with the adenovirus *Gai1* construct (“Ad-*Gai1*”), the adenovirus *Gai3* construct (“Ad-*Gai3*”) or the empty vector (“Vecor”), Different groups were cultured DMEM medium supplemented with BMP2 (20 ng/ml) for indicated time, the proteins listed were examined by Western blotting and phosphorylation (vs total proteins) was quantified (I-L). Quantification was performed from five replicate blot data (n=5, same for the blotting data in all Figures). Data were expressed as mean ± standard deviation (SD, same for all Figures).****P < 0.000, ***P < 0.001, **P < 0.01 vs. WT MEFs.

Figure 4. *Gai1/3* are required for BMP2-induced signaling activation in MC3T3-E1 and BMSCs. Stable MC3T3-E1 with the scramble control siRNA (“C-siRNA”), *Gai1* siRNA and/or *Gai3* siRNA, were treated with BMP2 (20 ng/mL) for applied time, and were tested by Western

blotting of listed proteins (A-B). Stable MC3T3-E1 with the scramble control shRNA (“Sc-shRNA”), *Gai1* shRNA and *Gai3* shRNA, were treated with BMP2 (20 ng/mL) for applied time, and were tested by Western blotting of listed proteins (C-D). BMSCs isolated from WT mice with the lenti-CRISPR/Cas9-*Gai1*-KO construct plus lenti-CRISPR/Cas9-*Gai3*-KO construct (“Cas9-*Gai1/3*-KO”), or the CRISPR/Cas9 control construct (“Cas9-C”), were treated with BMP2 (20 ng/mL) for applied time, and were tested by Western blotting of listed proteins (E, F). BMSCs were transfected with the adenovirus *Gai1* construct and adenovirus *Gai3* construct (OE-*Gai1/3*) or the empty vector. BMSCs in different groups were cultured in DMEM medium supplemented with BMP2 (20 ng/ml), the proteins listed were examined by Western blotting and phosphorylation was quantified (G-H). BMSCs isolated from WT mice were infected with the lentiviral *Gai1* shRNA and the lentiviral *Gai3* shRNA. Different groups were cultured and were treated with BMP2 (20 ng/ml) for 10 or 30 min, the proteins listed were examined by Western blotting and phosphorylation (vs total proteins) was quantified (I-J). n=5 per group. Data were expressed as mean $\pm$ standard deviation (SD, same for all Figures).****P< 0.000, ***P< 0.001, **P< 0.01.

Figure 5. *Gai1* and *Gai3* are required for osteogenic function mediated by BMP2. BMSCs isolated of from WT and *Gai1/3* DKO mice, and grown in the presence or absence of BMP2 (20 ng/mL). Cell activity (EdU staining) was examined at d5 (A). The expression of Runx2, OPN, Sp7 were examine by western blotting at different times(B). Runx2, OPN, Sp7, ALP mRNA were tested by qPCR at 1h (C). Extract BMSCs from WT and *Gai1/3*-DKO mice. Osteoblast differentiation was induced by medium containing or without BMP2. Mineralization (ALP and Alizarin Red staining) and quantification measured at d21 (D-E). BMSCs isolated of from WT mice were transfected the lentiviral *Gai1* shRNA and the lentiviral *Gai3* shRNA (sh-*Gai1/3*) , or the adenovirus *Gai1* construct and adenovirus *Gai3* construct (OE-*Gai1/3*), and grown in the presence or absence of BMP2 (20 ng/mL). Mineralization (ALP and Alizarin Red staining) and quantification measured at d21 (F-G). Statistical significance was obtained by Student’s t-tests (two-tailed). *p < 0.05; **p < 0.01; ***p < 0.001. Data are means $\pm$ SD.

Figure 6. Loss of *Gai1/3* inhibits the osteogenesis induced by BMP2 in vivo. Femoral fracture model was performed in "*Gai1/3*-CKD" and "Vehicle" mice, treat with or without BMP2 treat. Three-dimensional micro-CT images of the mouse femur fracture zone were taken 2 weeks after surgery, 3D μ CT renderings and quantification of Callus BMD, Callus number, Callus Volume, CV/TV of different groups (A-B). Representative images of H&E staining of femur fracture sections from mice at week2 post-surgery (C). Representative images of immunohistochemistry staining and semiquantitative analysis of OPN, Runx2 and Sp7 expression femur fracture sections from mice at week2 post-surgery (D-F). Alcian blue and Alizarin red staining of the whole skeletons, calvarium and clavicle of the newborn *Gai*^{BMSC+} mice and *Gai*^{BMSC-} mice mice, and statistical analysis (G-I). Statistical significance was obtained by Student’s t-tests (two-tailed). *p < 0.05; **p < 0.01; ***p < 0.001. Data are means $\pm$ SD.

