A multi-stem cell basis for craniosynostosis and calvarial mineralization

Craniosynostosis is a group of disorders of premature calvarial suture fusion. The identity of the calvarial stem cells (CSCs) that produce fusion-driving osteoblasts in craniosynostosis remains poorly understood. Here we show that both physiologic calvarial mineralization and pathologic calvarial fusion in craniosynostosis reflect the interaction of two separate stem cell lineages; a previously identified cathepsin K (CTSK) lineage CSC (CTSK⁺ CSC) and a separate discoidin domain-containing receptor 2 (DDR2) lineage stem cell (DDR2⁻ CSC) that we identified in this study. Deletion of Twist1, a gene associated with craniosynostosis in humans, solely in CTSK⁺ CSCs is sufficient to drive craniosynostosis in mice, but the sites that are destined to fuse exhibit an unexpected depletion of CTSK⁺ CSCs and a corresponding expansion of DDR2⁻ CSCs, with DDR2⁻ CSC expansion being a direct maladaptive response to CTSK⁺ CSC depletion. DDR2⁻ CSCs display full stemness features, and our results establish the presence of two distinct stem cell lineages in the sutures, with both populations contributing to physiologic calvarial mineralization. DDR2⁻ CSCs mediate a distinct form of endochondral ossification without the typical hematopoietic marrow formation. Implantation of DDR2⁻ CSCs into suture sites is sufficient to induce fusion, and this phenotype was prevented by co-transplantation of CTSK⁺ CSCs. Finally, the human counterparts of DDR2⁻ CSCs and CTSK⁺ CSCs display conserved functional properties in xenograft assays. The interaction between these two stem cell populations provides a new biologic interface for the modulation of calvarial mineralization and suture patency.

Given that CTSK⁺ CSCs (lineage (Lin)⁻ Thy1.2⁻ CD63⁻ CD200⁻ CD105⁻ CD51⁺ calvarial stem cells expressing a Ctsk-cre driven lineage reporter) contribute to physiologic calvarial mineralization, we hypothesized that defects in these cells may underlie calvarial disorders such as craniosynostosis. To examine this, we generated mice with a Ctsk-cre-driven conditional deletion of Twist1 (Twist1Cre), as TWIST1 loss of function causes Saethre-Chotzen syndrome, one of the most common craniosynostosis disorders in humans. Indeed, deletion of Twist1 in CTSK-lineage cells was sufficient to drive a suture fusion phenotype with a distribution consistent with this disorder, including fusion of the coronal (COR), lambdoid (LAM), squamosal (SQ) and occipitointerparietal (OIP) sutures (Fig. 1a,b and Extended Data Fig. 1a,b). We expected expansion and activation of CTSK⁺ CSCs to mediate fusion, but instead observed a marked depletion of CTSK-lineage cells at the sutures that were destined to fuse, and noted a correspondence between the degree of depletion and the completeness of fusion at each suture (Fig. 1c and Extended Data Fig. 1c,d), a finding that parallels other observations linking skeletal stem cell (SSC) abundance to suture patency in the Twist1⁺ craniosynostosis model. CTSK-lineage cells were present in identical numbers in E16.5 Twist1⁻/⁻ and wild-type control mice, but subsequently underwent postnatal apoptosis (Extended Data Fig. 1e,f). At sites displaying suture fusion and CTSK⁺ CSC dropout, CTSK⁺ CSCs and their derivatives disappeared and were replaced with another cell type not bearing the CTSK-lineage reporter (Fig. 1d and Extended Data Fig. 1g–k).

This dropout of CTSK⁺ CSCs was accompanied by a major shift in the form of bone formation in the sutures. We previously reported that CTSK⁺ CSCs are specialized for intramembranous bone formation,
Fig. 1 | Defects in CTSK+ CSCs cause craniosynostosis and the expansion of an alternative lineage. a. Micro-computed tomography (µCT) of skull of wild-type (WT) (Twist1<sup>fl/fl</sup>;Ctsk-cre;mT/mG, n = 6) and Twist1<sup>fl/fl</sup>;Ctsk-cre;mT/mG; iDTR<sup>Ctsk</sup> (P14) mice at P28. Red arrows show fusing sutures. IF, interfrontal; SAG, sagittal. b, Left, µCT of mouse skull at P30 showing histology sites (red lines). Immunofluorescence of LAM (middle) and OIP (right). Insets, nuclear fast red (NFR), safranin O (SaO), alcian blue (AB), DAPI, tdTomato COMP, CTSK–mGFP (P14) and CTSK–mGFP tdTomato (P21, P35). c, Immunofluorescence merged with bright-field images. d, Immunofluorescence of LAM in wild-type and Twist1<sup>fl/fl</sup>;Ctsk-cre;mT/mG mice at P14. d, Fluorescence-activated cell sorting (FACS) of total live CTSK<sup>+</sup> cells (top), CTSK<sup>+</sup> CSCs (Lin<sup>−</sup> Thy1<sup>−</sup> 6C3<sup>−</sup> CTSK–mGFP CD200<sup>−</sup> CD105<sup>−</sup> CD51<sup>−</sup>) (middle), and CTSK<sup>−</sup> putative CSCs (CTSK<sup>−</sup> pCSCs, Lin<sup>−</sup> Thy1<sup>−</sup> 6C3<sup>−</sup> CTSK–mGFP CD200<sup>−</sup> CD105<sup>−</sup> CD51<sup>−</sup>) (bottom) in microdissected sutures of wild-type (n = 12) and Twist1<sup>fl/fl</sup>;Ctsk-cre;mT/mG (n = 17) mice at P14. Lin indicates CD31, CD45 and Ter119. Absolute numbers and frequency of populations relative to total live cells are shown on the right. e, Immunostaining for COMP and Alcian Blue in LAM at P14. f, Endochondral ossification in Twist1<sup>fl/fl</sup>;Ctsk-cre;mT/mG mice at P7. g, FACS (left), total CTSK<sup>+</sup> cells relative to total live cells (middle) and immunofluorescence of LAM (right) of iDTR<sup>−</sup>;Ctsk-cre;mT/mG mice after treatment with diphtheria toxin (DT) or PBS (PBS, n = 4; DT, n = 3). h, µCT (left and middle) and 2D cut planes (right) from iDTR<sup>−</sup> (n = 5) and iDTR<sup>−</sup> (n = 6) mice at P21 and P35 after diphtheria toxin treatment. Red arrows, sites of suture fusion. i, Haematoxylin and eosin (H&E) staining of the sutures of iDTR<sup>−</sup> and iDTR<sup>−</sup> mice after diphtheria toxin treatment. j, FACS of CTSK<sup>−</sup> pCSCs and CTSK<sup>−</sup> pCSCs relative to total live cells from iDTR<sup>−</sup> mice (PBS, n = 4; DT, n = 4). Data are mean ± s.d.; unpaired two-tailed Student’s t-test (d, g, j) were repeated a minimum of three times on separate cohorts; images in a, c, e, g, i represent results from at least five biological replicates; b, f, are representative of a minimum of three independent experiments. Scale bars: 1 mm (µCT images), 100 µm (all other images). **** < 0.0001.

the major physiologic form of calvarial bone formation, in which bone is deposited directly without a cartilage template. Accordingly, dropout of CTSK<sup>−</sup> CSCs was accompanied by the pathologic induction of endochondral bone formation, a form of bone formation that is normally excluded from most sutures<sup>++</sup>, as seen through the presence of a cartilage template formed by non-CTSK-lineage cells that...
Fig. 2 | Identification of a sutural DDR2+ CSC. a. Immunofluorescence of DDR2+ cells in LAM from Ctsk-creERT2/mG mice at P7. b. FACS of the LAM suture from Ctsk-creERT2/mG mice at P7. Pregated on Lin THY1.2 6C3. c. Immunofluorescence of DDR2 in LMP from wild-type and Twist1CreERT2/mG mice at P14 (top) and in iDTRCre mice at P21 (bottom). d. Analysis of DDR2+ CSCs by FACS (Lin 6C3 THY1.2 CTSK-mGFP CD200 CD105 CD34 DDR2+) in the sutures of wild-type (n = 13), Twist1CreERT2 (n = 20) at P7 to P10 (top) and iDTRCre mice with PBS (n = 4) or diphtheria toxin (DT) (n = 3) treatment at P21 (bottom). Unpaired two-tailed Student’s t-test. e. Schematic of in vivo intramuscular serial transplantation. f. FACS analysis of DDR2+ CSC-derived cells after serial transplantation. Black arrows indicate parent-daughter gates. Red boxes delineate DDR2+ CSCs. Representative of four independent experiments. g. FACS analysis shows no interconversion between tdTomato+ DDR2+ CSCs and mGFP+ CTSK+ CSCs isolated from Ctsk-creERT2/mG mice after the first round of transplantation. h. Lineage tracing of sutural DDR2+ cells using Ddr2-creERT2/mG mice over an 18-month chase. i. FACS analysis showing total H2B–GFP label-retaining cells after 6 months of chase after doxycycline (Dox) treatment. J. GFP signal intensity histogram of Lin THY1.2 6C3 DDR2+ or DDR2− populations after 6 (n = 9) or 12 (n = 8) months of chase. Label-retaining cells (LRCs) were determined as gated. Green, CD200+ CD105− (CSCs); Blue, CD105+; Red, CD200− CD105−. k. Immunofluorescence of LAM from H2B–GFP mice after 6 months of chase. In all graphs, each dot represents an individual mouse. Data are mean ± s.d. Images in a, c, h, k are representative of at least three independent experiments. Scale bars: 100 µm (a, c, h, k), 50 µm (a, far right), 20 µm (c, h, right, k, inset).

was subsequently remodelled by osteoclasts and chondrocyte apoptosis into mineralized bone (Fig. 1e,f and Extended Data Fig. 1f). This cartilage was fully remodelled into bone at later timepoints, thereby distinguishing this endochondral ossification process from the formation of ectopic cartilage (Fig. 1b and Extended Data Fig. 1m,n). Together, these data show that a second calvarial lineage that is not derived from CTSK+ progenitors expands to become the dominant population at fusing sutural sites displaying endochondral ossification. Dropout of CTSK+ CSCs was sufficient to drive the expansion of this alternative non-CTSK population and suture fusion, as seen with inducible deletion of CTSK+ CSCs using diphtheria toxin administration in iDTR:Ctsk-cre (iDTRCre) mice. These mice displayed hypomineralization...
of the calvarium similar to that seen after conditionally deleting the osterix gene (Osx also known as Sp7) required for osteoblast generation in CTSK+ cells (Osx<sup>Cre<sub>+</sub></sup>), as well as partial suture fusion at similar sites to those fused in Twist<sup>1<sub>Ctsk<sub>+</sub></sub></sup> mice (Fig. 1g–j and Extended Data Fig. 2a–f). Similar to findings in Twist<sup>1<sub>Ctsk<sub>+</sub></sub></sup> mice, compensatory expansion of non-CTSK-lineage sutural cells preceded fusion in iDTR<sup>1<sub>Ctsk<sub>+</sub></sub></sup> mice. The iDTR<sup>1<sub>Ctsk<sub>+</sub></sub></sup> model produced a porosity phenotype in the cortices of long bones similar to that reported in Osx<sup>Cre<sub>+</sub></sub> mice, thereby validating that this model produced the expected loss of function in CTSK-expressing SCSs<sup>2</sup>. Additionally, an osteopetrotic phenotype representing deletion of CTSK-expressing osteoclasts was observed in long bones, thereby providing an internal control validating the expected iDTR effect (Extended Data Fig. 2g–i). However, owing to the expected turnover of mature osteoclasts, this osteoclast ablation effect was transient (Extended Data Fig. 2j). Although a contribution from osteoclasts to the fusion phenotype cannot be excluded, this is unlikely to be a primary driver of the observed phenotype, as blocking osteoclast activity with bisphosphonates did not produce suture fusion (Extended Data Fig. 2k). On the basis of the presence of a fusion phenotype in iDTR<sup>1<sub>Ctsk<sub>+</sub></sub></sup> but not in Osx<sup>Cre<sub>+</sub></sub> mice, we postulate that it is the absence of CTSK+ SCSs themselves and not the absence of more mature Osx-dependent osteoblast populations in the CTSK-lineage that drives the fusion phenotype.

