

Temporally regulated FGFR2-retinoic acid signaling mediates dura mater-suture mesenchyme interactions to prevent craniosynostosis in mice

Supplementary information

Detailed materials and methods:

Cell culture and conditioned medium (CM) system

Coronal suture mesenchymal cells from P5.5 control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice were isolated as previously described¹. Dura mater cells were obtained from P5.5 control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice using a modified version of this protocol. The coronal suture area was dissected under a Leica M60 microscope (Leica Microsystems, Wetzlar, Germany), carefully removing adjacent bones and periosteum. The dura mater was finely separated, and both tissues were washed thoroughly with PBS and mesenchymal cell culture medium (STEMCELL Technologies, Vancouver, Canada, 05513) containing Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, Massachusetts, USA, 15240062).

Tissues were minced with micro scissors and cultured in 6-well plates (Avantor, Radnor, Pennsylvania, USA, 10861-696) at 37 °C in 5% CO₂. After primary culture, dura mater cells were transferred to 24-well plates and cultured for 2-4 days. CM was generated through control siRNA (Thermo Fisher Scientific, 4390844), *Rbp1* siRNA (4390771-s201917, -s201918), or *Aldh1a3* siRNA (4390771- s81046, s81047) transfection per manufacturer's protocol, or without siRNA. CM was then collected for culture experiments. Other dura mater cells were treated with P38

inhibitor addezmapimod (MedChemExpress, HY-10256, 20 μ M) or DMSO and harvested for analysis after 1 week of culture.

After primary culture, coronal suture mesenchymal cells were dissociated with TrypLE (Gibco, Waltham, Massachusetts, USA, 12604021), filtered through 40 μ m Falcon strainers (Corning, Corning, New York, USA, 21008-949), centrifuged (500 g, 3 min), and resuspended in medium. Cells were seeded in 24-well plates and treated with one of the following: 1. Different groups of dura mater cell CM. 2. P38 inhibitor addezmapimod or DMSO. 3. Control siRNA. 4. *Aldh1a3* siRNA (4390771- s81046, - s81047). After one week of culture, cells were harvested for analysis.

MSC implantation surgery and sham surgery

The MSC implantation surgery was performed on *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice at P21.5, following a previously described method with minor modification¹. A rectangular cranial defect (0.3 to 0.4 mm wide) was created over the coronal suture region using a microsurgical drill. GLI1+ MSCs were suspended in a photocrosslinkable hydrogel composed of gelatin methacryloyl (GelMA), hyaluronic acid methacrylate², and type I collagen at a ratio of 10:2:1. The cell-laden hydrogel was then applied to the defect site and crosslinked *in situ* under UV light. The transplanted MSCs were GLI1+ suture mesenchymal stem cells isolated from coronal suture mesenchyme of P1m wild-type mice. The GLI1+ cell population in the suture mesenchyme is known to exhibit clonogenicity and multilineage differentiation capacity (osteogenic, chondrogenic, and adipogenic) and functions as a resident progenitor pool supporting cranial suture patency and regeneration¹.

The sham surgery was performed also on *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice at P21.5, following the previously described method¹. A rectangular cranial defect (0.3 to 0.4 mm wide) was created over the coronal suture region using a microsurgical drill. Without MSCs implantation, the incision was then sutured.

Intracranial pressure (ICP) measurement

ICP was measured in P3m to P3.5m control, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+};Aldh1a3^{fl/+}*, MSC-implanted *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice, and sham surgery-treated *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice following a previous study¹ with minor modifications³. Mice were anesthetized, and a burr hole was made in the right parietal bone. A trimmed pipette tip served as a guide for a fiber-optic pressure probe (FISO Technologies, Quebec City, Canada), which was inserted until it contacted the dura mater. The setup was sealed with caulking material, and ICP data were recorded using Evolution software (FISO Technologies).

Craniofacial shape analysis

Principal component analysis (PCA) was performed using MorphoJ 1.07a (Klingenberg T., United Kingdom)⁴, following methods described previously¹. The groups included P3.5m control, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+};Aldh1a3^{fl/+}* mice. Shape coordinates were aligned using Procrustes superimposition, a standard geometric morphometric technique, to place the landmark configurations into a common reference space. Landmark subsets were analyzed individually to assess shape differences in two distinct skull regions: the top and lateral portions of the calvarium.

Behavioral assays

Behavioral assays were performed on postnatal P3.5m control, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+};Aldh1a3^{fl/+}*, MSC-implanted *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, and sham surgery-treated *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. All mice acclimated to the testing room for ≥ 60 min. Experimenters were blinded to genotypes and treatments during both behavioral testing and data analysis (N = 20 per group, female : male ratio = 10 : 10).

Rotarod test

Motor learning ability was assessed using a rotarod apparatus (Panlab, Harvard Apparatus, Barcelona, Spain)¹. During the training phase, mice were first trained to stay on a rotating rod at 4 rpm for 60 s. During the testing phase, 24 h later, mice were subjected to an accelerating protocol (4-40 rpm over 5 min). Each mouse performed three trials/day for four consecutive days, with at least 15 min rest intervals between trials to prevent fatigue. The time before falling (latency to fall, s) was automatically recorded. Motor learning curves were constructed from performance over consecutive days.

Novel object test

Cognitive performance was measured in a square open-field arena¹. During the habituation phase, mice were allowed to explore the empty arena for 5 min. Two identical objects were placed symmetrically in the arena during the training phase, and mice again explored for 10 min. After 24 h, one object was replaced with a novel object of comparable size but different shape and material. Behavior was video-recorded and manually scored by blinded observers, with direct nose contact

or active sniffing counted as exploration. The preference index (%) was calculated as $(\text{Time}_{\text{novel}} - \text{Time}_{\text{familiar}}) / (\text{Time}_{\text{novel}} + \text{Time}_{\text{familiar}}) \times 100\%$, where time values represent the total duration of direct interaction with each object.

Three-chamber social interaction test

Sociability and social novelty were tested using a three-chamber Plexiglas box (Panlab)¹. Mice were allowed to habituate to the apparatus by freely exploring the empty chambers for 10 min. During the sociability testing phase, one side chamber contained a novel, age- and sex-matched mouse (Stranger 1) enclosed in a wire cage; the other chamber contained an empty cage. The test mouse was allowed to explore for 10 min. During the social novelty testing phase, a second unfamiliar mouse (Stranger 2) replaced the empty cage, and exploration continued for another 10 min. Time spent sniffing the mice in each wire cage was scored during each test phase. Preference indices were calculated as follows:

Sociability phase: $(T_{\text{stranger1}} - T_{\text{empty}}) / (T_{\text{stranger1}} + T_{\text{empty}}) \times 100\%$,

Social novelty phase: $(T_{\text{stranger2}} - T_{\text{stranger1}}) / (T_{\text{stranger2}} + T_{\text{stranger1}}) \times 100\%$.

Elevated plus maze test

Anxious behavior was assessed using a plus maze with 29.5×6 cm arms and 40 cm elevation (Panlab)¹. Each mouse was placed in the center zone facing an open arm and allowed to explore for 10 min. Behavior was tracked with Smart v3.0 software (Panlab). An arm entry was counted when all four paws entered an arm. Open arm exploration time was expressed as: $T_{\text{open arm}} / 10 \times 100\%$.

Y-maze spontaneous alternation test

Spatial working memory was assessed using a Y-maze with slight modifications from a previous protocol⁵. After one hour of acclimation, mice were placed at the end of one arm and allowed to freely explore for 8 min. Mice were recorded and an arm entry was counted when all four paws entered one arm of the Y. Spontaneous alternation between arms (%) was calculated as (Number of alternations / (Total arm entries - 2)) × 100%.

MicroCT imaging

Live mice were imaged using a Ct Lab In-vivo 90 micro-CT scanner (Rigaku, Tokyo, Japan). Radiographs were captured with a 70 kVp and 114 mA X-ray source. Three-dimensional reconstructions and cross-sectional images were analyzed using AVIZO 9.4.0 software (Thermo Fisher Scientific).