Figure 7. The adaptor protein Gab1, downstream of *Gai1/3*, mediates BMP2-induced signaling activation. WT, *Gai1/3* DKO MEFs, *Gai1*, *Gai2*, *Gai1* KO MEFs were treated with BMP2 (20 ng/mL) for applied time period, p-Gab1 and Gab1 were shown (A-D). *Gai1/3* DKO MEFs were transiently transfected with the adenovirus *Gai1* construct (“Ad-*Gai1*”), the

adenovirus *Gai3* construct (“Ad-*Gai3*”) or the empty vector (“Vec”), BMP2-induced *Gab1* activation was tested (E). BMSCs were transiently transfected with the adenovirus *Gai1* and *Gai3* construct (“OE-*Gai1/3*”), BMP2-induced *Gab1* activation was tested (F). BMSCs were transfected the lentiviral *Gai1* shRNA and the lentiviral *Gai3* shRNA (*Gai1/3*-shRNA), BMP2-induced *Gab1* activation was tested (G). WT MEFs or *Gab1* knockout MEFs were treated with BMP2 (20 ng/mL), BMP2 (20 ng/mL)-activated signalings were tested, and protein phosphorylation was quantified (H-J). Quantifications were from five biological replicates. Statistical significance was obtained by Student’s t-tests (two-tailed). **p* < .05; ***p* < .01; ****p* < .001. Data are means ± SD.

Figure 8. Mutants of *Gai1* and *Gai3* disrupt BMPR1A/B binding of adaptor proteins and prevent activation of downstream signaling. The *Gai3* protein is represented as a slate cartoon model, BMPR1A/BMPR1B/*Gab1* protein is shown as a cyan cartoon model, and their binding sites are shown as the corresponding-colored stick structure. When focusing on the binding region, the binding site is then shown as a presentation of the protein to which it belongs (A-C). BMSCs were treated with BMP2 (20 ng/mL) for 30 min, BMPRs-*Gai1/3*-*Gab1* association (“IP”) and expression (“Input”) was shown (D). BMSCs isolated from WT and DKO mice were treated with BMP2 (20 ng/mL) for 30 min, In situ PLA technology indicated endogenous BMPR1A/B-*Gab1* protein interactions in BMSCs. Red, positive interaction complexes (D). Scale bar: 10μm. Stable WT BMSCs, with the DN mutant-*Gai1* (murine) construct plus DN-*Gai3* construct (“DN-*Gai1/3*”) or the vector control (“Vec”), were treated with BMP2 (20 ng/mL) for 30 min. The association of BMPR1A, Grb1, Shc, *Gai1*, and *Gai3* was examined by co-immunoprecipitation (Co-IP) assays (F), and their expressions are shown in “Input” (F). The listed proteins in membrane fraction lysates and total cell lysates were examined (G). The osteogenic ability of BMSCs (“DN-*Gai1/3*”, “Vec”) was detected by ALP and Alizarin Red staining at d21 (H-I). Statistical significance was obtained by Student’s t-tests (two-tailed). **p* < .05; ***p* < .01; ****p* < .001. Data are means ± SD.

Figure S1. The construction of *Gai1/3*-CKD and *Colla1*-Cre; *Gai1/3*^{fl/fl} mice. Schematic of the AAV9 vector components (A). Western blot analysis and quantitative analysis of *Gai1* and *Gai3* in BMSCs (B). Breeding strategy of LysmCre; *Gai3*^{fl/fl} mice (C). qPCR analysis of *Gai1* and *Gai3* in LysmCre; *Gai3*^{fl/fl} BMSCs (D). *n* = 3 per group. All bar graphs are presented as the mean ± SD. ns, *p* > 0.05; ****p* < 0.001.

Figure S2. Overexpression of *Gab1* cannot restore the signal transduction disorder and osteogenic function limitation caused by *Gai1/3* deficiency. *Gai1/3* DKO BMSCs were transiently transfected with the adenovirus *Gab1* construct (“Ad-*Gab1*”), the adenovirus *Gai3* construct (“Ad-*Gai3*”) or the empty vector (“Vecor”), Different groups were cultured DMEM medium supplemented with BMP2 (20 ng/ml) for indicated time, the proteins listed were examined by western blotting and phosphorylation (A). Osteoblast differentiation was induced by medium containing or without BMP2. Mineralization (ALP and Alizarin Red staining) and quantification measured at d21 (B-C). . *n* = 3 per group. All bar graphs are presented as the mean ± SD. ns, *p* > 0.05.

Figure S3. *Gai1* had binding sites with BMR1A/BMR1B/Gab1. The *Gai1* protein is represented as a slate cartoon model, BMR1A / BMR1B/Gab1 protein is shown as a cyan cartoon model, and their binding sites are shown as the corresponding-colored stick structure. When focusing on the binding region, the binding site is then shown as a presentation of the protein to which it belongs (A-C). BMSCs were treated with BMP2 (20 ng/mL) for 10, 30 min, BMPRs-*Gai1*-Gab1 association (“IP”) and expression (“Input”) was shown (D).