On the basis of these results, we hypothesized that a second calvarial stem cell exists within the pool of non-CTSK-lineage sutural cells. To identify this putative second non-CTSK calvarial stem cell, we identified candidate markers by transcriptional analysis, immunostaining and flow cytometry. Among these candidates, the transmembrane collagen receptor DDR2 was prioritized on the basis of reports that Ddr2 is expressed in the sutural cells and that Ddr2-mutant mice display calvarial mineralization defects<sup>11</sup>. Additionally, we evaluated several other candidate markers that displayed inadequate selectivity in labelling the non-CTSK cell fraction or were found to mark populations without robust bone-formation capacity (Extended Data Fig. 3a–g). DDR2 marked a sutural population that did not overlap with CTSK-lineage cells and was present at sites of sutural endochondral ossification (Extended Data Fig. 2a,b and Extended Data Fig. 3h). Consistent with the expansion of non-CTSK-lineage cells observed above, both Twist<sup>1<sub>Ctsk<sub>+</sub></sub></sup> and iDTR<sup>1<sub>Ctsk<sub>+</sub></sub></sup> mice, as well as the Twist<sup>i<sub>Ctsk<sub>+</sub></sub></sup> craniosynostosis model, all displayed marked expansion of a DDR2+ population within the pool of

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Fig. 4 | DDR2+ CSCs contribute to calvarial mineralization and suture fusion. a, b, µCT of the skulls of DTA and DTA<sup>lox/lox</sup> (a), and Osx<sup>Cre</sup> and Osx<sup>Cre</sup> (b) mice at P14, 7 days after tamoxifen treatment (DTA, n = 5; DTA<sup>lox/lox</sup>, n = 6; Osx<sup>Cre</sup>, n = 7; Osx<sup>Cre</sup> <sup>lox/lox</sup>, n = 6). c, Schematic showing orthotopic transplantation of FACS-purified CTSK+ CSCs and DDR2+ CSCs into four-week-old suture-ablated mice. d, µCT of skulls 8 weeks after transplantation. Black arrowhead shows the primary implantation site. Red arrows show fusing sutures. e, 2D cut plane of skull 16 weeks after transplantation of indicated populations. Regions outlined in red are enlarged below. Asterisks indicate fused sutures. f, g, 2D cut plane of skull 16 weeks after transplantation of indicated populations. Regions outlined in red are enlarged below. g, CTSK+ CSCs and iDTR;CTSK+ CSCs were isolated from P7–P10 Cre<sup>+/–</sup> Tm<sup>Cre</sup>/mG or iDTR<sup>Cre</sup>/mT/mG mice, respectively. Recipient mice were administered diphtheria toxin (DT) eight weeks after transplantation. h, µCT of skulls 16 weeks after transplantation (control, n = 6; CTSK<sup>+</sup>, n = 6; DDR2+, n = 5; CTSK<sup>+</sup> and DDR2+, n = 5; iDTR:CTSK<sup>+</sup> and DDR2+, n = 4), red arrows indicate fusion sites. i, H&E staining (top) and fluorescence imaging of the primary implantation site 16 weeks after transplantation, with diphtheria toxin administration. Asterisks indicate the region of fusion. j, Fused LAM (left) or OIP (right) suture length as a proportion of total LAM or OIP suture length on the ipsilateral side, n = 3 independent experiments. Data are mean ± s.d.; one-way ANOVA with Tukey’s post-test (j). Images in e, f, h, i are representative of a minimum of three independent experiments. Scale bars: 100 µm (f, right, i), 1 mm (all µCT images).

sutural non-CTSK-lineage cells. This population of sutural DDR2+ cells included a subpopulation bearing the same surface immunophenotype as CTSK+ CSCs, Lin<sup>Thy1.2</sup> CD200<sup>+</sup> CD105<sup>+</sup> CD51<sup>+</sup> cells that we hypothesized to represent a calvarial stem cell type expressing DDR2 (DDR2+ CSCs) (Fig. 2c, d and Extended Data Fig. 3i–l).

We used serial transplantation, analysis of differentiation hierarchy, lineage tracing and label-retention studies, in addition to in vitro colony formation, clonal multipotency and serial mesosphere formation assays to confirm that putative DDR2+ CSCs are indeed stem cells. DDR2+ CSCs also displayed in vitro features of stemness. Single-cell-derived clones of DDR2+ CSCs were capable of in vitro tri-lineage differentiation into osteoblasts, adipocytes and, separately, chondrocytes. DDR2+ CSCs also displayed in vitro fibroblast colony-forming unit (CFU-F) activity and osteoblast colony-forming unit (CFU-Ob) activity, similar to that of CTSK+ CSCs. Finally, DDR2+ CSCs and CTSK+ CSCs displayed serial mesosphere formation capacity, a correlate of stemness in skeletal cells<sup>14</sup> (Extended Data Fig. 4a–g). In vivo transplantation showed that DDR2+ CSCs, but not other DDR2+ populations, were able to both self-renew and generate all of the other DDR2+ populations present in the native suture after the first round of transplantation. When re-isolated after the first round of transplantation and subjected to the second round of transplantation, DDR2+ CSCs were again capable...
of similar self-renewal and differentiation (Fig. 2e,f and Extended Data Fig. 4h,i), demonstrating serial transplantation and self-renewal capacity similar to that of other SSC populations identified in long bones13. Finally, DDR2+ CSCs that self-renewed after the first two rounds of transplantation were again capable of bone formation after re-isolation and a third round of transplantation. Both DDR2+ CSCs and CTSK+ CSCs formed mineralized bone organoids after each round of re-isolation. In line with the model of the suture containing two separate stem cell populations, we observed DDR2+ CSCs and CTSK+ CSCs—an additional, separate label-retaining cells—in the fraction of DDR2+ CSCs bearing surface markers consistent with CTSK+ CSCs (Lin CD200+ CD105 THY1.2 6C3 cells). As it was not possible to use GFP-based genetic reporters of the CTSK lineage together with the H2B–GFP system, we sought alternative markers of the CTSK+ CSC lineage to confirm the additional presence of label-retaining CTSK+ CSCs, identifying that galectin-1 immunostaining displayed high concordance with Ctsk-cre based lineage reporters (Extended Data Fig. 5p). Using galectin-1, the label retention in DDR2+ CSCs was confirmed to localize to CTSK-lineage galectin-1+ cells and therefore represent labelling of CTSK+ CSCs (Extended Data Fig. 5q). Thus, there are separate pools of calvarial label-retaining cells corresponding to both DDR2+ CSCs and CTSK+ CSCs, consistent with the presence of two stem cell types in the calvarium.

Next, we evaluated whether DDR2+ CSCs are capable of differentiating into bone-forming osteoblasts and whether they mediate endochondral ossification accounting for the inappropriate sutural endochondral ossification seen in Twist1Ctsk mice. Indeed, DDR2+ CSCs isolated by FACS and transplanted to the kidney capsule of secondary hosts formed robust bone organoids (Fig. 3a,b and Extended Data Fig. 6a,b). This bone was derived from graft DDR2+ CSCs, as osteocytes and osteoblasts in the organoid bore the tdTomato marker of graft DDR2+ CSCs (Fig. 3c). Additionally, no interconversion between DDR2+ CSCs and CTSK+ CSCs could be observed as there was no induction of Ctsk-cre-driven mGFP expression in the DDR2+ CSCs over time. Similarly, CTSK+ CSC-derived cells displayed no induction of DDR2 expression (Fig. 3c and Extended Data Fig. 6c,d).

Whereas CTSK+ CSCs mediated the expected intramembranous ossification after transplantation, DDR2+ CSCs underwent endochondral ossification consistent with being mediators of the inappropriate sutural endochondral ossification preceding suture fusion in Twist1Ctsk mice (Fig. 3d,e and Extended Data Fig. 6e–i). However, whereas endochondral ossification is typically intrinsically coupled with the...
Fig. 6 | Identification of human DDR2+ CSCs. a, H&E (n = 14) and Alcian Blue staining (a; n = 7), immunohistochemistry (b; n = 7) and immunofluorescence (c,d) of human SAG craniosynostosis specimens. e, In vivo serial transplantation of hDDR2+ CSCs (left) and hDDR2– CSCs (right). FACS analysis shows self-renewal and differentiation of the CSCs. FACS from initial donor (top), primary transplants (middle) and secondary transplants (bottom). Black and red arrows indicate parent–daughter gates and transplanted populations, respectively. Insets, µCT images of bone organoids derived from the tertiary transplants. Flow cytometry histograms of dye-dilution proliferation analysis. g, CFU-F analysis of hDDR2+ CSCs, hDDR2– CSCs and other human calvarial non-stem populations (n ≥ 3 independent experiments). h, Bright-field images of the initially seeded, primary, secondary and tertiary mesenchymal cells (left) and quantification of cell numbers per sphere (right; n ≥ 9). i, Left, a xenograft bone organoid in the renal capsule of an NSG–GFP host mouse. µCT images of hDDR2+ CSC or hDDR2– CSC-derived bone organoids over time after transplantation. j, von Kossa and Safranin O staining, showing endochondral ossification in xenograft bone organoids derived from hDDR2+ CSCs. k, Immunostaining of xenograft bone organoids derived from hDDR2+ CSCs implanted into the renal capsule of NSG–GFP hosts. Top left, confocal image showing the graft stained as indicated. Top middle and right, enlarged view of the region indicated in the left image. Bottom, COL2A1+ chondrogenic cells (left) and MMP13+ apoptotic cells (right) in xenograft organoids derived from hDDR2+ CSCs. Data are mean ± s.d. One-way (g) or two-way (h) ANOVA with Tukey’s post-test. FACS analysis (e,f) and images (a–d,g,k) are representatives of at least three independent experiments. Scale bars: 200 µm (a, left); 25 µm (a, middle and right; b); 100 µm (c,d,j,k); 20 µm (d, inset); 50 µm (g); 50 µm (h); 1 mm (all µCT images).

Recruitment of haematopoietic marrow elements129, DDR2+ CSCs mediate a distinct form of endochondral ossification without recruitment of haematopoietic stem cells, consistent with their inability to recruit long-term haematopoietic stem cells (Fig. 3f,g and Extended Data Fig. 6j). Although the aetiology of this difference in haematopoietic support capacity is likely to be multifactorial, DDR2+ CSC-derived organoids did not include cells expressing the haematopoietic niche factor CXCL12 that were present in organoids derived from long bone SSCs20 (Extended Data Fig. 6k). Likewise, DDR2-lineage cells displayed reduced expression of other mediators of haematopoietic support relative...
to femoral SSCs, including Cxcl12, Angpt1 and Kitlg (Extended Data Fig. 6l). Together, this indicates that a third fundamental form of bone formation, endochondral ossification without haematopoiesis, exists alongside traditional intramembranous and endochondral ossification, and DDR2+ CSCs are specialized for this third form of bone formation.