Histology, immunofluorescence and RNAscope *in situ* hybridization (ISH)

Coronal suture samples of mice were fixed overnight at 4 °C in 4% PFA, then decalcified in EDTA for 2 days to 3 weeks depending on the age of mice. Samples were dehydrated through graded sucrose (15%, 30%, and 30% with 50% OCT, each overnight at 4 °C), embedded in OCT (Sakura, Tokyo, 4583), and cryosectioned at 8 µm (Leica Microsystems, CM1850). Sections were mounted on SuperFrost Plus slides (VWR, Radnor, Pennsylvania, USA, 48311-703) and stored at -80 °C.

For hematoxylin and eosin (H&E) staining, standard protocols were followed. For immunostaining, sections were permeabilized with blocking buffer (1% BSA, 2% goat serum, 0.3% Triton X-100 in PBS) for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies:

anti-SP7 (Abcam, Cambridge, Cambridgeshire, United Kingdom, ab22552, 1:100), anti-RUNX2 (Cell Signaling Technology, Danvers, Massachusetts, USA, 12556, 1:100), anti-RBP1 (Proteintech, Rosemont, Illinois, USA, 22683-1-AP, 1:150), anti- β -Gal (Abcam, ab9361; 1:100), and anti-p-P38 (Cell Signaling Technology, 4511, 1:100). After washing, the slides stained with anti-SP7 (Abcam), anti-RUNX2 (Cell Signaling Technology), anti-RBP1 (Proteintech), and anti-p-P38 (Cell Signaling Technology) were incubated for 2h at room temperature with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Waltham, Massachusetts, USA, A11008, 1:200). The slides stained with anti- β -Gal (Abcam, ab9361; 1:100) were incubated for 2h at room temperature with the secondary antibody goat anti-chicken IgY (H+L) HRP (Thermo Fisher Scientific, PA1-28798, 1:200), washed, and then incubated with TSA Plus Cyanine 3 kit (Quanterix, Billerica, Massachusetts, USA, NEL744001KT, 1:500). After washing, all slides were incubated with DAPI for 10 min (Thermo Fisher Scientific, 62248, 1:1000), and then mounted in Epremedia mounting medium (Thermo Fisher Scientific, 9990402).

For ISH, sections were processed using the RNAscope multiplex fluorescent v2 assay (Advanced Cell Diagnostics, Newark, California, USA, 323100) according to the manufacturer's protocol. Probes (Advanced Cell Diagnostics) used included *Gli1* (311001), *Fgfr2* (1238761-C2), *Rbp4* (508501), *Rbp7* (589151), *Aldh1a2* (447391), *Aldh1a3* (501201), *Rarg* (463111), *Shh* (314361), *Ihh* (413091-C3), *Dhh* (415031), *Ptch1* (402811), *Gli2* (405771), and *Gli3* (445511).

Images were captured with a Keyence BZ-X810 microscope (Keyence Corporation, Osaka, Japan) at 20 $\times$ magnification using DAPI, GFP, Cy3, and Cy5 filters with BZ-X810 Viewer software

(Keyence), and analyzed with Z-X810 Analyzer software (Keyence) and ImageJ 1.54p software (National Institutes of Health, Bethesda, MD, USA).

EdU labelling

EdU (50 µg EdU per 1 g body weight) was intraperitoneally injected 3 days before harvesting samples. Click-It EdU cell proliferation kit (Invitrogen, C10337) was used for EdU signal detection.

RNA sequencing analysis

Two independent RNA sequencing experiments were conducted: one comparing P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* and control mice, and the other comparing P3.5 and P7.5 control mice. Under a microscope, coronal sutures were dissected with less than 0.2 mm of adjacent bone, and the underlying dura mater was carefully separated. Suture mesenchyme and dura mater tissues were collected into 1.5 mL tubes, and RNA was extracted using the RNeasy Micro Kit (QIAGEN, Germantown, Maryland, USA, 74034). RNA integrity was confirmed (RNA integrity numbers > 7.0) using an Agilent 2100 Bioanalyzer (Santa Clara, California, USA).

cDNA library preparation and sequencing were performed at University of California, Los Angeles Technology Center for Genomics & Bioinformatics using the NovaSeq High Output system (75 bp paired-end reads, three or four biological replicates per group). Raw reads were trimmed, aligned to the mm39 genome in Partek Flow (Partek Inc., St. Louis, Missouri, USA), and normalized. Differential expression analysis was done using Differential Expression Sequencing 2 (DESeq2) with Wald test and Benjamini-Hochberg correction ($p < 0.05$). Heatmaps were generated via two-

way hierarchical clustering (Euclidean distance, average linkage). Pathway enrichment was performed using Ingenuity Pathway Analysis (QIAGEN, v127006219).

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad, 1725271) on a Bio-Rad CFX96 Real-Time System. The primers included:

Gli1 (forward: 5'-CCAAGCCAACCTTTATGTCAGGG-3',

reverse: 5'-AGCCCGCTTCTTTGTTAATTTGA-3'),

Rbp1 (forward: 5'-CTGAGCAATGAGAATTCGAGGA-3',

reverse: 5'-GCGGTCGTCTATGCCTGTC-3'),

Aldh1a3 (forward: 5'-GGGTCACACTGGAGCTAGGA-3',

reverse: 5'-CTGGCCTCTTCTTGGCGAA-3'),

Rarg (forward: 5'-GGAGCAGGCTTCCCATTCG-3',

reverse: 5'-CATGGCTTATAGACCCGAGGA-3').

Western blot

For tissue samples, coronal suture mesenchyme and underlying dura mater were harvested separately from P7.5 control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. For cell samples, cultured coronal suture mesenchymal cells or dura mater cells were treated with P38 inhibitor adenzmapimod or DMSO for 0.5 hour before collection. Proteins were extracted using RIPA buffer (Cell Signaling

Technology, 9806) with protease inhibitors (Thermo Fisher Scientific, 1861278). Tissues were sonicated on ice for 10 min, and cells lysed on ice for 20 min. Lysates were centrifuged (14,400 rcf, 4 °C) to remove debris, and protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227).

Samples were mixed with 4× Laemmli buffer (Bio-Rad, 1610747), boiled at 98 °C for 10 min, resolved on 10% SDS-PAGE gels, and transferred to 0.45 µm PVDF membranes (Millipore Sigma, Burlington, Massachusetts, USA, ISEQ00005). Membranes were blocked in 5% milk in TBST for 1 hour at room temperature, then incubated overnight at 4 °C with primary antibodies (Cell Signaling Technology, 1:1000): anti-p-PLCγ1 (8713), anti-PLCγ1 (2822), anti-p-AKT (4060), anti-AKT (2920), anti-p-JNK (9255), anti-JNK (9252), anti-p-P38 (4511), anti-P38 (8690), anti-p-ERK (4370), anti-ERK (4695), or anti-β-ACTIN (4970).

Following TBST washes, membranes were incubated with HRP-conjugated goat anti-rabbit (Bio-Rad, 1706515) or anti-mouse (Bio-Rad, 1706516) secondary antibodies (1:1000, 2 h, room temperature). Protein bands were detected using SuperSignal West Femto substrate (Thermo Fisher Scientific, 34094) and imaged using an iBright 1500 system (Thermo Fisher Scientific).

ELISA

RA concentrations were measured by ELISA (MyBioSource, San Diego, California, USA, MBS706971) according to the manufacturer's instructions. Four sets of comparisons were performed: 1. P3.5 control, P7.5 control, and P7.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. 2. P7.5 control, P7.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, and P7.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+};Aldh1a3^{fl/+}* mice. 3. CM from P5.5 control dura mater cells with control siRNA, P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* dura mater cells

treated with control siRNA, P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* dura mater cells treated with *Rbp1* siRNA, and P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* dura mater cells treated with *Aldh1a3* siRNA. 4. Cells and their cultured medium from P5.5 control coronal suture mesenchymal cells with control siRNA, P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* coronal suture mesenchymal cells treated with control siRNA, and P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* coronal suture mesenchymal cells treated with *Aldh1a3* siRNA. Tissue samples and CM were collected and processed for protein extraction as described above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, California, USA). To assess significance, either a two-tailed Student's t-test or ANOVA (analysis of variance) was applied, according to the number of groups being compared. A significance threshold of $p < 0.05$ was used for all tests. Data are presented as mean $\pm$ SEM, with N=3 samples in each experimental group, unless otherwise specified in the manuscript or figure legends.