Figure 1

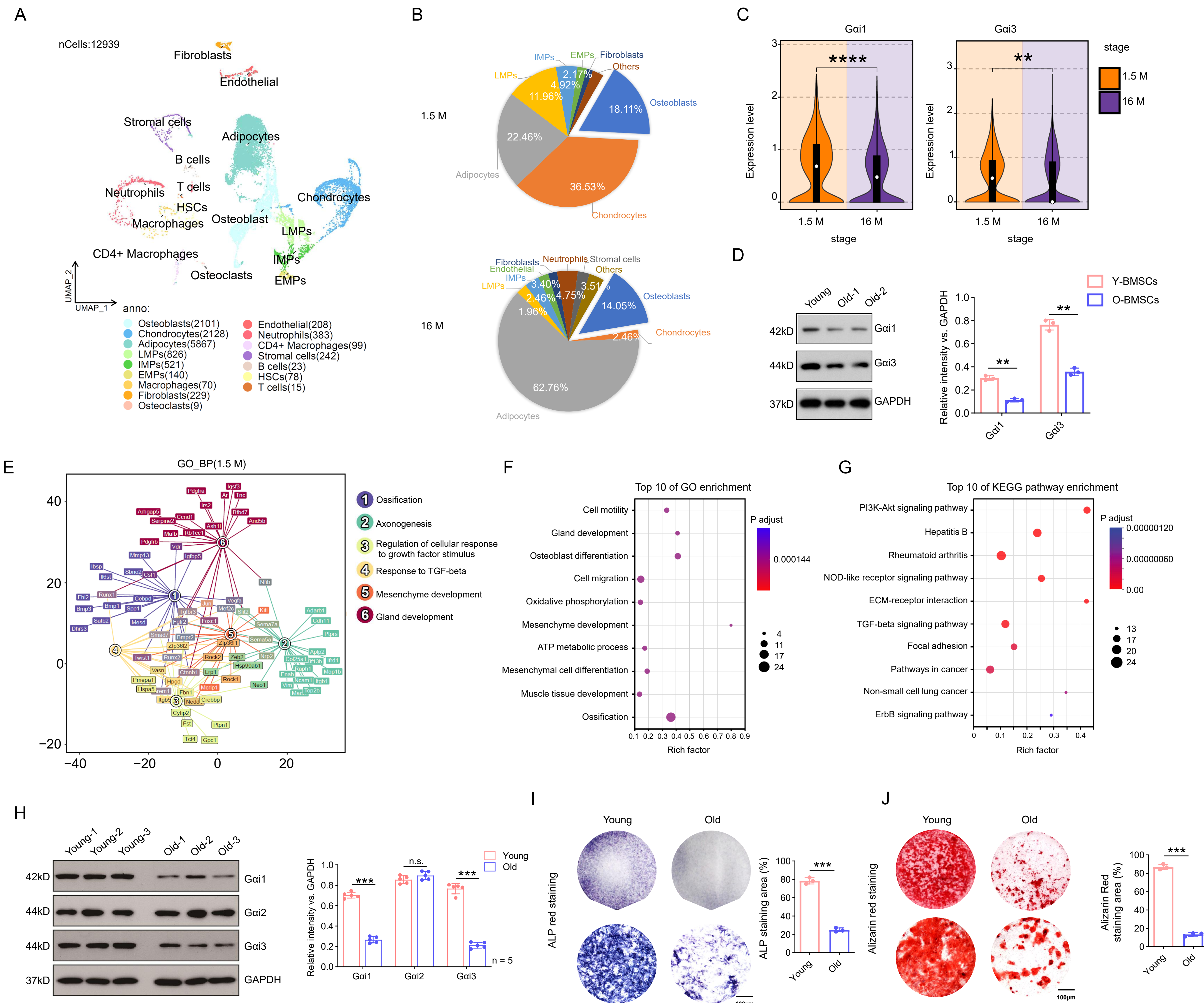


Figure 2

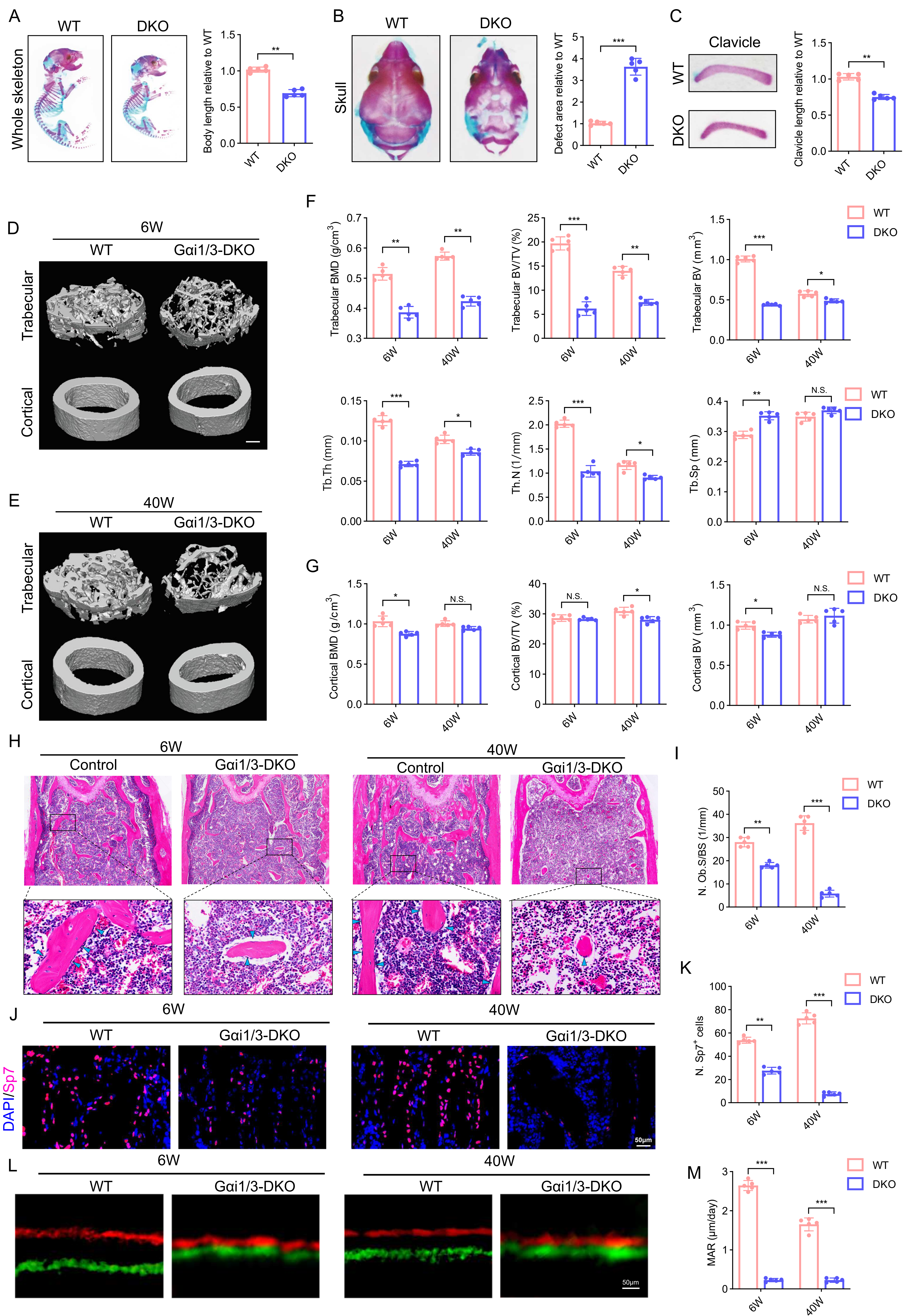