DDR2+ CSCs and CTSK+ CSCs display broadly distinct transcriptional profiles, reinforcing the notion that DDR2+ CSCs and CTSK+ CSCs are distinct cell types (Fig. 3b). Consistent with the chondrogenic capacity of DDR2+ CSCs, these cells expressed higher levels of classically chondrocyte-associated transcripts such as Sox9, Col2a1 and Acan, although these transcripts were also present at lower levels in CTSK+ CSCs. Both CTSK+ CSCs and DDR2+ CSCs expressed several transcripts associated with SSCs at other skeletal sites, including Myc, Runx2, Kif4 and Neogenin1,21,22. Of note, many of these shared genes were also present in both periosteal and endosteal stem cells in addition to growth plate resident SSCs in long bones, indicating convergence on a set of SSC-defining genes shared among multiple SSC types at different skeletal sites. GLII was expressed in both CTSK+ CSCs and DDR2+ CSCs, suggesting that this schema resolves the pool of GLII-lineage calvarial progenitors into multiple discrete stem cell types. Similarly, DDR2+ CSCs and CTSK+ CSCs also co-expressed other genes previously used to define calvarial progenitor populations, including Axin223 and Prrx123, indicating that the DDR2+ CSC versus CTSK+ CSC definitions cut across several established schemas for calvarial progenitors. DDR2+ CSCs and CTSK+ CSCs also co-expressed several genes involved in embryonic sutureogenesis, including Mxsp724, Mxsp2, Acta2, Lgr3 and Lrig1 (Fig. 3i and Extended Data Fig. 7a–c). Finally, a number of genes associated with human craniosynostosis are expressed at higher levels in DDR2+ CSCs, including Efnb1, Il1h, Mxs2, Fgffr2 and Fgfr3, suggesting a potential role of DDR2+ CSCs in the associated disorders (Extended Data Fig. 7d). These DDR2+ CSC and CTSK+ CSC expression signatures mapped onto distinct populations in several single-cell RNA-sequencing (scRNA-seq) datasets of mouse calvarium, providing additional support for the co-existence of DDR2+ CSC and CTSK+ CSC lineages in the calvarial sutures (Extended Data Fig. 7e–f).

In addition to a role in craniosynostosis, the DDR2+ CSC lineage makes an important contribution to calvarial mineralization distinct from that of the CTSK+ CSC lineage. Inducible postnatal deletion of DDR2+ CSCs in Ddr2-creER;Dta mice resulted in enlargement of the anterior fontanelle owing to hypomineralization of the frontal and parietal bones, and severe hypomineralization of the interparietal bone (Fig. 4a). Similarly, deletion of Osx specifically in DDR2+ CSCs (Osxcre; Ddr2-creER) resulted in calvarial hypomineralization similar to that seen in Ddr2-creER;Dta mice (Fig. 4b). Notably, DDR2+ CSCs, as mice with the spontaneously occurring smallie mutation that causes a loss of DDR2 expression (Ddr2spl mice) display calvarial hypomineralization, which is especially evident in the parietal and frontal bones widened sutures. Ddr2sple mice also displayed an absence or reduction in the cartilage structures in the lateral portions of the interparietal and occipital bones, implicating the DDR2+ CSC lineage in the formation of the cartilaginous elements of the calvarium (Extended Data Fig. 8a–c).

Using an orthotopic transplantation model in which the native lambdoid suture is surgically ablated and replaced by a defined set of cells implanted into the ablation site (Fig. 4c and Extended Data Fig. 8d), implantation of DDR2+ CSCs was sufficient to promote fusion in a manner that is restrained by co-implantation with CTSK+ CSCs (Fig. 4d,e and Extended Data Fig. 8e,f). DDR2+ CSCs, but not CTSK+ CSCs, were sufficient to mediate fusion both at the implantation site (the LAM suture) and in an extended region of the SAG, OIP and SQ sutures adjacent to the implantation site, despite both populations being capable of robust engraftment (Fig. 4f and Extended Data Fig. 8g–i). This fusion in adjacent regions reflected the migration of graft DDR2+ CSCs to these sites, in addition to the retention of graft cells at the primary implantation site. Consistent with the finding that depletion of CTSK+ CSCs was sufficient to induce suture fusion, co-implantation of CTSK+ CSCs with DDR2+ CSCs prevented the fusion-promoting activity of DDR2+ CSCs, while not affecting their ability to engraft. This fusion-suppressing ability of CTSK+ CSCs reflects continuous, direct regulation and not induced death or differentiation of DDR2+ CSCs as co-implantation of CTSK+ CSCs and DDR2+ CSCs followed by post-transplantation ablation of the implanted CTSK+ CSCs using a diphtheria toxin system was able to restore the fusion activity of DDR2+ CSCs (Fig. 4g–j and Extended Data Fig. 8j,k).

Further investigation revealed Igf1 as a candidate CTSK+ CSC-derived factor regulating the expansion and fusion-promoting activity of DDR2+ CSCs. Within these CSC populations, Igf1 was largely expressed on CTSK+ CSCs, and its cognate receptor Igf1r was largely expressed on DDR2+ CSCs (Extended Data Fig. 9a). In addition to the number of Igf1 expressing CTSK+ CSCs being decreased in Twist1cre lots, mice, the per cell expression of Igf1 was downregulated in the residual remaining Twist1cre lots CTSK+ CSCs (Extended Data Fig. 9b). Injection of recombinant Igf1 over the calvarium was able to partially prevent the fusion phenotype in Twist1cre lots mice, thus repressing Igf1 was sufficient to ameliorate the Twist1cre lots craniosynostosis phenotype (Fig. 5a,b) and Extended Data Fig. 9c). Moreover, CTSK+ CSC-derived Igf1 was necessary to prevent suture fusion as Igf1fl/fl;Ctsk-cre (Igf1fl/fl; Ctskcre) mice displayed spontaneous suture fusion and expansion of DDR2+ CSCs, mirroring observations in Twist1cre lots mice (Fig. 5c–f and Extended Data Fig. 9d,e). Thus, in this context, Igf1 is a CTSK+ CSC-derived regulator of DDR2+ CSCs, although other such regulators may exist.

Human counterparts of DDR2+ CSCs were present in the sutures of individuals with craniosynostosis, establishing the clinical relevance of this SSC type. Consistent with previous studies31,33 and observations in Twist1cre lots mice, calvarial specimens from patients with sporadic craniosynostosis contained abundant DDR2+ cells and displayed inappropriate endochondral ossification with cartilage undergoing active remodelling to bone (Fig. 6a–c and Extended Data Fig. 10a–c). Human sutural tissue also included both DDR2+ and DDR2− cells bearing a surface immunophenotype nearly identical to that of mouse DDR2+ CSCs and CTSK+ CSCs (Lin−THY1−CD200+CD105− cells that are DDR2+ or DDR2−, hereafter referred to as hDDR2+ CSCs and hDDR2− CSCs) (Extended Data Fig. 10d). Given the inability to utilize CTSK-based lineage reporters in humans, hDDR2+ CSCs were used here as a proxy for mouse CTSK+ CSCs. hDDR2+ CSCs and hDDR2− CSCs exhibited conserved gene expression features similar to those of their respective mouse counterparts, including expression of Igf1r on hDDR2+ CSCs (Fig. 6d and Extended Data Fig. 10e,f). Of note, this definition of hCSSCs also largely overlapped with another set of markers used to define stem cells in human fetal long bones (PdpnCD146CD164CD73+34). Thus, hDDR2+ and hDDR2− CSCs exhibit a convergent immunophenotype that fulfills criteria from multiple schema for identifying SSCs.

Both hDDR2+ CSCs and hDDR2− CSCs exhibited robust functional evidence of stemness, as both were able to respectively generate all of the skeletal human DDR2+ and DDR2− populations present in the initial donor suture tissue in addition to undergoing proliferative self-renewal over the course of three rounds of serial transplantation (Fig. 6e,f and Extended Data Fig. 11a–f). hDDR2+ and hDDR2− CSCs were also able to serially form bone organoids in xenograft systems over the course of three successive rounds of transplantation and re-isolation (Fig. 6e). Similarly, hDDR2+ CSCs and hDDR2− CSCs displayed in vitro features of stemness, including CFU-F activity, clonal multipotency and the capacity to serially form mesospheres (Fig. 6g,h and Extended Data Fig. 11g–i). Consistent with observations that mouse DDR2+ CSCs are specialized for endochondral ossification, hDDR2+ CSC-derived organoids included both graft-derived osteoblasts and chondrocytes and characteristically included the transient presence of an initial cartilage template that underwent vascular invasion and remodeling to mineralized bone (Fig. 6i–k and Extended Data Fig. 11j,k).
In summary, in this Article we find that the calvarial sutures contain two distinct stem cell types. One is a CTsk CSC that is specialized for intramembranous bone formation. The second is a DDR2+ CSC that characteristically mediates endochondral ossification without marrow formation. These definitions provide a starting point for further iterative refinement of stem cell identity in the calvarium. This also provides support for an emerging consensus that skeletal physiology broadly reflects a diverse set of distinct progenitor cell types. Interactions between these two cell types are critical for the pathogenesis of craniosynostosis and suture patency, as dropout of CTsk CSCs triggers a maladaptive expansion and activation of DDR2+ CSCs, which in turn results in inappropriate endochondral ossification in the sutures and ultimately, inappropriate fusion. This model also potentially explains widespread findings from the clinic and animal models that calvarial hypomineralization can accompany craniosynostosis. Counterparts of both of these cell types are also present in human calvarial sutures. Given their role in craniosynostosis, agents that inhibit DDR2+ CSCs or otherwise block their compensatory expansion offer an attractive strategy for adjunct medical therapy of craniosynostosis. Moreover, this provides an example of how multiple SSC lineages can co-exist and interact at a single site, with disruptions in these stem cell–stem cell interactions serving as a new mechanism for skeletal disease.

**Online content**

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-023-06526-2.
Methods

Animals

Ctsk-cre mice were a gift from S. Kato and T. Nakamura \textsuperscript{41}, Twist1\textsuperscript{fl/fl} mice (016832-UNC) were obtained from the Mutant Mouse Resource & Research Centers \textsuperscript{42}. Osx\textsuperscript{fl/fl} mice were a gift from B. de Crombrugghe \textsuperscript{43} and Ddr2\textsuperscript{cre} mice on the C57BL/6J background were obtained from R. T. Franceschi \textsuperscript{10}. mT/mG (007676), ROSA26iDTR (007900), ROSA-DTA (009669), MIP-GFP (006564), NSG-eGFP (021937), pTRE-H2B-GFP (005104) and iGf\textsuperscript{l} (016831) mice were purchased from Jackson Laboratories. ROSA:LN:LTA mice (Jackson Laboratories, 011008) were a gift from L. Puglielli \textsuperscript{44}. Ddr2-cre ERE mice were generated by R. T. Cowling and B. H. Greenberg with assistance from the Transgenic Core and Embryonic Stem Cell shared resource at UCSD \textsuperscript{45}. In brief, a MerCreMer cassette was inserted in-frame into exon 2 of Ddr2 using homologous recombination following a strategy similar to one previously described \textsuperscript{46}. Ddr2-creER mice were crossed with mT/mG mice for lineage tracing studies. Recipient heterozygous MIP-GFP mice were generated by breeding homozygous MIP-GFP males with wild-type C57BL/6J females. All mice were maintained on a C57BL/6J background throughout the study. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Weill Cornell Medical College institutional animal care and use committee (IACUC). All animal experiments followed all relevant guidelines and regulations.

Administration of chemical reagents

iDTR:Ctsk-cremiT/mG pups were given 50 mg of diphtheria toxin (Sigma, D0564) intraperitoneally as indicated in Extended Data Fig. 2a. PBS was used as a vehicle. For functional inhibition of osteoclasts, C57BL/6J mice were given alendronate sodium trihydrate (1 mg kg\textsuperscript{-1}; Sigma, A4978) intraperitoneally as illustrated in Extended Data Fig. 2k. For lineage tracing analysis, Ddr2-creER activity was induced at P2. Tamoxifen (100 mg kg\textsuperscript{-1}; Sigma, T5648) was prepared in corn oil and subcutaneously injected into animals for 2 consecutive days as described in Extended Data Fig. 5a. To investigate the physiologic contribution of DDR2 CSCs, ROSA-DTA, and Osx\textsuperscript{fl} mice were crossed with Ddr2-creERmT/mG mice and tamoxifen was delivered subcutaneously into P7 pups. Recombinant IGF1 (1 mg kg\textsuperscript{-1}; R&D Systems, 291-G1) was subcutaneously administered over the calvarium of Twist1\textsuperscript{fl/fl} mice at P3, as described in Extended Data Fig. 9c. PBS was used as a vehicle. Where these agents were utilized, mice receiving control versus experimental treatments were randomized from among all applicable mice.