References:

- 1 Yu, M. *et al.* Cranial suture regeneration mitigates skull and neurocognitive defects in craniosynostosis. *Cell* **184**, 243–256.e218 (2021). <https://doi.org/doi:10.1016/j.cell.2020.11.037>
- 2 Huang, Y. *et al.* Type 2 fibroblast growth factor receptor signaling preserves stemness and prevents differentiation of prostate stem cells from the basal compartment. *J Biol Chem* **290**, 17753–17761 (2015). <https://doi.org/10.1074/jbc.M115.661066>
- 3 Murtha L, M. D., Spratt N. Epidural intracranial pressure measurement in rats using a fiber-optic pressure transducer. *J Vis Exp* **62**, 3689 (2012). <https://doi.org/10.3791/3689>
- 4 CP, K. MorphoJ: an integrated software package for geometric morphometrics. *Mol Ecol Resour* **11**, 353–357 (2011). <https://doi.org/10.1111/j.1755-0998.2010.02924.x>
- 5 Kraeuter, A. K., Guest, P. C. & Sarnyai, Z. The Y-maze for assessment of spatial working and reference memory in mice. *Methods Mol Biol* **1916**, 105–111 (2019). https://doi.org/10.1007/978-1-4939-8994-2_10

Fig. S1:

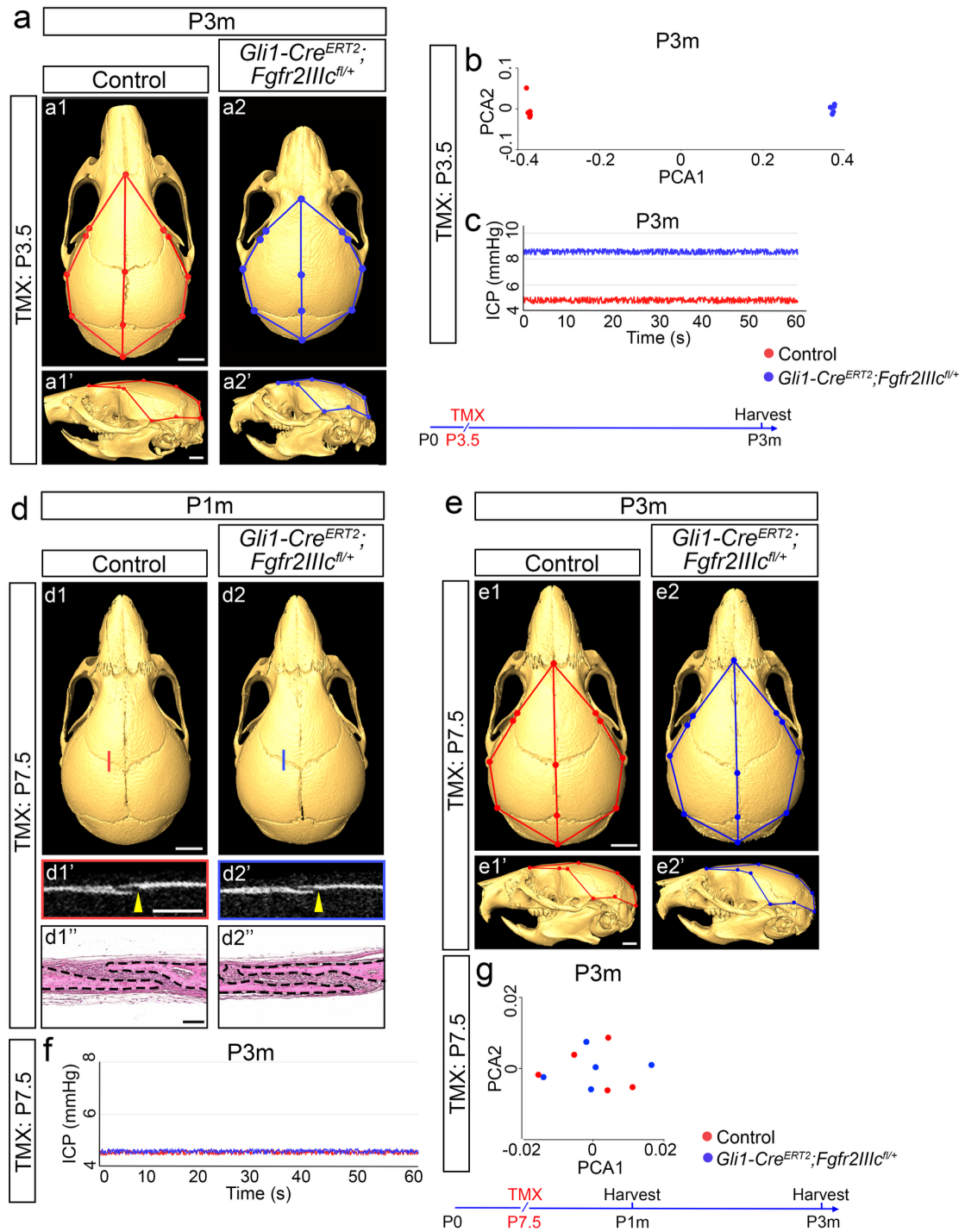


Fig. S1. *Fgfr2* overactivation in GLI1+ progenitors at P7.5 fails to perturb coronal suture or skull development. (a, b) Skull shapes of P3m control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice after tamoxifen induction at P3.5 in axial (a1, a2) and sagittal (a1', a2') views, and the related principal component analysis (PCA) dot plot (b). (c) Intracranial pressure (ICP) measurement over a 60-second period in mice after tamoxifen induction at P3.5. (d) MicroCT images of the skull (d1, d2), coronal suture ortho-slices (d1', d2'), and H&E staining of the coronal sutures (d1'', d2'') of P1m control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice after tamoxifen induction at P7.5. Triangles highlight the patent coronal sutures. (e, g) Skull shape of P3m mice after tamoxifen induction at P7.5 in the axial (e1, e2) and sagittal (e1', e2') views, and the related PCA dot plot (g). (f) ICP measurement over a 60-second period in mice after tamoxifen induction at P7.5. Scale bars: 50 μ m for (d1'', d2''), 1 mm for (a, d1, d2, d1', d2', e). Data are mean $\pm$ SEM, N=20 in (a, b), N=6 in (d), and N=5 in (c, e-g). For *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice in (d), phenotype penetrance = 0.

Fig. S2:

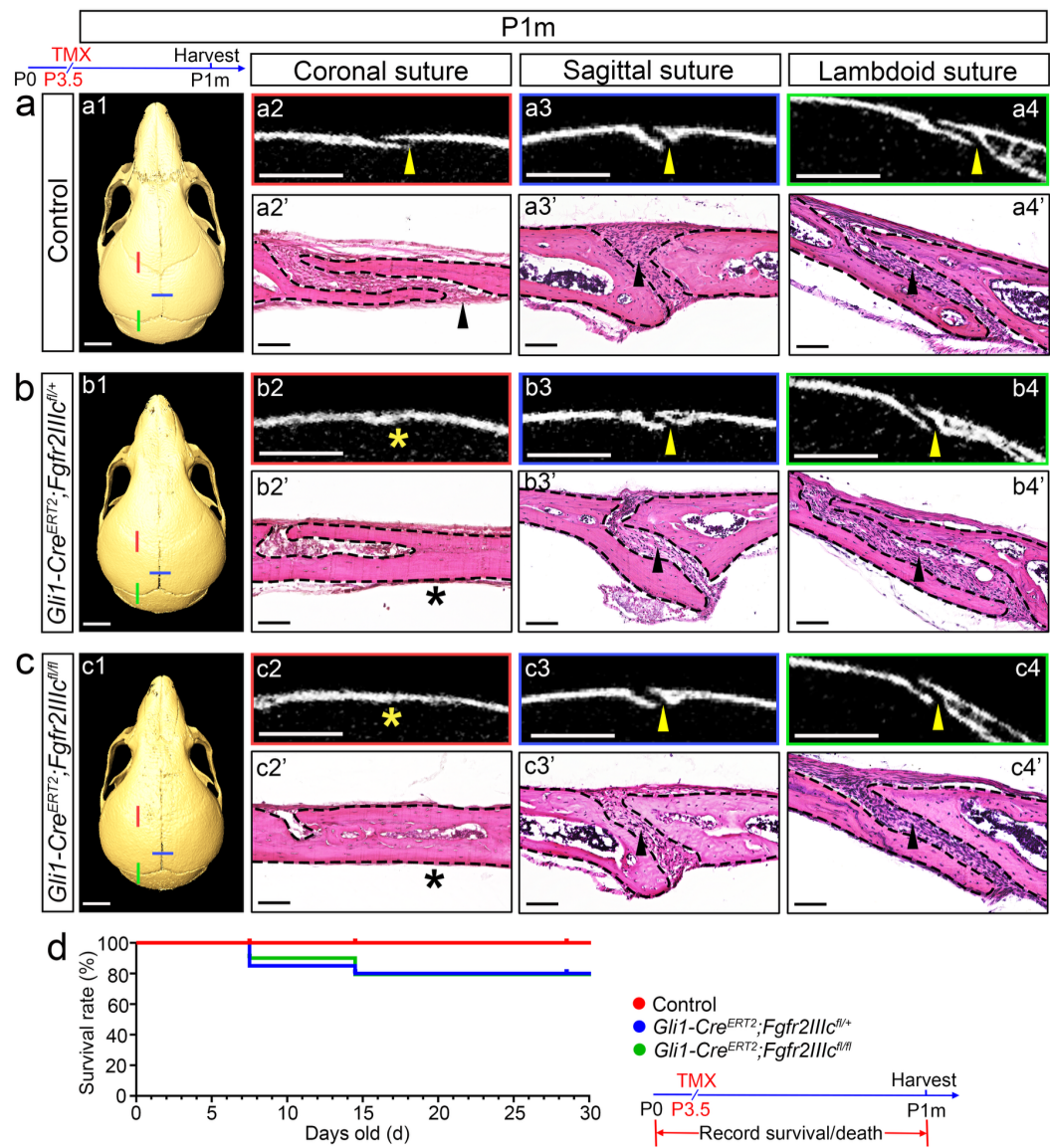


Fig. S2. Heterozygous and homozygous *Fgfr2* mutations lead to coronal suture-specific craniosynostosis with comparable survival rates. (a-c) MicroCT images of control, *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}*, and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/fl}* mice showing skull (a1-c1), coronal suture ortho-slices (a2-c2), H&E staining of the coronal sutures (a2'-c2'), sagittal suture ortho-slices (a3-c3), H&E staining of the sagittal sutures (a3'-c3'), lambdoid suture ortho-slices (a4-c4), and H&E staining of the lambdoid sutures (a4'-c4') at P1m after tamoxifen induction at P3.5. Triangles highlight patent sutures, while asterisks highlight fused sutures. (d) Kaplan-Meier survival curves with log-rank test showing postnatal survival from P0 to P1m for the three groups. No significant differences in survival rate were observed between *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/fl}* mice. Scale bars: 50 μ m for H&E staining, 1 mm for MicroCT images. N=20 for (a, b), and N=6 for (c). For (b, c), phenotype penetrance = 100% for coronal suture, while phenotype penetrance = 0 for both sagittal and lambdoid sutures. N=20 in each group at P0 for (d).

Fig. S3:

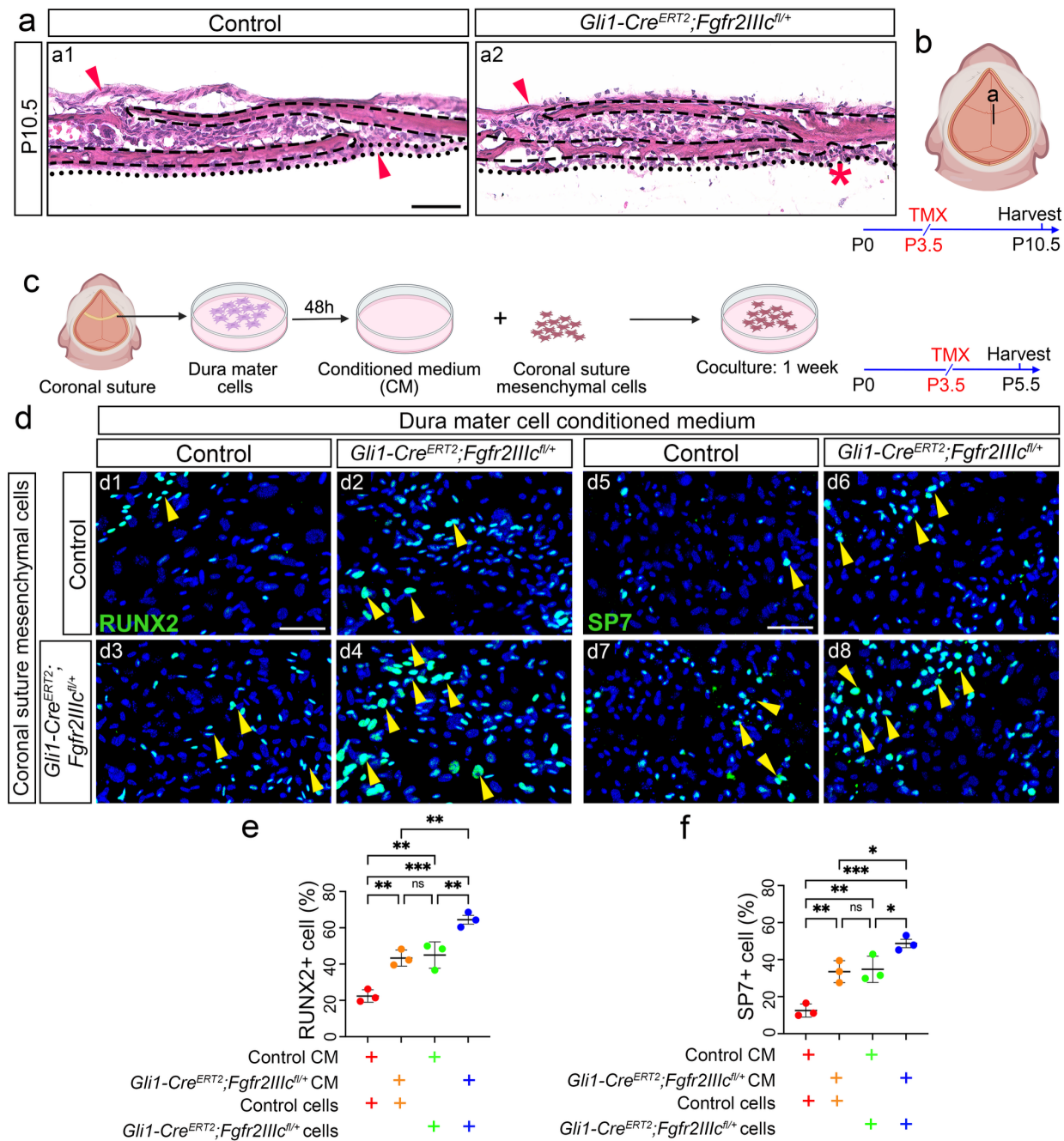


Fig. S3. Both coronal suture mesenchymal cells and dura mater cells contribute to the osteogenic activity change in *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. (a) H&E staining of coronal sutures for P10.5 control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. Dotted/dashed lines highlight frontal bone, parietal bone, and dura mater. Triangles highlight the patent coronal suture, while the asterisk highlights fused site of coronal suture in *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. (b) Diagram of sectioning site and orientation for (a). (c) Diagram showing the conditioned medium (CM) co-culture system. (d) Immunostaining of RUNX2 (d1-d4) and SP7 (d5-d8) expression in the coronal suture mesenchymal cells after CM treatment. Triangles highlight RUNX2⁺ or SP7⁺ cells. (e) Quantification of RUNX2⁺ cell percentage after CM treatment. (f) Quantification of SP7⁺ cell percentage after CM treatment. Scale bars: 50 μ m. Data are mean $\pm$ SEM, N=3. *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant, calculated by one-way ANOVA with Tukey's multiple comparisons test (e, f). Illustrations were created with BioRender.com for (b, c).