Figure 3

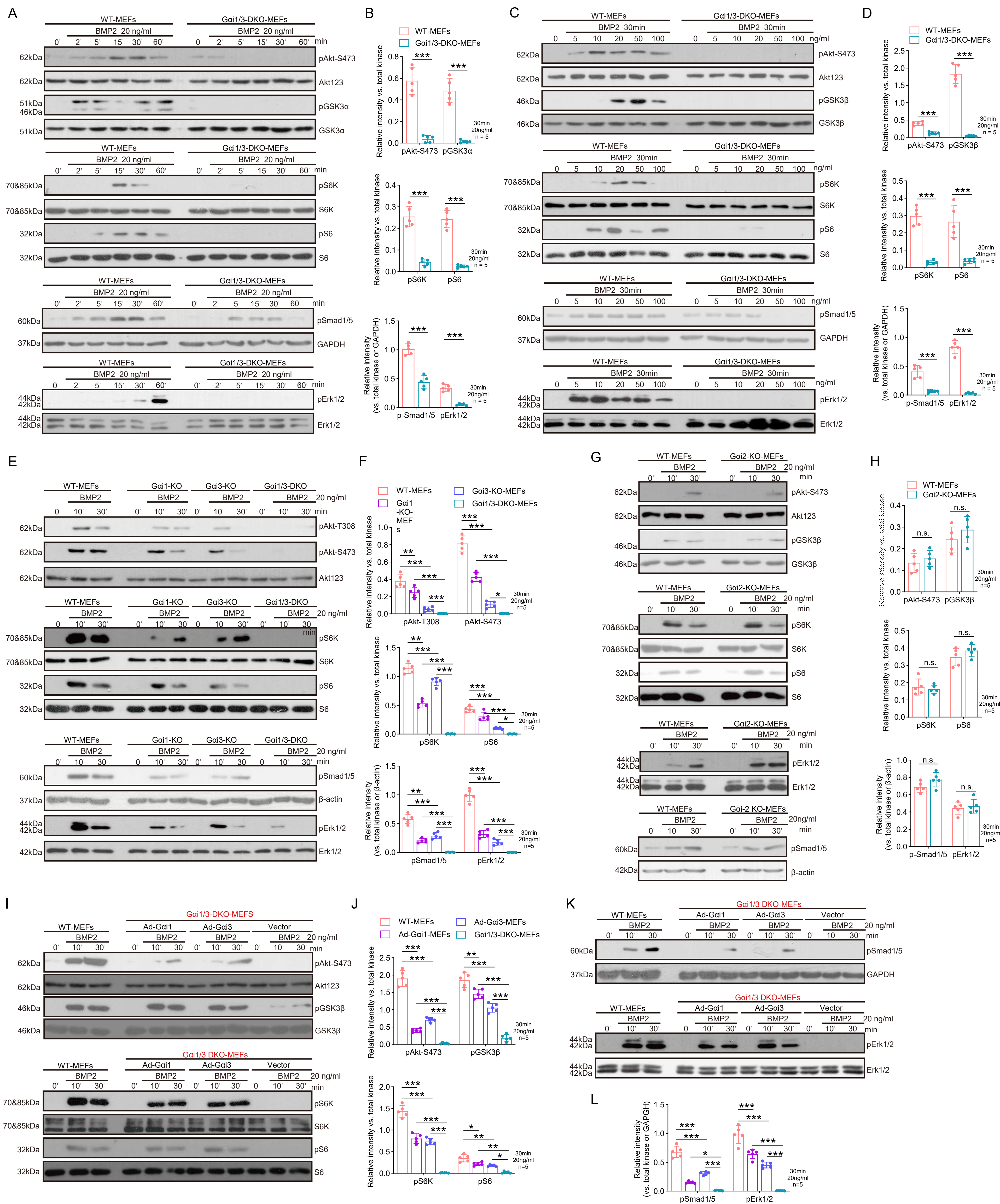