Histone H2B–GFP labelling-retention system

pTRE-H2B–GFP mice were previously described \textsuperscript{46}. To label slow-cycling cells under the control of a tetracycline response element, pTRE-H2B–GFP mice were crossed with ROSA:LN:LTA (tet-off) mice. Eight-week-old mice were fed with a doxycycline rodent diet (200 mg kg\textsuperscript{-1}; Bio-Serv, S3888) for 6- and 12-month chase periods. Litters without doxycycline exposure were used as controls. Additional controls consisted of mice that were continuously exposed to doxycycline, including during the embryonic period by providing doxycycline to pregnant female mice. These mice were used as a negative control for H2B–GFP expression.

Human specimens

This study was carried out under the institutional review board (IRB) protocol (no. 1402014802), with all patients providing informed consent through the Center for Neurogenetics at Weill Cornell Medical Center. Human calvarial tissue used for scientific analyses was obtained from 37 cases of craniosynostosis surgical repair procedures. Participants were 70% who were male. Detailed annotation of the clinical specimens including age, sex and the primary diagnosis is provided in Supplementary Table 1. All human tissue experiments followed all relevant guidelines and regulations. Written consent was provided by the parents or guardians of all study participants.

Isolation of suture cells

For mouse samples, microdissected calvarial sutures were subjected to both mechanical and enzymatic digestion. The collected tissues were minced using razor blades and digested for up to 20 min with digestion cocktail buffer containing Collagenase P (1 mg ml\textsuperscript{-1}; Roche, 11213857001) and Dispase II (2 mg ml\textsuperscript{-1}; Roche, 04942078001) in medium containing 2% serum at 37 °C with agitation. Medium containing 2% serum was added to the digest and the tubes were centrifuged to pellet cells. The supernatant was carefully removed, and the pellet was resuspended in DNase I (2 units per ml; Roche, 04716728001) solution and briefly incubated for 5 min at 37 °C. Medium was added to the tube and the digested tissue was resuspended thoroughly by pipetting and then filtered through a 70-μm nylon mesh. Tubes were centrifuged and the resulting cell pellet was subjected to FACS. For human calvarium specimens, tissues were subjected to mechanical and enzymatic digestion with Collagenase A (1 mg ml\textsuperscript{-1}; Roche, 10103386001) and Dispase II (2 mg ml\textsuperscript{-1}) in a medium containing 2% serum for 30 min at 37 °C under agitation, and the cells were then isolated following the same procedure as described above.

Fluorescence-activated cell sorting

Prepared single-cell suspensions were washed twice with ice-cold FACS buffer (2% serum + 1 mM EDTA in PBS) and incubated with Fc blocking buffer (1:100 dilution; BD Bioscience, 53342 for mouse and 564765 for human) for 15 min at 4 °C. Primary antibody dilutions were prepared in Brilliant Stain Buffer (BD Bioscience, 563794). Cells were incubated in the dark for 40 min at 4 °C with primary antibody solution, washed 2 times with FACS buffer, and incubated with secondary antibody solution for 20 min, if needed. Detailed information of all antibodies for FACS of murine and human samples is stated in the Reporting Summary. Validation for cell sorting and the specificity of selected antibodies was initially performed using HEK-293 cells (ATCC, CRL-1573). Cells were then washed several times and resuspended in FACS buffer with DAPl (BD Bioscience, 564907). FACS was performed using a Becton Dickinson Aria II equipped with five lasers (BD Bioscience). Beads (Invitrogen, 01-3333-42) were used to set initial compensation. Fluorescence minus one (FMO) controls were used for additional compensation and to assess background levels for each stain. Gates were drawn as determined by internal FMO controls to separate positive and negative populations for each cell surface marker. Typically, 2.5 million events were recorded for each FACS analysis, and the data were analysed using FlowJo (v10.6.1). To better depict the FACS gating strategy, arrows have been added to the plot to illustrate parent/daughter gates. Additionally, extended data figures have been provided to show the full details of the FACS profiles and gating strategies used (Extended Data Figs. 1g, 3i, 4h,1,l, 5n, 10d and 11b,c,e).

Primary cell culture and clonal differentiation assays

The clonal multipotency of sorted cell populations was evaluated under normoxic conditions. Mouse primary cells were grown in a complete MesenCult medium (Stem Cell Technologies, 05501) with stimulatory supplements (Stem Cell Technologies, 05502 and 05500). After initial cell plating, cells were left undisturbed and allowed to grow at 37 °C under humidified conditions for a week. Half of the medium was then removed every three days. Cells were passaged once they were 60–70% confluent using a cell dissociation reagent (Gibco, A1105-01). Cells sorted from human calvarial tissue were cultured under conditions similar to those described above using a commercial medium preparation (Stem Cell Technologies, 05401) with stimulatory supplements (Stem Cell Technologies, 05402). The differentiation potential of ten colonies derived from single FACS-isolated cell populations was...
examined. In brief, single sorted cells were plated at a density of 100 cells in 6-well culture plates and allowed to form individual colonies. Initial dispersion of the plated cells as single cells was confirmed by light microscopy. Each of the selected colonies was extracted using a cloning cylinder. The extracted cells were regrown for 3 days in 12-well plates and then allowed to differentiate under both osteogenic and adipogenic conditions as described below. For osteogenic differentiation and Alizarin Red S staining, sorted cells were expanded and then allowed to differentiate using an osteogenic differentiation medium (Gibco, A1007201) for 21 days. The medium was changed every other day. At the end of this period, cells were washed with cold PBS and fixed with 4% paraformaldehyde (PFA) for 30 min on ice. Cells were washed with distilled water and stained with Alizarin Red S for 2 min. Cells were then washed thoroughly with water and air-dried before microscopic visualization. Adipogenic differentiation studies were conducted with sorted cells using methods similar to those described above. In brief, cells were allowed to differentiate in an adipogenic differentiation medium (Gibco, A1007001). Fresh medium was added every other day for a total of 14–20 days. The medium was removed, and cells were washed with PBS and fixed with 4% PFA for 30 min at room temperature. Cells were rinsed again with PBS and stained for 30 min with Oil Red O working solution (3:2 dilution with water). After washing five times with PBS, cells were then observed under a light microscope. For chondrogenic differentiation and Alcian Blue staining, cells were grown as pellet culture. Ten thousand cells in chondrogenic differentiation medium (Gibco, A1007101) were centrifuged at 1,500 rpm for 5 min in 15-ml conical tubes. Cultures were re-fed every 3 days and allowed to differentiate for a minimum of 14 days. At the endpoint of this study, the medium was removed, and pellets were collected and washed carefully with PBS and fixed with 4% PFA for 30 min, then embedded in OCT compound. After cryo-sectioning, slides were stained for 30 min with 1% Alcian Blue solution, washed three times with 0.1 N HCl, and then with PBS.

Mesosphere assays
Mesosphere assays were performed as described previously. In brief, single cells isolated by FACS from mouse or human calvarial sutures were plated at a density of 100 cells per cm² and allowed to grow in ultra-low adherence culture dishes (Stern Cell Technologies, 27145). Plates were incubated at 37°C with 5% CO₂ and left undisturbed for a week. Half of the medium was replaced every five days. Ten days later, mesosphere formation was observed using microscopy, and mesospheres were dissociated into single cells using Accutase solution (Gibco, A11105-01) and subsequently re-plated to generate secondary and tertiary mesospheres.

Culture and differentiation for CFU-F and CFU-Ob
Freshly FACS-isolated mouse cells were seeded at a clonal density of 2 × 10³ cells per well in six-well culture plates with MEM α (Gibco, A10499001) containing 10% serum, and triplicate cultures were established. Half of the medium was replaced every 3 days. CFU-F was measured after 10 days of culture by crystal violet staining and CFU-Ob was measured at 28 days of culture by alizarin red S staining after light fixation with 4% PFA. The number of colonies comprising at least 50 cells was counted in each well on day 10 (CFU-F) and day 28 (CFU-Ob). For human cells, cells were seeded at a clonal density of 1 × 10⁵ cells per well in 6-well culture plates and then subjected following the same procedure as described above.

μCT scans and analysis
μCT analysis was conducted on a Scanco Medical μCT 35 system at the Citigroup Biomedical Imaging Core as previously described. In brief, a Scanco Medical μCT 35 system with an isotropic voxel size of 7 μm and 20 μm was used to image the distal femur and tibia, and skull, respectively. Scans were conducted in PBS and used an X-ray tube potential of 85 kVp, an X-ray intensity of 0.145 mA, and an integration time of 600 ms. μCT analysis was performed by an investigator blinded to the genotypes of the animals.

Renal capsule transplantation for bone organoids
Freshly FACS-purified mouse tdTomato-expressing DDR2⁺ CSCs and mGFP-expressing CTSK⁺ CSCs from Ctsk-cre;mT/mG mice were transplanted underneath the renal capsules of 8 to 10-week-old MIP-GFP mice. To avoid the potential immunogenicity of GFP itself, all transplantation studies were conducted in MIP-GFP hosts that have baseline immunologic tolerance to GFP variants. For human DDR2⁺ CSCs and DDR2⁺ CSCs, cells freshly isolated by FACS were transplanted under the renal capsule of 8 to 12-week-old immunodeficient NSG-eGFP mice. Ten thousand to twenty thousand mouse or human cells were implanted underneath the renal capsule. The number of cells implanted for serial transplantation studies is included in the corresponding figure legends. Renal capsule transplantation was performed as previously described. In brief, mice were anaesthetized and shaved on the left flank and abdomen before sterilization at the surgical site. The kidney was externalized through a 1 cm incision and a 2 mm pocket was made in the renal capsule. A matrigel plug (Corning, 356231) containing the cells of interest was implanted underneath the capsule and the hole was sealed using a cauterizer before replacing the kidney back into the body cavity. More than 75 donor mice were euthanized to collect the desired number of CSCs for every organoid-based transplantation experiment. Animals were euthanized by CO₂ after the indicated experimental duration, then kidneys were lightly fixed with 4% PFA for 6 h and mineralized bone formation was detected by μCT scans and fluorescent stereomicroscopes. Samples were subjected to infiltration, embedding and sectioning as described below. Human xenografts derived from hDDR2⁺ CSCs were detected by both staining with human-specific nuclear antigen and observing the absence of a host-expressed GFP transgene.