Fig. S4:

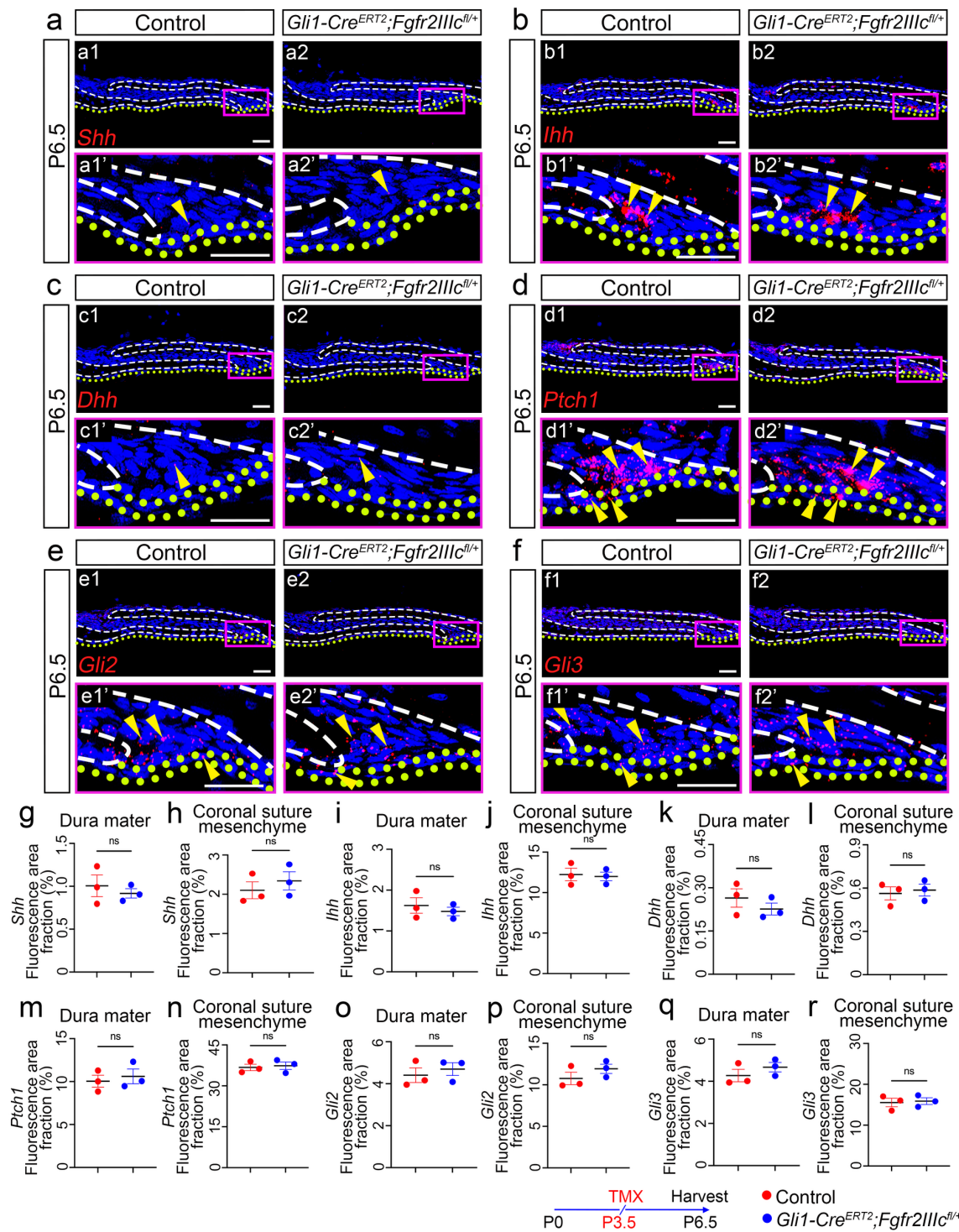


Fig. S4. Expression of Hedgehog signaling components *Shh*, *Ihh*, *Dhh*, *Ptch1*, *Gli2*, and *Gli3* are not significantly altered upon FGFR2 overactivation. (a - f) RNAscope analyses of *Shh* (a), *Ihh* (b), *Dhh* (c), *Ptch1* (d), *Gli2* (e), and *Gli3* (f) expression in coronal sutures of control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. Dotted/dashed lines outline the frontal bone, parietal bone, and dura mater. Triangles highlight gene expression in dura mater and coronal suture mesenchyme. (g-r) Quantification of the *Shh* (g, h), *Ihh* (i, j), *Dhh* (k, l), *Ptch1* (m, n), *Gli2* (o, p), and *Gli3* (q, r) RNAscope-positive area in the dura mater and coronal suture mesenchyme, expressed as the percentage of area occupied by *Shh*, *Ihh*, *Dhh*, *Ptch1*, *Gli2*, and *Gli3* signal, representing relative gene expression. Scale bars: 20 μ m. Data are mean $\pm$ SEM, N=3. ns: not significant, calculated by two-tailed unpaired t test (g-r).

Fig. S5:

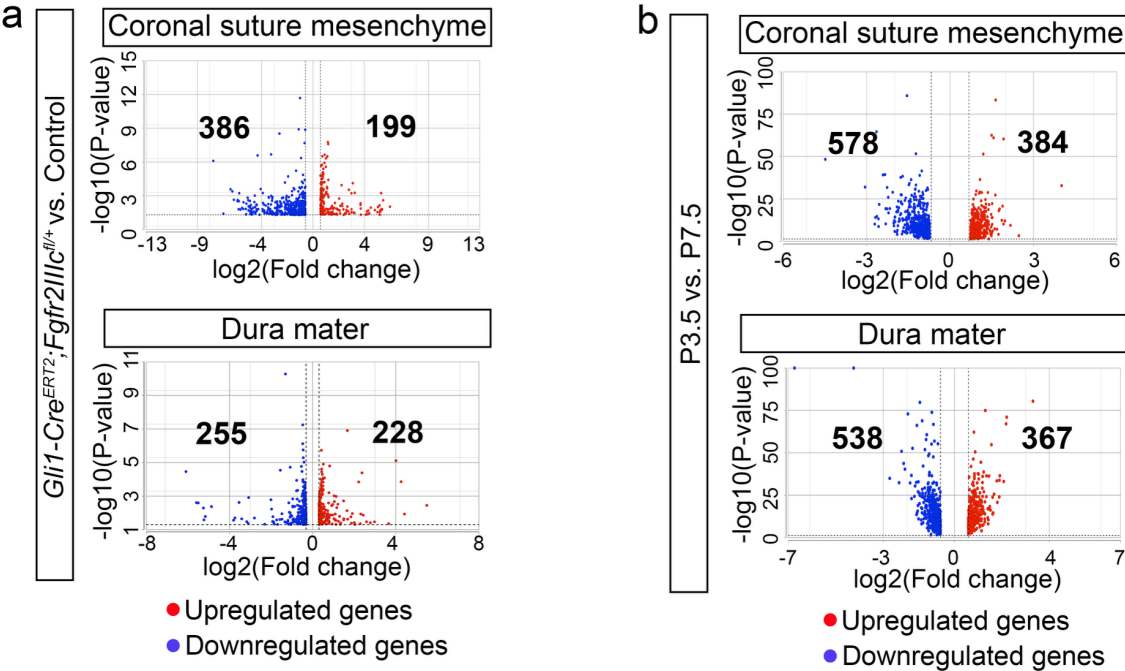


Fig. S5. Control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice display distinct transcriptomic heterogeneity across different postnatal developmental stages. (a, b) Volcano plots of differentially expressed genes in bulk RNA-seq analysis of coronal suture mesenchyme and dura mater tissue from P5.5 *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* and control mice after tamoxifen induction at P3.5 (a), as well as from P3.5 and P7.5 control mice (b). The differentially expressed genes were selected using cutoffs of $p < 0.05$ and fold change < -1.2 or > 1.2 . N=3.