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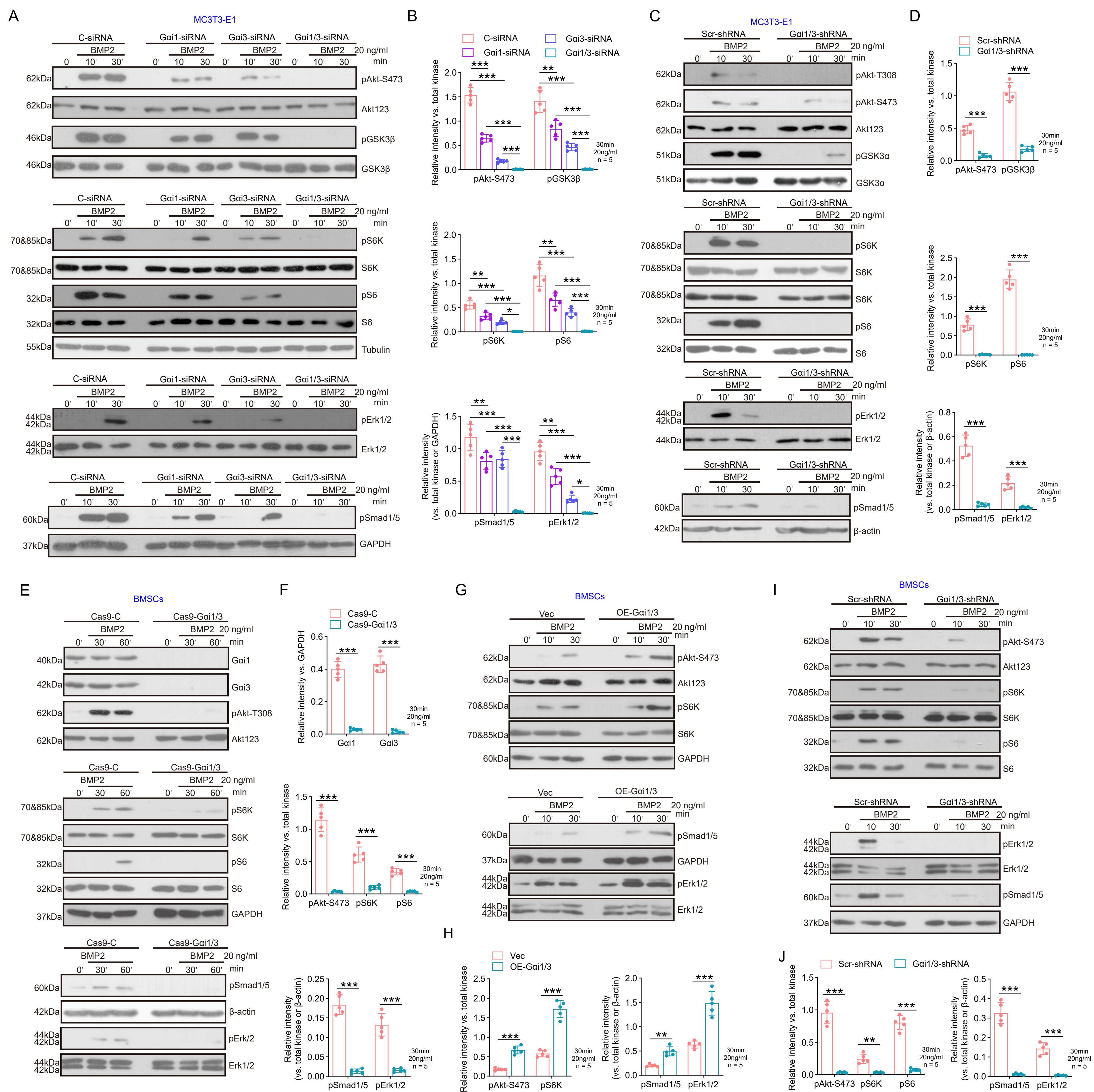