Intramuscular transplantation for determination of stemness and differentiation hierarchy
In validation studies, we found that the intramuscular site was more suitable than the kidney capsule for serial transplantation studies, as it was easier to physically separate graft tissue from surrounding host parenchyma during re-isolation after each round of transplantation (data not shown). Intramuscular transplantation was performed in 6- to 8-week-old MIP-GFP or immunodeficient NSG-eGFP mice. A 1-mm longitudinal incision was made on the right hindlimb and the right anterolateral femur was exposed. The vastus lateralis muscle was dissected and a 2- to 3-mm muscle pouch was surgically created. A surgifoam absorbable gelatin sponge (Ethicon, 1972) containing sorted cell populations of interest was placed into the muscle pouch. For human DDR2⁺ CSCs and DDR2⁺ CSCs, cells isolated by FACS were labelled with DiD lipophilic cell membrane-labelling dye AF647 (Invitrogen, V22887) prior to transplantation. The overlying fascia was closed using 4-0 polyglactin 910 absorbable sutures (Ethicon, J386), and wound clips were used to close the skin incision. For serial transplantation studies, animals were euthanized by CO₂ narcosis 7–10 days post-surgery, and the muscle housing the grafts was dissociated for re-isolation of graft cells by FACS for the second round of transplantation. After digestion of the primary grafts, the resulting single-cell suspension was subsequently processed for sorting as described above. Cell populations of interest self-renewing after the first round of transplantation were again re-isolated and transplanted into new hosts. For mouse studies, 2 to 3 × 10^⁶ DDR2⁺ CSCs were initially transplanted into primary recipients. 1 to 3 × 10^⁶ DDR2⁺ CSCs were re-isolated from the primary transplants and then re-transplanted. Fewer than approximately 1 × 10⁵ DDR2⁺ CSCs were re-isolated from the secondary transplants. Grafts were explanted and analysed seven days after transplantation. For human studies, 2 to 5 × 10⁶ hDDR2⁺ CSCs and hDDR2⁺ CSCs were initially transplanted into immunodeficient NSG-eGFP primary recipients. Twenty thousand to fifty thousand hDDR2⁺ CSCs and hDDR2⁺ CSCs were re-isolated from
the primary transplants and then transplanted. One thousand to three thousand hDDR2− CSCs and hDDR2+ CSCs were re-isolated from the secondary transplants and then transplanted into the kidneys of tertiary recipients. Grafts were explanted and analysed 10 days (for FACS) and 21 days (for bone organoid formation) after transplantation.

**Suture ablation model**

Four-week-old MIP-GFP mice were anaesthetized and had the skin overlying the skull shaved before sterilization of the surgical site. A scalpel was used to carefully remove the periosteum of the skull prior to removing the lambdoid sutures and surrounding bones on the right side of the skull using a micro hand drill with 0.2 mm drill bits (Stoelting, 58610). Cells populations of interest (2 × 10^5 cells) were isolated by FACS from calvarial sutures of Ctsk-cre/m1/mG mice and were mixed with 2 μl of a gel mixture (GelMA:Matrigel:Collagen1, 6:3:1) (Cellink, IK3051020303, Corning, 356231, Gibco, A1048301, respectively) prior to implantation at the defect site. The gel mixture, with or without cells, was cross-linked by exposure to UV light for a few seconds after transplantation and the skin incision was closed using an absorbable suture. Animals were euthanized by CO2, and the specimens were collected after 8 and 16 weeks.

**Sample preparation for cryo-sectioning and imaging**

Freshly extracted mouse samples were fixed with 4% PFA for 4–6 h at 4 °C. Samples were washed with PBS and decalcified with daily changes of 0.5 M EDTA for 1–5 days depending on the age of the samples. Samples were incubated with infiltration medium (20% sucrose + 2% polyvinylpyrrolidone in PBS) with rocking until they sank to the bottom of the tube. Embedding was performed with a customized embedding medium (OCT + 15% sucrose in PBS) and samples were preserved at −80 °C. Sections 10–20 μm in thickness were cut using a Leica cryostat.

**Immunohistochemistry**

Immunohistochemical analysis was conducted on Zeiss instruments and systems at the Optical Microscopy Core using the previously described method. In brief, frozen samples were thawed at room temperature and rehydrated with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 min, and blocked for 30 min with 5% donkey serum in PBS. Dilutions of primary antibodies (1:50–1:100) were freshly prepared in 0.3% Triton X-100 in PBS. Samples were incubated overnight with primary antibodies at 4 °C and, then washed three times with PBS. Secondary antibodies (1:1,000 dilution) were added to samples for 1 h, followed by washing three times with PBS. Samples were finally mounted with ProLong Gold antifade reagent with or without DAPI (Invitrogen, P36931). Detailed information of all antibodies used for the immunohistochemistry of murine and human samples is presented in the Reporting Summary. Imaging was performed with a Zeiss LSM 880 laser scanning confocal microscope. All data were processed using Zeiss ZEN 2.3 SPI software. In Fig. 6a,b and Extended Data Fig. 10a, formalin-fixed paraffin-embedded (FFPE) human calvarium specimens were used for histology and stained with the antibodies as indicated in images.

**Bulk RNA sequencing**

Total mRNA was freshly extracted from FACS-isolated CSC populations from P7 Ctsk-cre mice (n = 5) using RNasey Plus Micro Kit (Qiagen, 74034). Total RNA integrity was checked using a 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis and amplification were performed by SMART-Seq v4 ultra-low input RNA kit (Takara Bio) starting with 1 ng of total RNA from each sample. One hundred and fifty picograms of qualified full-length double-strand cDNA was used and processed for Illumina library construction with the Nextera XT DNA Library Preparation Kits (Illumina). Then the normalized cDNA libraries were pooled and sequenced on an Illumina NovaSeq6000 sequencer with pair-end 50 cycles. Bulk RNA sequencing was performed on a total of 10 FACS-isolated CSC populations (5 CTSK+ and 5 DDR2+) from mice, and a counts matrix was obtained. DESeqDataSet was generated using this unnormalized counts matrix. Differential expression analysis was performed using the DESeq function and results tables were generated using the results function to compare the DDR2+ and CTSK+ groups. Log fold change shrinkage was performed using the IcfShrink function with the apeglm method for visualization and ranking of genes. Results were exported as .csv files (one with all the genes, and one with the subset of genes with adjusted P value < 0.05). Two-dimensional principal component analysis plots of the samples were generated by the first two principal components using the plotPCA function. Normalized read counts were used for generating heat map plots. The data were visualized by a heat map with hierarchical clustering in R v3.6.2 with the pheatmap package v1.0.12. GO analysis was performed using DAVID Bioinformatics Resource tools v6.8.

**Analysis of scRNA-seq data**

Single-cell RNA sequencing data from public datasets were analysed by Seurat v4.3.0 for standard cell clustering and visualization workflows. In brief, the clustering in Ayturk et al. was used as reported in that paper for the analysis, without modification. For the Farmer et al. report, clustering analysis was repeated due to the distinct experimental focus of the present Article. FindNeighbors() and FindClusters() functions were used for this clustering. In both cases, filtering based on mitochondrial and ribosomal RNA content was not modified from the analysis performed in the initial studies reporting this data. The AddModuleScore() function was performed to annotate all cells for both CTSK+ and DDR2+ gene expression signatures. The gene list comprising the CTSK+ and DDR2+ expression signatures was generated by taking the top 50 differentially expressed genes in each of these cell types in the bulk RNA sequencing data in Fig. 3i. This gene list was further trimmed by evaluating the expression of all genes in the Ayturk et al. and Farmer et al. scRNA-seq datasets, genes displaying negligible expression were dropped from the gene signature as noncontributory. Components of the gene expression signatures used for this score are individually displayed in Extended Data Fig. 7e (iii), f (iii), and the full list of genes comprising these scores is provided in Supplementary Table 2. Expression of selected individual components of these gene expression signatures, focusing on genes with biologic relevance to craniosynostosis, is displayed in violin plots in Extended Data Fig. 7e (iii), f (iii). For visualization of the clusters and the projection of the CTSK+ and DDR2+ gene expression signature score on these cells, UMAP was used for the Farmer et al. and L-distributed stochastic neighbour embedding was used for the Ayturk et al. reports to mirror the data visualization methods used in the initial reports and thereby facilitate comparison to the analysis originally reported.

**Statistical analyses**

All data are shown as mean ± s.d. as indicated. For comparisons between two groups, unpaired, two-tailed Student’s t-tests were used. For comparisons of three or more groups, one-way or two-way ANOVA was used if normality tests passed, followed by Tukey’s multiple comparison test for all pairs of groups, unless otherwise specified. GraphPad PRISM v8.1.2 was used for statistical analysis. P < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

**Reporting summary**

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

**Data availability**

RNA-seq data have been deposited at the Gene Expression Omnibus (GEO) under accession number GSE232652. Source data are provided with this paper.
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Author contributions S.B. designed, conducted, and analysed the majority of experiments. M.B.G. and S.D. supervised the project. M.B.G., S.D. and S.B. conceived the project. S.B. and A.R.Y. performed all mouse surgeries. S.B., A.R.Y. and J.S. conducted experimental repeats. S.B. and J.S. contributed to methodology development for serial transplantation and the label-retention system and data collection. S.B., J.S., A.R.Y., and M.C. maintained and genotyped all mice. J.M., T.B. and P.B. supervised or conducted cell sorting by flow cytometry. L.H., S. Lalani, and T.Z. performed RNA-seq and scRNA-seq data analysis. D.J.P., M.E.R., T.A.I., C.E.H. and S. Lakhanl provided access to clinical specimens and supervised human studies. M.C., Z.L., B.R.S. and K.W.M. helped with human specimen processing. S.B. and M.B.G. prepared the manuscript. All authors read and approved the manuscript. R.T.F., R.T.C., F.F.M., C.G. and B.H.G. assisted with mouse cross generation and participated in data analysis and interpretation. J.S., A.R.Y., M.C. and B.R.S. performed μCT scans and analysis.

Competing interests The authors declare no competing interests.

Additional information Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-023-06526-2. Correspondence and requests for materials should be addressed to Shawon Debnath or Matthew B. Greenblatt. Peer review information Nature thanks Andrei Chagin and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Reprints and permissions information is available at http://www.nature.com/reprints.
Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Analysis of skeletal CTSK lineage cells in the calvarial sutures of wildtype (WT) and Twist1<sup>−/−</sup> mice. a, µCT images of the skulls of WT and Twist1<sup>−/−</sup> mice (P0 WT, n = 6 Twist1<sup>−/−</sup>, n = 8; P7 WT, n = 8 Twist1<sup>−/−</sup>, n = 5; P14 WT, n = 6 Twist1<sup>−/−</sup>, n = 6; P21 WT, n = 6 Twist1<sup>−/−</sup>, n = 5; P56 WT, n = 9 Twist1<sup>−/−</sup>, n = 6). Twist1<sup>−/−</sup> mice display 100% penetrance of fusion at the squamous (SQ), occipitointerparietal (OIP), and lambdoid (LAM) sutures from 14 days of age and onwards. Partial penetrance of coronal (COR) suture fusion (16-40%) is observed. b, H&E images of the OIP suture in WT and Twist1<sup>−/−</sup> mice at P21. The black arrow indicates the fused suture. c, Fluorescence images of the major sutures including the Interfrontal (IF), coronal (COR), sagittal (SAG), and lambdoid (LAM) sutures in WT (Twi st1+/+;Ctsk-CRE;mTmG) and Twist1<sup>−/−</sup> (Twi st1fl/fl;Ctsk-CRE;mTmG) mice at P21. d, FACS plots (left) and absolute and relative quantification (right) of the amount of skeletal CTSK lineage cells (CD31−CD45−Ter119−CTSK-mGFP+) from the major sutures of WT (n = 4) and Twist1<sup>−/−</sup> (n = 4) mice at P14. Lin indicates CD31, CD45, and Ter119 for all FACS analyses. e, FACS plots and absolute cell number of the skeletal CTSK lineage cells from the calvarial sutures of WT (n = 4) and Twist1<sup>−/−</sup> (n = 4) mice at E16.5. f, FACS plots (left) and absolute and relative quantification (right) of apoptotic cells within the skeletal CTSK lineage cells from the major sutures of WT (n = 4) and Twist1<sup>−/−</sup> (n = 4) mice at P14. Lin indicates CD31, CD45, and Ter119 for all FACS analyses. g, Gating strategy for FACS analysis of CTSK<sup>+</sup> CSC and CTSK<sup>−</sup> putative CSC populations in the sutures of WT (Ctsk-CRE;mTmG) mice. h, Twi st1 gene expression levels in FACS-isolated CTSK<sup>+</sup> CSCs and CTSK lineage cells of WT (n = 3) and Twist1<sup>−/−</sup> (n = 3) mice as determined by RT-PCR. QuantStudio 6 Flex RT-PCR Software v1.3 was used for mRNA analysis. i, FACS analysis of the frequency of cell populations in the calvarial suture. i) CTSK CSCs and CTSK putative CSCs (CTSK<sup>−</sup>pCSCs) relative to total skeletal lineage cells in the sutures of WT (n = 12) and Twist1<sup>−/−</sup> (n = 17) mice. ii) Absolute and relative quantification of the amount of CTSK<sup>+</sup>PP1 (Lin Thy-1.2 6C3 CTSK<sup>−</sup>CD200<sup>−</sup> CD105<sup>−</sup>) and PP2 (Lin Thy-1.2 6C3 CTSK CD105<sup>−</sup>) populations in the sutures of WT (n = 7) and Twist1<sup>−/−</sup>mTmG (n = 9) mice. j, Images of immunostaining for Ki-67 of the LAM sutures in WT (n = 3) and Twist1<sup>−/−</sup>mTmG (n = 3) mice at P10. Quantification of the number of tdTomato and Ki-67 double-positive cells per high power field in the LAM and OIP sutures (bottom). k, Confocal fluorescence imaging for Osteocalcin (OCN) staining of the COR (i) and LAM sutures (ii) in WT and Twist1<sup>−/−</sup>mTmG mice. Two regions in the LAM sutures from each group (n = 4) were measured for quantification. l, Confocal immunofluorescence imaging for hallmarks of active endochondral ossification including staining for osteocalcin (OCN), chondrocytes (COL2A1, COMP), molecular mediators of endochondral ossification (MMP13), markers of hypertrophic chondrocyte apoptosis (Cleaved Caspase-3 (CC3)), and vascular invasion (CD31) in consecutive slices of the OIP sutures of Twist1<sup>−/−</sup>mTmG mice at P7. The insets show high magnification of the region demonstrating that sutural cartilage templates derived from non-CTSK lineage cells in Twist1<sup>−/−</sup> mice. m, n, Timecourse Safranin O staining (red) and representative low-power (i) and high-power (ii) images of the OIP (m) and LAM (n) sutures in WT (n = 3) and Twist1<sup>−/−</sup> (n = 3) mice. In the OIP and LAM sutures, the penetrance of this sutural endochondral ossification phenotype is 100% in Twist1<sup>−/−</sup>mTmG mice at P10. Far-right, enlarged view of the box in each panel. Each of dots shown in the graph represents an individual mouse. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (d, e, h, k), two-way ANOVA with Sidak’s corrections for multiple comparisons test (f). All images and FACS plots are representative of at least three independent experiments. Scale bars are denoted in images.
Extended Data Fig. 2 | In vivo depletion of CTSK+ cells using an inducible DTR (iDTR) system.