Fig. S6:

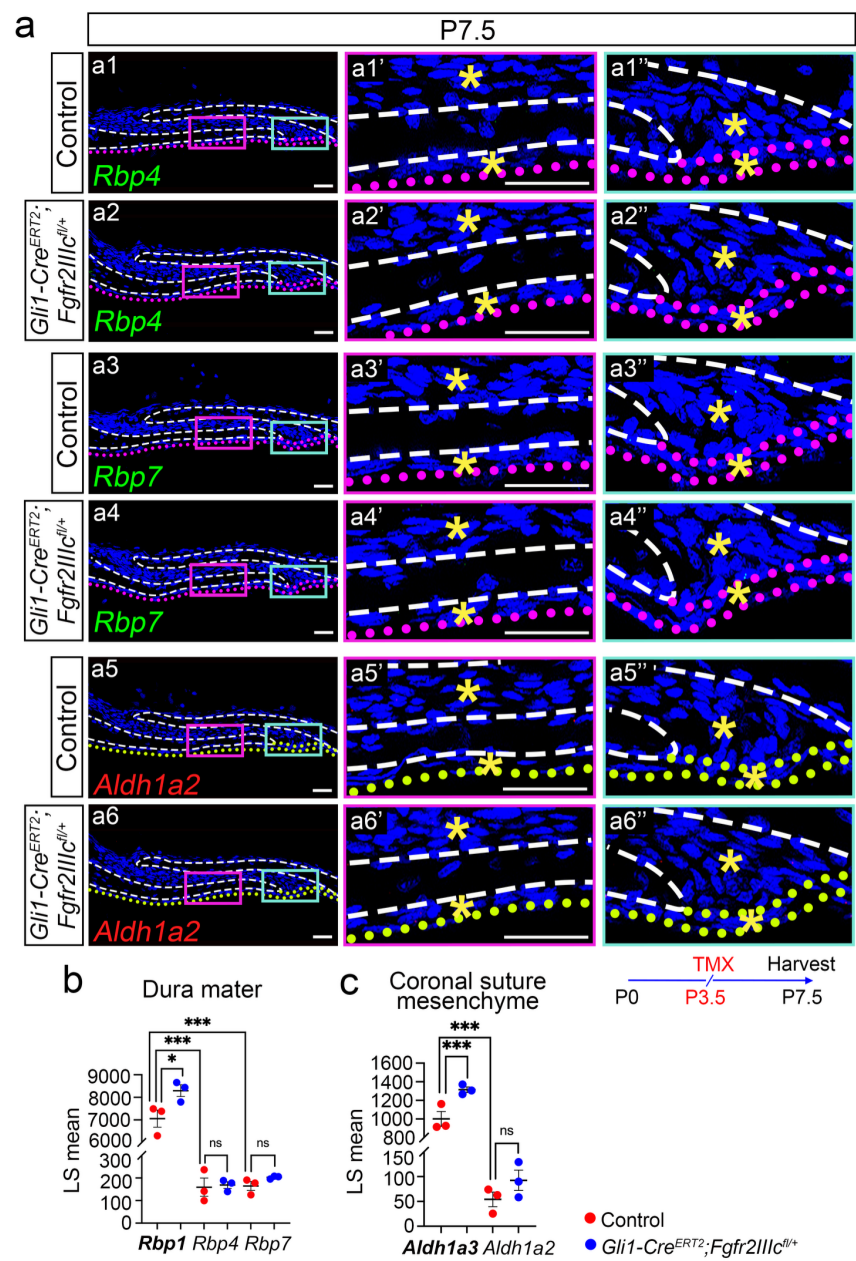


Fig. S6. RA signaling-related genes *Rbp4*, *Rbp7*, and *Aldh1a2* are rarely expressed in the coronal suture. (a) RNAscope analyses of *Rbp4* (a1-a1'', a2-a2''), *Rbp7* (a3-a3'', a4-a4''), and *Aldh1a2* (a5-a5'', a6-a6'') expression in the coronal sutures of P7.5 control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice after tamoxifen induction at P3.5. Dotted/dashed lines outline the frontal bone, parietal bone, and dura mater. Asterisks highlight lack of gene expression in dura mater and coronal suture mesenchyme. (b) The Least Squares mean (LS mean) values of *Rbp1*, *Rbp4* and *Rbp7* in the dura mater comparing control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. (c) The Least Squares mean (LS mean) values of *Aldh1a3* and *Aldh1a2* in the coronal suture mesenchyme comparing control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. Scale bars: 20 μ m. Data are LS Mean, N=3. *p < 0.05, ***p < 0.001, ns: not significant, calculated by Wald test followed by Benjamini-Hochberg correction to control the false discovery rate (b, c).

Fig. S7:

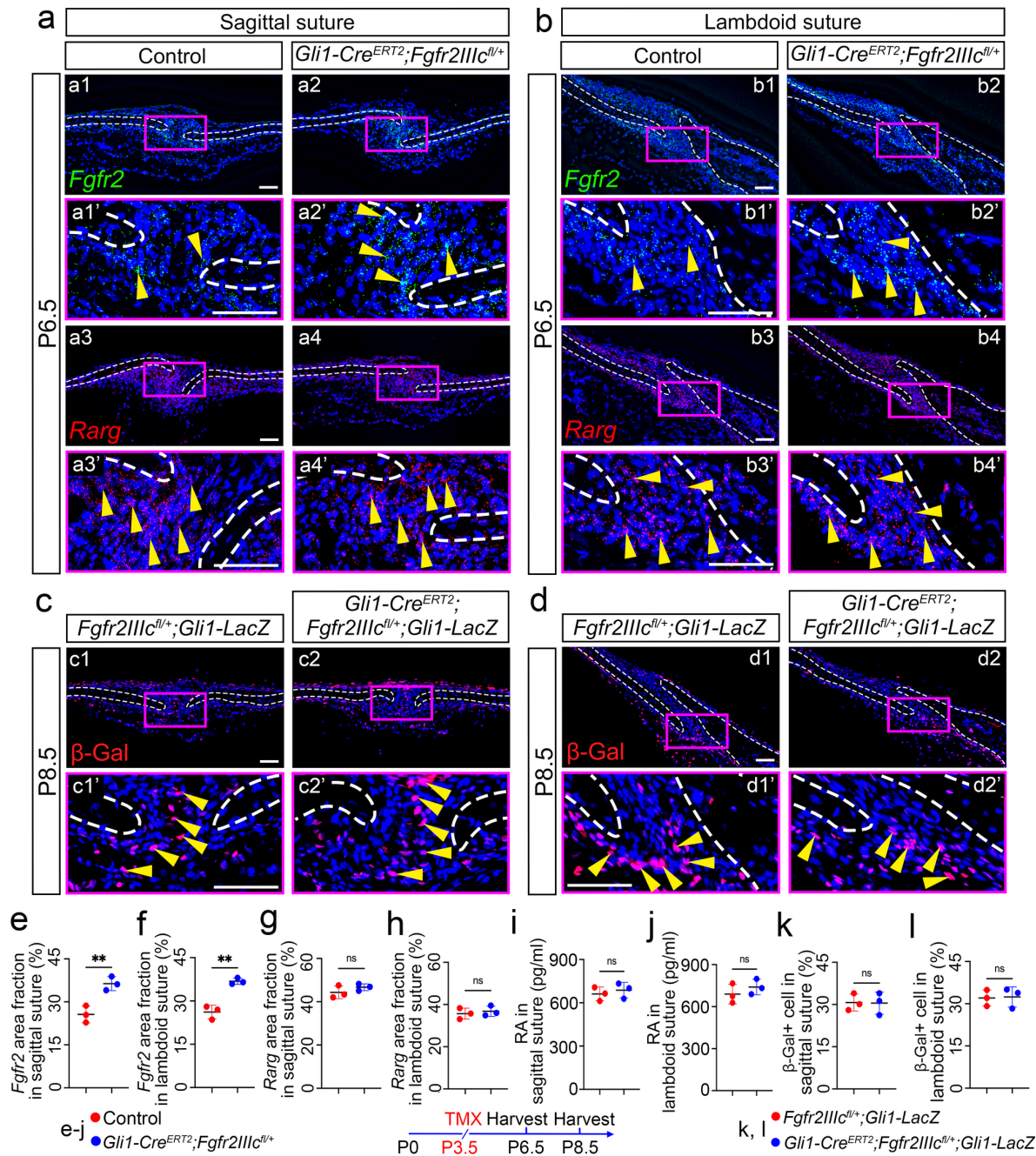


Fig. S7. FGFR2 overactivation occurs in sagittal and lambdoid sutures but does not upregulate RA signaling or deplete GLI1+ progenitors. (a) RNAscope analyses of *Fgfr2* (**a1, a2, a1', a2'**) and *Rarg* (**a3, a4, a3', a4'**) expression in sagittal sutures at P6.5 after tamoxifen induction at P3.5 in control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice. Dashed lines outline the parietal bones. Triangles highlight the cells with *Fgfr2* or *Rarg* expression. **(b)** RNAscope analyses of *Fgfr2* (**b1, b2, b1', b2'**) and *Rarg* (**b3, b4, b3', b4'**) expression in lambdoid sutures at P6.5 in control and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* mice after tamoxifen induction at P3.5. Dashed lines outline the parietal and interparietal bones. Triangles highlight the cells with *Fgfr2* or *Rarg* expression. **(c, d)** Immunostaining of β -Gal in sagittal **(c)** and lambdoid **(d)** sutures at P8.5 after tamoxifen induction at P3.5 in *Fgfr2IIIc^{fl/+};Gli1-LacZ* and *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+};Gli1-LacZ* mice. Dashed lines outline the parietal and interparietal bones. Triangles highlight the β -Gal+ cells. **(e-h)** Quantification of the *Fgfr2* **(e, f)** and *Rarg* **(g, h)** RNAscope-positive area in the sagittal and lambdoid sutures, expressed as the percentage of area occupied by *Fgfr2* or *Rarg* signal, representing relative gene expression. **(i, j)** Quantification of RA concentration by ELISA in the sagittal **(i)** and lambdoid **(j)** sutures. **(k, l)** Quantification of the β -Gal+ cell percentage in the sagittal **(k)** and lambdoid **(l)** sutures. Scale bars: 20 μ m. Data are mean $\pm$ SEM, N=3. **p < 0.01, ns: not significant, calculated by two-tailed unpaired t test **(e-l)**.

Fig. S8:

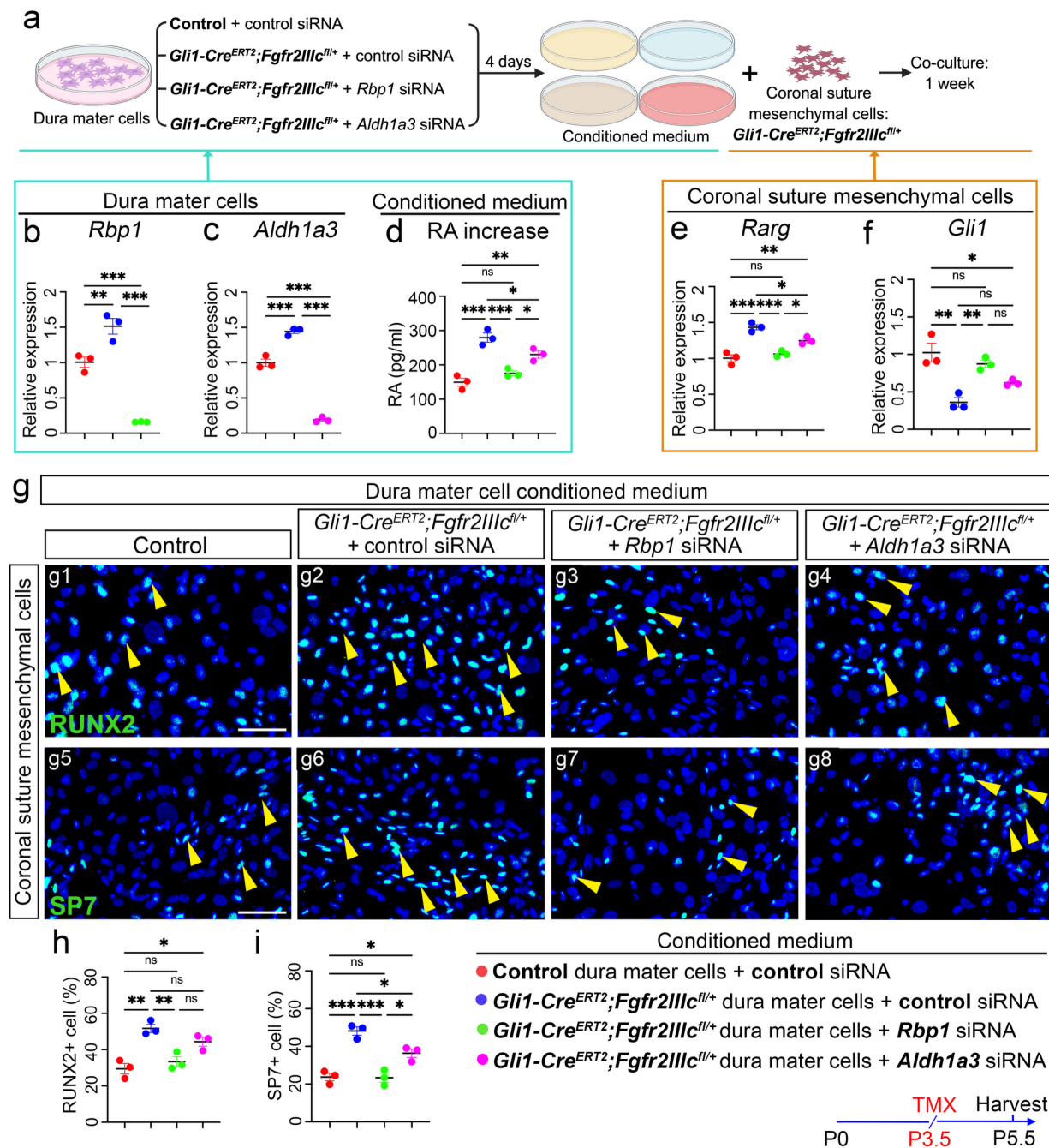


Fig. S8. Dura mater modulates osteogenic differentiation of the coronal suture mesenchymal cells via RA signaling *in vitro*. (a) Diagram of the CM cell culture system. (b, c) Quantification of the real-time qPCR of *Rbp1* (b) and *Aldh1a3* (c) expression in the dura mater cells. (d) Quantification of RA concentration by ELISA in the CM. (e, f) Quantification of the real-time qPCR of *Rarg* (e) and *Gli1* (f) expression in *Gli1-Cre^{ERT2};Fgfr2IIIc^{fl/+}* coronal suture mesenchymal cells treated with different CM. (g) Immunostaining of RUNX2 (g1-g4) and SP7 (g5-g8) expression in the coronal suture mesenchymal cells treated with different CM. Triangles highlight RUNX2⁺ or SP7⁺ cells. (h, i) Quantification of RUNX2⁺ (h) and SP7⁺ (i) cell percentages after CM treatment. Scale bars: 50 μ m. Data are mean $\pm$ SEM, N=3. *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant, calculated by one-way ANOVA with Tukey's multiple comparisons test (b-f, h, i). Illustration created with BioRender.com for (a).