Figure 5

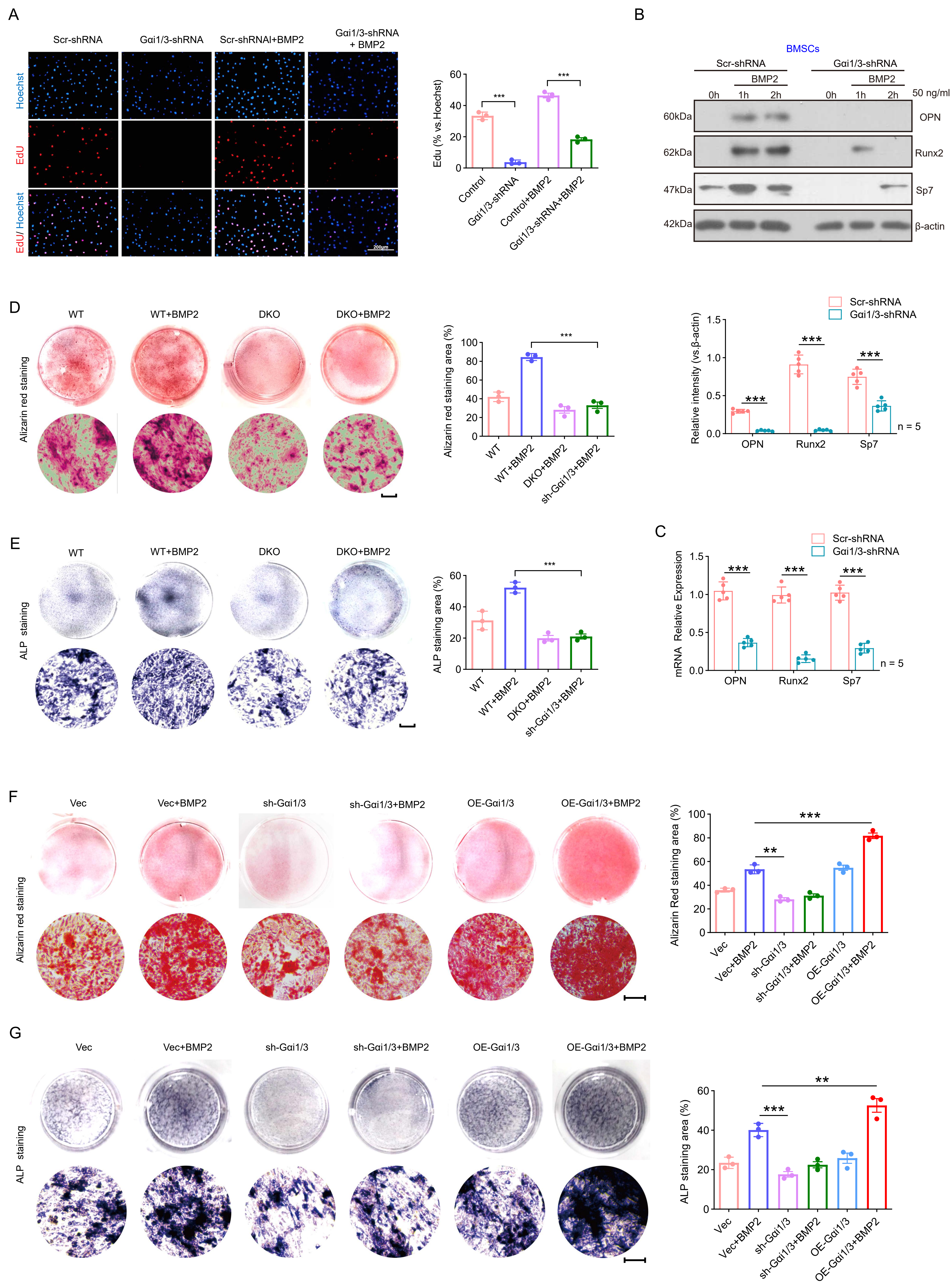


Figure 6

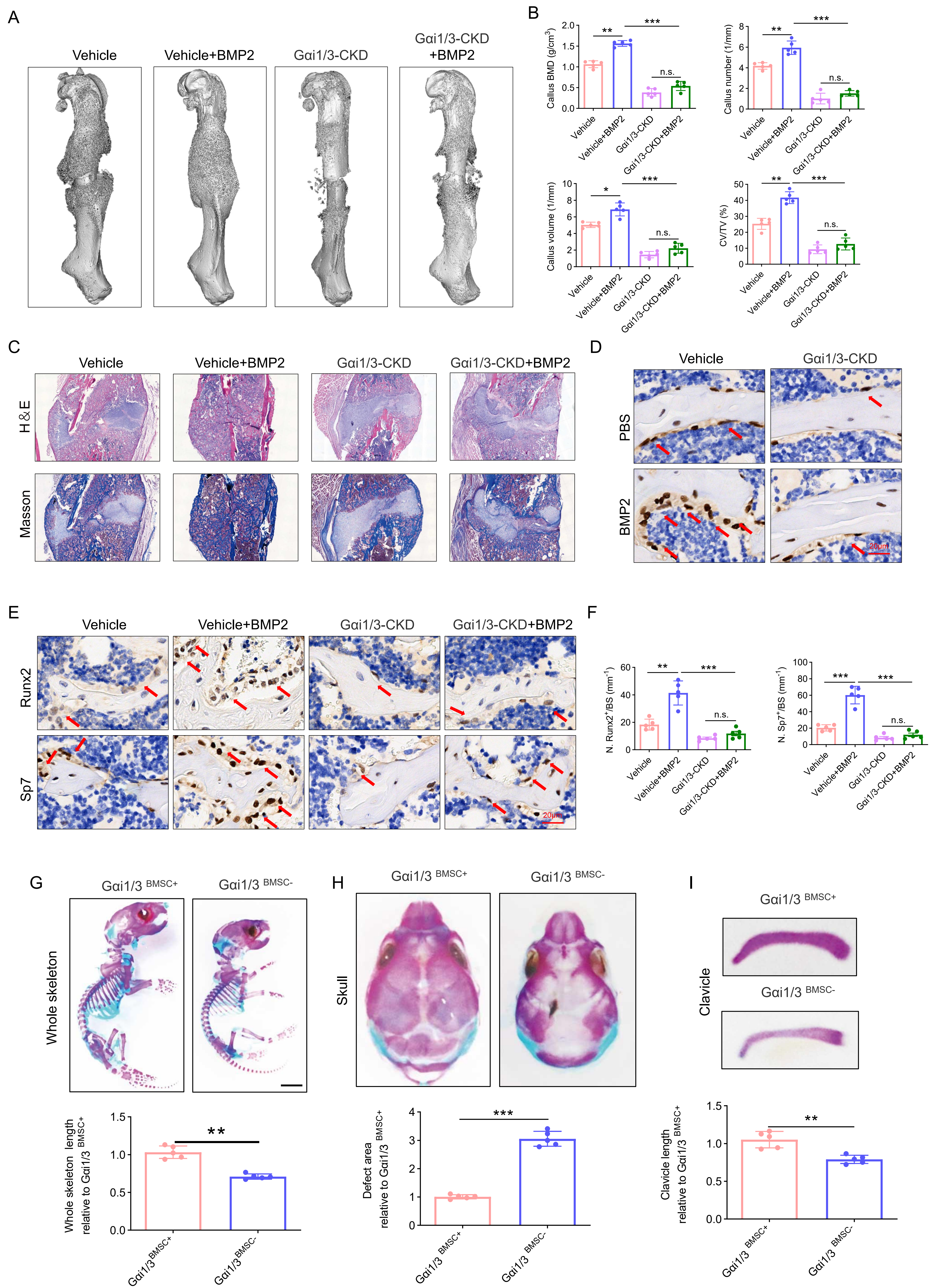