a, Schematic representation of the experimental design and gross appearance of ROSA26iDTR and iDTR\textsuperscript{Ctsk} mice before and after DT administration. The red arrow in image indicates an abnormal dome-shaped skull.

b, Immunostaining for Cleaved Caspased-3 (CC3) in the sutures of iDTR\textsuperscript{Ctsk};mTmG mice with PBS or DT. n = 3 independent experiments. Green, CTSK+ (mGFP) cells; Red, non-CTSK lineage (tdTomato) cells; Magenta, CC3.

c, FACS analysis of the absolute amount of CTSK+ cells and CTSK− pCSCs in the sutures of iDTR\textsuperscript{Ctsk} mice with PBS (n = 4) or DT (n = 4) administration (i). Normalized absolute quantification of the indicated cell types by analyzing the same number of cells passing the FSC/SSC, doublet, and live gates in all specimens (ii).

d, µCT scan 3D images of the skull of ROSA26iDTR and iDTR\textsuperscript{Ctsk} mice at P11, P21, and P35 after DT administration. Red arrows indicate suture defects including abnormal fusion and hypomineralization (ROSA26iDTR, n = 5; iDTR\textsuperscript{Ctsk}, n = 5).

e, µCT scan 2D images of the skull with an enlarged view of the region outlined in blue of ROSA26iDTR and iDTR\textsuperscript{Ctsk} mice at P21 and P35.

f, Confocal immunofluorescence images of Ki-67 and quantification of the number of tdTomato and Ki-67 double-positive cells per high-power field of the OIP and LAM sutures in iDTR\textsuperscript{Ctsk};mTmG mice at P14 after PBS (n = 3) or DT (n = 3) administration. Dots in graphs represent an individual mouse.

g-i, Representative µCT images of the cortical (g), trabecular bone (h), and tibias (i) in ROSA26iDTR and iDTR\textsuperscript{Ctsk} mice after DT administration. Arrows show cortical defects including uneven periosteal surfaces and a double cortex. Quantification of bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) of trabecular bones in ROSA26iDTR (n = 4) and iDTR\textsuperscript{Ctsk} (n = 4) mice at P21 after DT administration (h). j, Representative confocal fluorescent images of CTSK-expressing osteoclasts in iDTR\textsuperscript{Ctsk};mTmG mice at P35 after PBS or DT administration. Red arrows indicate CTSK+ osteoclasts in the interparietal bones. Quantification of the number of osteoclasts per high-power field (HPF) of the sutures iDTR\textsuperscript{Ctsk};mTmG mice after PBS or DT administration. Two or three regions from each group (n = 3) were measured for quantification. k, Experimental scheme for functional inhibition of osteoclast activity using alendronate (1 mg/kg) and µCT scan images of the long bones and skulls of C57BL/6 J mice at P42 after alendronate treatment. (Vehicle (PBS), n = 3; Alendronate, n = 5). ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (c, f, h, j). All images and FACS plots are representative of at least three independent experiments. Scale bars are denoted in images.
Extended Data Fig. 3 | Characterization of DDR2+ CSCs. a, Candidate cell surface markers from gene expression analysis of FACS-purified CTSK+ CSC and CTSK− pCSC populations determined by RNA-Seq. b, Ddx2 mRNA expression in FACS-isolated CTSK− pCSCs of WT (n=6) and Twist1+/- (n=6) mice analyzed by RT-PCR. c, Immunostaining for candidate cell surface markers including NGFR (CD271), NCAM1 (CD56), PDGFRα (CD140α), and MCAM (CD146) in the calvarial suture of Ctsk-Cre;mTmG mice at P7. Experiments were repeated 3 independent times. d, Flow cytometry for CD271, CD140α, and CD146 versus CTSK lineage cells in the calvarial sutures. e, Flow cytometry gating strategy for CTSK-negative DDR2+ CSCs in the calvarial sutures of P7 Ctsk-Cre;mTmG mice. Black arrows indicate parent/daughter gates. j, Flow cytometry analysis of the percentage of CD51+ cells within CTSK− pCSC, CTSK+ CSC, and DDR2+ CSC populations (n=4). k, Quantification of the percentage of DDR2+ CSCs within the pool of the CTSK putative stem cell fraction in the sutures of WT (n=5) and Twist1+/- (n=5) mice as determined by flow cytometry. l, FACS plots showing skeletal DDR2+ cells (i) and DDR2+ CSCs (ii) in the sutures of WT, Twist1+/-, and Twist1+-/ mice at P12. ****P<0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (c, k). Images in d, f, h are representative of a minimum of three biological replicates, and FACS plots are representative of three independent experiments. Scale bars are denoted in images.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Determination of the stemness and differentiation hierarchy of DDR2 + CSCs. 

a, Fluorescence images of a timecourse of single cell-derived clonal expansion in FACS-purified CTSK+ CSCs and DDR2 + CSCs isolated from Ctsk-Cre;mTmG mice (i) and no induction of Ctsk-Cre driven mGFP was observed in DDR2 + CSCs (ii). b, Alcian Blue (i), Alizarin Red S (ii), and Oil Red O (iii) staining for in vitro clonal multipotency. 10 single FACS-isolated CTSK+ CSCs or DDR2 + CSCs were expanded and able to undergo both osteoblast and adipocyte differentiation. All cells were derived from the expansion of a single FACS-isolated CTSK+ CSC or DDR2 + CSC. Bar graphs show the clonal efficiency demonstrating that all colonies were capable of both osteoblasts and adipocytes differentiation from the same clone of cells. 10 colonies were examined in triplicate experiments. c, Images showing the fibroblast colony-forming units (CFU-F) derived from FACS-isolated CTSK+ CSCs or DDR2 + CSCs. The number of CFU-F colonies per well was quantified at day 10 after seeding (n = 5 single FACS-isolated CTSK+ CSCs or DDR2 + CSCs). d, In vitro limiting dilution analysis and CFU-F formation. Dilutions of 500, 200, 100, 50, 20, and 10 CTSK+ CSC or DDR2 + CSCs were seeded onto 6-well culture plates. e, Clonal expansion and differentiation into osteoblasts in FACS-isolated CTSK+ CSCs and DDR2 + CSCs. Colonies were stained for Alizarin Red S at day 28. The number of CFU-Ob colonies per well was quantified (n = 6 single FACS-isolated CTSK+ CSCs or DDR2 + CSCs). f, Bright-field and fluorescence images of initial seeded, primary, secondary, and tertiary mesenchymal spheres derived from CTSK+ CSCs (GFP, green), DDR2 + CSCs (RFP, red), and non-stem fraction of calvarial cells (Thy-1.2+ here) in CTSK+ or DDR2 + lineage isolated from Ctsk-Cre;mTmG mice. g, Quantification of individual cell numbers per sphere (top) and the percentage of cells able to form spheres (bottom) for CTSK+ CSCs, DDR2 + CSCs, CTSKThy-1.2+, and DDR2Thy-1.2+ cells. n = 3 independent experiments. h, Experimental strategy for in vivo stemness and differentiation hierarchy study and gating strategy showing serially transplanted cell populations including DDR2 + CSCs (i), DDR2Thy-1.2 + CD63 + CD200 + CD105+ (ii), DDR2Thy-1.2 + CD63 + CD200CD105 (iii), and DDR2Thy-1.2 + CD63 −CD105 (iv) of the calvarial suture of the primary donor (Ctsk-Cre;mTmG mice). Freshly FACS-isolated 2×10⁴ cells were transplanted into the muscle of primary recipients. i, FACS analysis of serially transplanted cells derived from (i), (ii), (iii), and (iv) populations after the first and second rounds of intramuscular transplantation. 1 to 3×10⁴ cells after the first round of transplantation were re-isolated and re-implanted into the muscle of secondary recipients. FACs plots marked by black boxes show differentiation of the indicated populations after the first round of transplantation and plots marked by red boxes show these populations in secondary recipients after the second round of transplantation. Black and red arrows indicate parent/daughter gates and transplanted populations, respectively. Experiments underwent 5 independent repeats. j, µCT images of bone formation from transplanted CTSK+ CSCs and DDR2 + CSCs under the renal capsule of recipients after each round of serial transplantation. The number of transplanted cells is indicated in images. k, FACs plots of tdTomato-expressing DDR2 + CSC and mGFP-expressing CTSK+ CSC populations isolated from Ctsk-Cre;mTmG mice after the first round of transplantation. Collagen sponges without cells were used as a negative control. l, FACS plots demonstrating that CTSK+ CSCs did not induce expression of DDR2 over the course of serial transplantation and that CTSK+ CSCs were capable of self-renewal and generating the entire lineage of CTSK lineage cells present in initial donors after the first round of transplantation. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (c, d), two-way ANOVA with Tukey’s multiple comparisons test (g). Images in a-f, j are representative of a minimum of three biological replicates, and FACs plots are representative of at least three independent experiments. Scale bars are denoted in images.
Extended Data Fig. 5 | Long-term lineage tracing and label-retention studies in the calvarial suture. a, Experimental design for lineage tracing of tamoxifen (TAM)-induced Ddr2-CreER;mTmG mice pulsed at P2. b, Confocal fluorescence imaging of the calvarial suture in Ddr2-CreER;mTmG mice before TAM induction. c, Short-term chase of calvarial DDR2 lineage cells in the area of lambdoid sutures in Ddr2-CreER;mTmG mice. Low-power (top) and high-power images (bottom) of the suture mesenchyme, calvarial bone, and marrow showing DDR2 lineage sutural resident cells and bone-adjacent osteoblasts 10 days after an initial pulse. Green, DDR2 lineage (mGFP) cells; Red, non-DDR2 lineage (tdTomato) cells; Blue, DAPI. d, Long-term chase for 6 (d) or 18 months (e) to visualize calvarial DDR2 lineage cells in Ddr2-CreER;mTmG mice. f, Timecourse fluorescence imaging of osteoblasts (i) and CD200-expressing cells (ii) derived from DDR2 lineage cells (green) in the lambdoid sutures of Ddr2-CreER;mTmG mice. g, Flow cytometry plot showing the concordance between DDR2 antibody staining and Ddr2-CreER pulse-chase studies in calvarial lineage cells. In addition to DDR2 expression on skeletal H2B/GFP high labeling in the calvarial sutures after dox exposure (H2B+/tTA, n = 7). Controls of mice that were continuously exposed to dox (tTA, n = 2; H2B, n = 2; H2B/tTA, n = 5), including during the embryonic period by providing dox to pregnant female mice, continuing dox exposure while pups were breastfeeding and then subsequently continuing direct dox exposure after weaning. Freq. frequency. n, o, Gating strategy for H2B-GFP+nuc label-retaining cells within DDR2’ CSC and DDR2’ CSC populations and offset histogram overlays displaying GFP fluorescence in Lin Thy-1.2 6C3 DDR2’ or DDR2’ populations including CD200’ CD105’ (CSC), CD200’CD105’, and CD200’ CD105’ after 6 (i) or 12 month (ii) chase periods. Black arrows indicate parent/daughter gates. p, Representative images (left) and quantification (right) of immunostaining for Galectin-1 (Gal-1) overlapping with CTSK+ (mGFP) cells in Ctsk-Cre;mTmG mouse calvarium at P14 (n = 5). Green, CTSK’ (mGFP) cells; Magenta, Galectin-1; Blue, DAPI. A relative amount of Galectin-1’ cells overlapping with CTSK’ (mGFP) or non-CTSK lineage (tdTomato) cells was quantified per high power field. q, Representative immunostaining images for Galectin-1 in the calvarial sutures of H2B-GFP mice after 12 months of dox exposure. Co-localization is shown by white arrows. Green, H2B-GFP; Red, Galectin-1; Blue, DAPI. Each of dots shown in the graph represents an individual mouse. ****P < 0.0001, P values are shown. Mean ± s.d., two-tailed Student’s t-test (p). One-way ANOVA with Tukey’s multiple comparisons test (j, m). Images in b, f, p, q and FACS plots are representative of at least three independent experiments. Scale bars are denoted in images.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Functional characterization of DDR2+ CSCs.