Fig. S9:

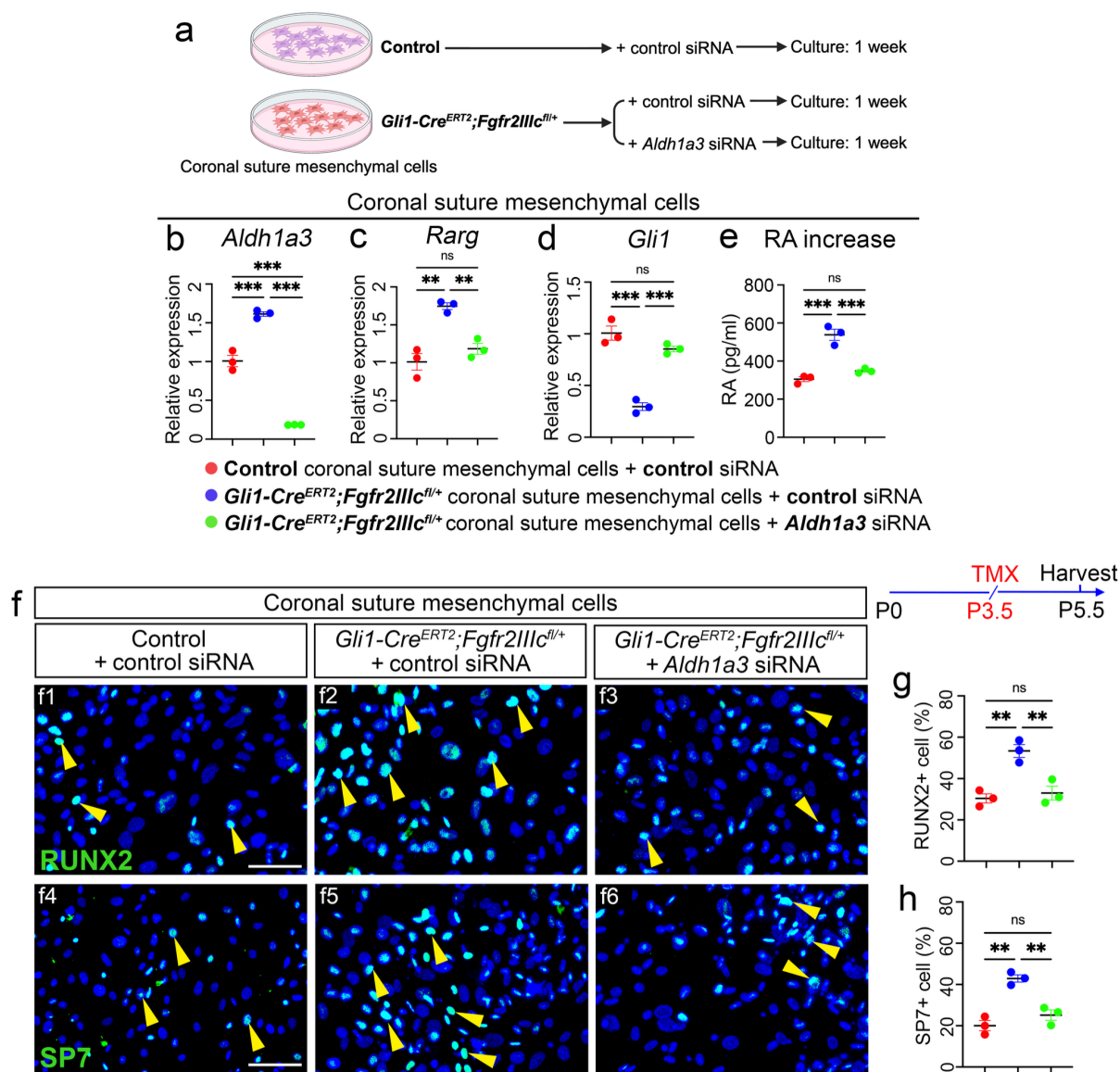


Fig. S9. RA synthesis gene *Aldh1a3* upregulates osteogenic differentiation of the coronal suture mesenchymal cells *in vitro*. (a) Diagram of the cell culture system. (b-d) Quantification of the real-time qPCR of *Aldh1a3* (b), *Rarg* (c), and *Gli1* (d) expression in coronal suture mesenchymal cells. (e) Quantification of RA concentration by ELISA in the coronal suture mesenchymal cells. (f) Immunostaining of RUNX2 (f1-f3) or SP7 (f4-f6) expression in the coronal suture mesenchymal cells after siRNA treatment. Triangles highlight RUNX2⁺ or SP7⁺ cells. (g, h) Quantification of RUNX2⁺ (g) and SP7⁺ (h) cell percentages. Scale bars: 50 μ m. Data are mean $\pm$ SEM, N=3. **p < 0.01, ***p < 0.001, ns: not significant, calculated by one-way ANOVA with Tukey's multiple comparisons test (b-e, g, h). Illustration created with BioRender.com for (a).

Fig. S10:

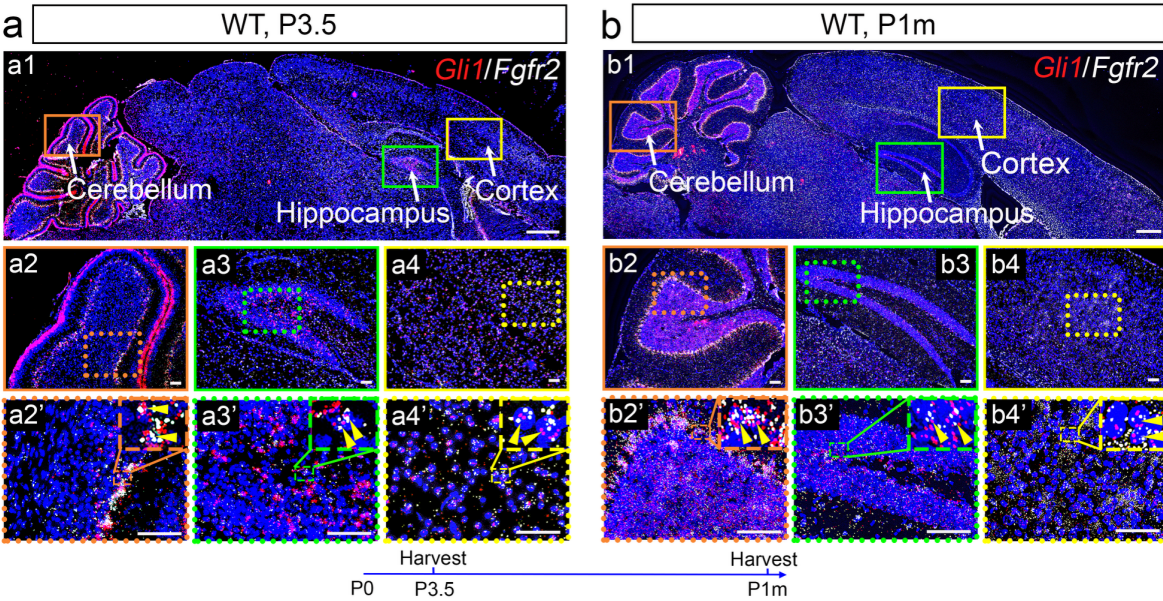


Fig. S10. *Gli1* and *Fgfr2* are co-expressed in the brain at both P3.5 and P1m. (a) RNAscope analyses of *Gli1* and *Fgfr2* in the brain (**a1**), with coexpression notably in the cerebellum (**a2, a2'**), hippocampus (**a3, a3'**), and cortex (**a4, a4'**) of P3.5 control mice. Triangles highlight the cells with coexpression of *Gli1* and *Fgfr2*. **(b)** RNAscope analyses of *Gli1* and *Fgfr2* in the brain (**b1**), with coexpression notably in the cerebellum (**b2, b2'**), hippocampus (**b3, b3'**), and cortex (**b4, b4'**) of P1m control mice. Triangles highlight the cells with coexpression of *Gli1* and *Fgfr2*. Scale bars: 500 μm for (**a1, b1**), 50 μm for the other figures. N=3.