Figure 7

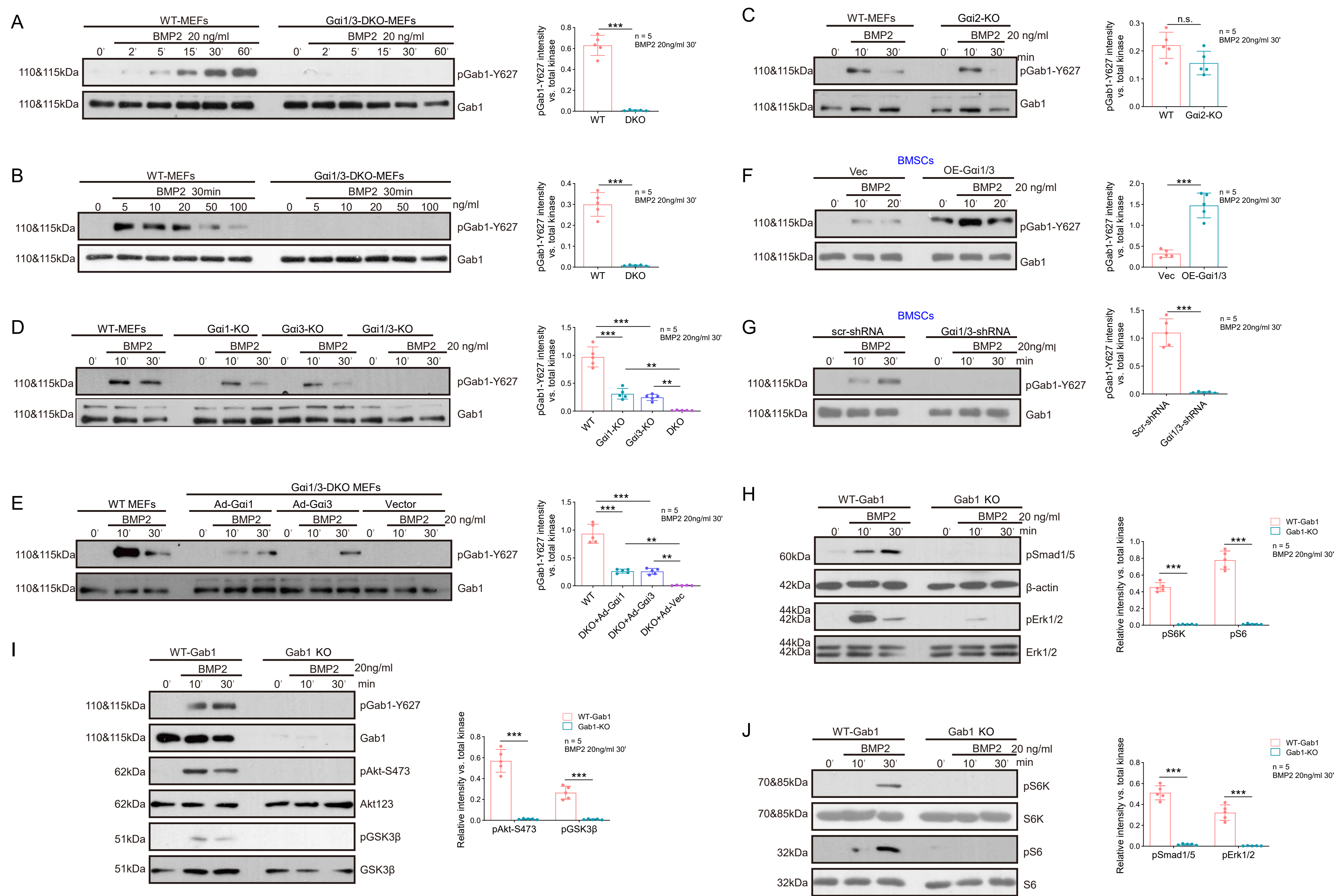