a, Quantification of bone volume (BV) when equal numbers of CTSK+ CSCs and DDR2+ CSCs were individually isolated from WT (left, n = 6) or Twist1Ctsk (right, n = 6) mice 4 weeks after transplantation. Each of dots shown in the graph represents an individual graft. b, Representative images of von Kossa staining (top, black) for mineralized bone and Safranin O staining (bottom, red) for cartilage in adjacent serial sections from renal capsule bone organoids derived from CTSK+ CSCs and DDR2+ CSCs isolated from Twist1Ctsk mice 4 weeks after transplantation. c, Fluorescence images of grafts derived from DDR2+ CSCs (red) isolated from Ctsk-Cre;mTmG mice by FACS 4 or 8 weeks after transplantation into the renal capsule of secondary recipients. Green, CTSK+ mGFP; Red, Primary graft (DDR2+ CSCs); Blue, DAPI. d, Representative immunostaining images for DDR2 (magenta) in the graft 6 weeks after transplantation of CTSK+ CSCs (green) into the renal capsule. e, Images of von Kossa and Safranin O staining in the adjacent serial sections of the same renal capsule bone organoid grafts derived from CTSK+ CSC or DDR2+ CSCs 2 weeks after transplantation (i). Quantification of the area of von Kossa and Safranin O staining is provided in ii. f–i, Endochondral ossification with active cartilage remodeling is seen in the grafts derived from DDR2+ CSCs (red) via immunostaining for Osteocalcin (green) and CD31 (magenta) 4 weeks after transplantation (f), COMP (green) and MMP13 (magenta) 2, 4, and 8 weeks after implantation (g), DDR2+ CSCs and their derivatives (white) and COL2A1 (magenta) (h), Osteocalcin (green) and SOST (white) at 8 weeks post-transplantation (i) into the renal capsules of primary recipients. Higher magnification images are provided with the corresponding Roman numerals. j, k, Endochondral ossification without recruitment of hematopoietic elements in DDR2+ CSC-derived bone organoids: Immunostaining for hematopoietic cells with CD45 (green) and CD34 (magenta) in mouse calvarium at P21 (i), grafts derived from freshly sorted femoral skeletal stem cells (SSCs) (ii), or DDR2+ CSCs (iii) (j). Immunostaining for CXCL12 (green) in the grafts derived from femoral skeletal stem cells (SSCs, red) (left) or DDR2+ CSCs (red) (right) (k) 4 weeks after the renal capsule transplantation. Co-localization is shown by white arrows. l, RT-PCR analysis of hematopoietic niche factors including Cxcl12, Angpt1, and Kitlg in FACS-isolated femoral SSCs (n = 3) and DDR2+ CSCs (n = 3). Each of dots shown in the graph represents the cells of interest isolated from an individual mouse. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (a, e (ii), l). All images shown in this figure are representative of a minimum of three independent experiments. Scale bars are included in microscopy images.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Transcriptional analysis of CTSK+ CSCs and DDR2+ CSCs isolated from mouse calvarial sutures. a, Bar graphs showing normalized read counts of *Gli1, Runx2, Nestin, Prx1, Axin2, Cd200, Fgfr3, Sox9, Sox6, Lgals1, Thbs4*, and *Scube2* as determined by RNA-Seq analysis of FACS-isolated CTSK+ CSCs (n = 5) and DDR2+ CSCs (n = 5) in the calvarial suture at P7. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test. b, Immunostaining for GLI1 in the calvarial sutures of *Ctsk-Cre;mTmG* (i) and *Ddr2-CreER;mTmG* (ii) mice at P14. Green, CTSK+ (mGFP) (i); DDR2+ (mGFP) (ii); Magenta, GLI1; Blue, DAPI. Images represent the results of 3 independent experiments. Scale bars are included in microscopy images. c, Hierarchical clustering of expression heatmaps showing differentially expressed genes associated with bone development, including endochondral ossification (GO:0001958), positive or negative regulation of cartilage development (GO:0061036; GO:0061037, respectively), regulation of chondrocyte differentiation (GO:0032330) by Gene Ontology Biological Process analysis from RNA-Seq in FACS-purified CTSK+ CSCs and DDR2+ CSCs at P7. d, Heatmap generated from murine bulk RNA-Seq analysis of sorted CTSK+ CSCs and DDR2+ CSCs showing differentially expressed genes associated with human craniosynostosis. e, f, scRNA-Seq analysis using public datasets published by Farmer et al. 202153 (e) and Ayturk et al. 202054 (f). i) UMAP (e) and tSNE (f) plots of calvaria single cells. ii) Pseudocoloring of the corresponding UMAP (e) or tSNE (f) plots for the CTSK+ CSC and DDR2+ CSC combined gene expression score used to identify cells expressing a CTSK+ CSC-like or a DDR2+ CSC-like transcriptional profile. iii) Violin plots showing the per cluster CTSK+ CSC and DDR2+ CSC score, including normalized expression of selected genes comprising the CTSK+ CSC score (left) and the DDR2+ CSC score (right).
Extended Data Fig. 8 | Analysis of Ddr2 slie mice and orthotopic transplantation of calvarial stem cells. a, µCT scan 3D images of the skull of Ddr2wt/wt (WT) and Ddr2 slie/slie (Ddr2 slie) mice at P7 and P14. (P7 WT, n = 7 Ddr2 slie, n = 8; P14 WT, n = 5 Ddr2 slie, n = 6). b, RT-PCR analysis of Ddr2 expression in Ddr2wt/wt (n = 3), Ddr2 slie/wt (n = 3), and Ddr2 slie/slie (n = 3) mice. Each of dots shown in the graph represents an individual mouse. P values are shown. Mean ± s.d., one-way ANOVA with Tukey's multiple comparisons test. c, Alcian Blue staining of the lambdoid sutures in WT and Ddr2 slie mice at P7. Nuclear Fast Red was used as a counterstain. Red boxes images: whole-mount P3 skull preparations with Alcian Blue staining of WT (n = 8) and Ddr2 slie (n = 9) mice. Red lines in images indicate the sectioning area and direction. d, Surgical field and µCT scan 3D image after ablation of the right lambdoid (LAM) suture in 4-week-old MIP-GFP mice. e, µCT scans and stereomicroscopic fluorescence images of mouse skulls at 8 (e) or 16 weeks (f) post-transplantation. mGFP-expressing CTSK CSCs and tdTomato-expressing DDR2 CSCs isolated from the calvarial sutures of P7 to P10 Ctsk-Cre;mTmG mice. Each indicated population was implanted into the suture ablation area after carrier encapsulation. The control group was implanted with only the carrier gel mixture without cells. Red arrows in µCT images indicate the fusing and fused sutures. g-i, Confocal fluorescence imaging of graft cells, showing the presence of CTSK+CSC (green) and DDR2+CSC (red) graft cells both at the initial site of implantation (LAM, g) and migration of graft cells from the initial site of implantation to adjacent sutures (OIP, h; SQ, i) at 8 weeks post-transplantation. Each red line in µCT images indicates the sectioning area and direction. The asterisk in the image denotes site of suture fusion. j, 3D reconstruction of µCT scans of the skull 16 weeks after orthotopic transplantation with subsequent DT administration. (Control, n = 6; CTSK+, n = 6; DDR2+, n = 5; CTSK+ and DDR2+, n = 5; IDTR; CTSK+ and DDR2+, n = 4). Red arrows indicate the fusing and fused sutures. k, Fluorescence images of the sutures in mice receiving DDR2+CSC or co-transplanted iDTR; CTSK+CSC and DDR2+CSC grafts 16 weeks after orthotopic transplantation with subsequent DT administration. tdTomato and GFP visualization showing engrafted cells at sites of newly formed cranial bone. Each red line in µCT images indicates the sectioning area and direction. Green, CTSK+ (mGFP); Red, DDR2+ (tdTomato); Blue, DAPI. Images are representative of at least five (a, c) biological replicates and four (e-k) independent experiments. Scale bars are denoted in images.
Extended Data Fig. 9 | IGF1 secreted by CTSK+ CSCs suppresses fusion activity. a, Heatmap showing differential expression of candidate signaling mediators (i) and graphs displaying normalized read counts of Igf1 and Igf1r in CTSK+ CSCs and DDR2+ CSCs determined by RNA-Seq at P7 mice (n = 5). b, RT-PCR analysis of Igf1 gene expression in CTSK+ CSCs and CTSK lineage cells (Thy-1.2+/CD90+ here) from WT (n = 3) and Twist1tmCtsk (n = 3) mice at P10. c, Schematic of the experimental design for subcutaneous injection of recombinant IGF1 over the calvarium of Twist1tmCtsk mice. d, e, Characterization of Igf1fl/fl;Ctsk-Cre (Igf1Ctsk) mice; µCT scan 3D images (d) of the skull of WT and Igf1Ctsk mice at P28. Red arrows indicate fusing and fused sutures. µCT scans of 2D images (e, left) and 2D cut planes (e, right). The asterisk in e indicates fusing sutures. Each of dots shown in the graph represents an individual mouse. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (a(ii)), one-way ANOVA with Tukey’s multiple comparisons test (b). Scale bars are denoted in images.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Endochondral ossification in human craniosynostosis and characterization of human DDR2 + CSCs. a, Histological analysis of active cartilage remodeling occurring at sites of active suture fusion using H&E (top) and Alcian Blue (bottom) staining of FFPE calvarial specimens from patients with craniosynostosis. The specimen displayed represents a portion of the lambdoid suture. Higher magnification images are provided with the corresponding Roman numerals indicated in lower magnification images. b, Representative images demonstrating active endochondral ossification in patients with craniosynostosis: human calvarial specimens were immunostained for DDR2 (green) and Osteocalcin (red) (b) and DDR2 (green), COL2A1 (red), and Osteopontin (OPN, magenta) (c). d, FACS plots for human calvarium tissues. Lin indicates CD31, CD45, and CD235a staining. Red boxes indicate hDDR2 − CSCs and hDDR2 + CSCs. Orange boxes indicate markers defining human SSC populations (PDPN + CD146 − CD164 + CD73 +) reported in human fetal long bones14. Black arrows demonstrate parent/daughter gates. Experiments underwent a minimum of 5 independent repeats. e, Heatmaps showing gene expression features of human DDR2 − or DDR2 + CSCs displaying conservation of gene expression signatures observed in their murine counterpart cell types (murine CTSK − CSCs or DDR2 + CSCs, respectively), including expression of IGF1 on hDDR2 − CSCs and IGF1R on hDDR2 + CSCs as analyzed by RT-PCR. hDDR2 − CSCs and hDDR2 + CSCs were freshly isolated by FACS from five individual patients with craniosynostosis. The color scale (from low expression in blue to high expression in red) depicts the log2 fold change of gene expression. The sequence of the primers is provided in Supplementary Table 3. f, Representative images of immunostaining for DDR2 (green) and IGF1R (red) in human calvarial tissues of three individual patients with craniosynostosis. Higher magnification images are provided. Results are representative of a minimum of five (a–d) and three (f) independent experiments. Scale bars are denoted in images.
Extended Data Fig. 11 | See next page for caption.
Extended Data Fig. 11 | Determination of self-renewal, differentiation hierarchy, and functional characterization of hDDR2+ CSCs. 