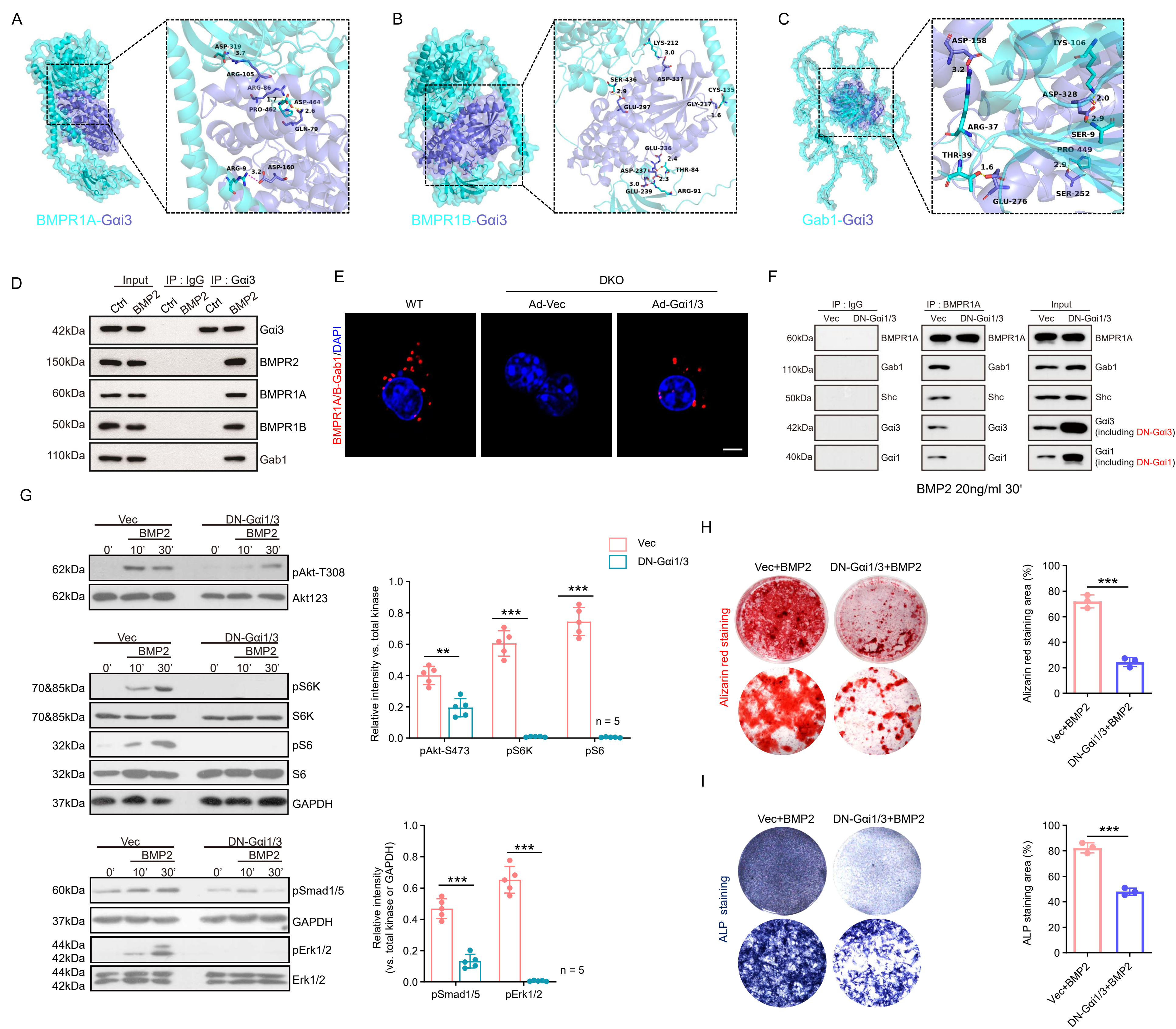


Figure 8



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