a, Schematic representation of the intramuscular transplantation model for human sutural cells. 

b, FACS plots showing the differentiation of the indicated input populations including hDDR2− CSCs (i), hDDR2+ CSCs (ii), hDDR2+ CD200 (iii), and matrigel only (iv) in primary recipients immunodeficient NSG-EGFP mice 10 days after intramuscular transplantation. 1 to 3×10^4 hDDR2− CSCs and hDDR2+ CSCs were initially transplanted into immunodeficient NSG-EGFP primary recipients. 1 to 5×10^3 hDDR2+ CSCs were re-isolated from the primary transplants. Matrigel without cells was used as a control. Black arrows demonstrate parent/daughter gates. Experiments underwent 3 independent repeats. 

c, Flow cytometry plots showing gating strategy for human DDR2+ and DDR2− CSCs with human-specific cell surface markers including human beta-2 microglobulin (β2M) and pan-human leukocyte antigen complex (HLA-ABC) in human calvarium. 

d, A schematic representation of in vivo serial transplantation studies of hDDR2− CSCs and hDDR2+ CSCs. To test the proliferative self-renewal and differentiation capacity of hDDR2− CSCs and hDDR2+ CSCs, freshly FACS-isolated cells were labeled with a lipophilic cell membrane dye prior to the intramuscular transplantation. Gross images of the primary grafts (arrowheads) explanted 10 days after transplantation. e, FACS plots showing that transplanted hDDR2− CSCs (top plots) and hDDR2+ CSCs (bottom plots) underwent multiple rounds of proliferation during the assays without interconversion in the primary grafts (i) or secondary grafts (ii). 

f, Immunostaining for the proliferation marker Ki-67 (green) in hDDR2− CSCs and hDDR2+ CSCs (red, stained by cytoskeleton protein, α-Tubulin) isolated after the second round of transplantation. g, In vitro limiting dilution analysis and CFU-F formation. Dilutions of 500, 200, 100, 50, 20, and 10 hDDR2− CSC or hDDR2+ CSCs were seeded onto 6-well culture plates. Colonies were stained with Crystal violet and analyzed 10 days after seeding. h, Images of Alizarin Red S (upper, red) and Alcian Blue (lower, blue) staining for in vitro clonal multipotency. All cells were derived from the expansion of a single FACS-isolated hDDR2− CSC or hDDR2+ CSC. i, Quantification of the percentage of cells able to form mesospheres from hDDR2− CSCs, hDDR2+ CSCs, hDDR2+ CD200−, and hDDR2+ CD200+ cells. 

n = 3 independent experiments. 

j, Quantification of the area of von Kossa and Safranin O staining in the renal capsule after transplantation of hDDR2− CSCs and hDDR2+ CSCs at 2 weeks post-transplantation. n = 3 independent experiments. 

k, Cellular and molecular features of endochondral ossification of hDDR2− CSCs: immunostaining for skeletal cell type markers and key markers of endochondral bone development including Osteocalcin (i) and Endomucin (EMCN) (ii) in human xenografts derived from FACS-isolated hDDR2− CSCs. Images in f–h represent five individual experiments and k represent a minimum of three independent experiments. 

Plots in b, c, e are representative of results from three independent experiments. ****P < 0.0001, P values are shown. Mean ± s.d., unpaired, two-tailed Student’s t-test (j), two-way ANOVA with Tukey’s multiple comparisons test (i). Scale bars are included in microscopy images.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
A SCANCO Medical microCT 35 system (v4.05, Switzerland) was used for scanning and 3D analysis. Fluorescence imaging was performed with a Zeiss LSM 880 laser scanning confocal microscope. Transplanted grafts were imaged by a Leica M205 FCA fluorescence stereo microscope. QuantStudio 6 Flex RT-PCR Software v1.3 was used for mRNA analysis. FACS data was collected with a BD FACSARia II and LSRFortessa. BD FACSDiva Software version 9.0.1 (build 2020 02 06 11 31) UDI: (01)00382906642868(10)901 Firmware Version 1.87 was used for the BD FACSAria. BD FACSDiva Software version 9.0 (build 2019 09 17 11 11) UDI: (01)00382906641175(10)90 Firmware Version 1.87 was used for the BD LSRFortessa. Normalized cDNA libraries prepared from FACS-isolated CTSK+ CSCs or DDR2+ CSCs were sequenced on an Illumina NovaSeq6000 sequencer.

Data analysis
GraphPad PRISM software (v8.1.2) was used for statistical analysis. A SCANCO Medical microCT 35 system (v4.05) was used for scanning and 3D analysis. All imaging data were processed using Zeiss ZEN 2.3 SP1 FP3 software (black, v14.0.18.201). Stereomicroscope images were processed by Leica Application Suite X (LAS X) software (v3.6.0.2014). FACS data was analyzed using FlowJo (v10.6.1). RStudio (v1.3.1093), pheatmap (v1.0.12) was used for analysis of bulk RNA sequencing data. Single-cell RNA sequencing data using public datasets published was analyzed by Seurat (v4.3.0).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.
Data
Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
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- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All relevant raw data has been made available. RNA-Seq data has been deposited at the Gene Expression Omnibus (GEO) under accession number GSE232652.

Field-specific reporting
Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

| Sample size | Generally, sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10-20% of the expected mean. Alpha and Beta were set to the standard values of .05 and 0.2, respectively. |
| Data exclusions | No exclusions. |
| Replication | Yes, all experiments reported in the manuscript were replicated to confirm reproducibility, and biological replicates were performed as stated in the figure legends. 1) FACS analysis of mouse samples included more than 50 independent replicates from Ctsk-Cre mice and 25 independent replicates from Floxed Twist1;Ctsk-Cre mice. FACS analysis from IDTR;Ctsk-Cre, Cdk2-CreER, Twist1+/-, and Floxed Igf1;Ctsk-Cre mice was replicated at least three times at indicated time points. H2B-GFP label retention studies were performed with 9 replicates after a 6 month chase and 8 replicates after a 12 month chase for flow cytometry analysis. 2) Analysis of human calvarium included 37 independent specimens. 3) All transplantation studies were performed at least in triplicate. 4) Animal studies were replicated across at least 3 independent cohorts. 5) Histological analysis on mouse samples included a minimum of 3 independent experiments. 6) In vitro studies including clonal expansion, differentiation, CFU-F, CFU-Ob, and mesensphere formation assays were performed in triplicate and the experiment was repeated 3 times. |
| Randomization | For all transplantation experiments, the recipient animals for each specific cell population transferred were randomized after selecting a group of sex-appropriate mice. For all animal studies, the animals were randomized after selecting a group of sex-appropriate mice. For immunohistochemistry, the slides for each specific groups were randomized after sample processing. |
| Blinding | Yes, μCT analysis, clonal differentiation assays, and immunohistochemistry were performed by investigators who were blinded to the nature of the mice/specimens under analysis (both what specific mouse strains or treatment groups were in the experiment and whether any individual mouse/specimen belonged to control versus experimental groups). |

Reporting for specific materials, systems and methods
We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
| --- | --- |
| Antibodies | |
| Eukaryotic cell lines | |
| Palaeontology and archaeology | |
| Animals and other organisms | |
| Human research participants | |
| Clinical data | |
| Dual use research of concern | |

**Methods**

| n/a | Involved in the study |
| --- | --- |
| ChIP-seq | |
| Flow cytometry | |
| MRI-based neuroimaging | |
Antibodies

Validation

The following validation methods were conducted: 1) isotype controls were used, 2) for IHC, the secondary fluorescent antibody was added alone without the primary antibody, 3) the manufacturer has validated these antibodies for use in the same species and assay format, 4) for flow cytometry, compensation beads (Invitrogen, cat. 01-3333-41 and 01-2222-42) were used to set initial compensation. Additionally, 5) cross-instrument comparisons of FACS panel performance were used for additional validation.
Eukaryotic cell lines

Policy information about cell lines

Validation for cell sorting and the specificity of selected antibodies was initially performed using HEK-293 cells from ATCC (cat. CRL-1573), a commercially available human embryonic kidney cell line.

Authentication

These cells were used directly from the manufacturer (ATCC) who performed authentication. These cell line was not authenticated by the authors.

Mycoplasma contamination

Cells were tested for mycoplasma using the PlasmoTest™ - Mycoplasma Detection Kit from InvivoGen (cat. rep-pt1) and PCR analysis. All cells were confirmed to be free of mycoplasma.

Commonly misidentified lines

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Cathepsin K-Cre (Ctsk-Cre) mice were a gift from S. Kato (University of Tokyo) and T. Nakamura (Tokyo Dental College), Floxed Twist1 (Twist1fl/fl, Stock 008172), mTmG (Stock 007676), ROSA26:DTA (Stock 007900), ROSA-DTA (Stock 009669), MIP-GFP (Stock 006864), NOA scid gamma (NSG)-EGFP mice (Stock 021937), pTRE-H2BGFP (Stock 005104), Floxed Igf1 (Igf1fl/fl, Stock 016831) mice were purchased from Jackson Laboratories, ROSA:LN:LTA mice (JAX, Stock 011008) were a gift from L. Puglielli (University of Wisconsin-Madison). Ddr2-CreER (MerCreMer) mice were generated by R. T. Cowling and B. H. Greenberg with assistance from the Transgenic Core and Embryonic Stem Cell shared resource at UCSD. Detailed information is described in methods section of the manuscript. Recipient heterozygous MIP-GFP mice were generated by breeding homozygous MIP-GFP males with wildtype C57Bl/6j females. All mice were maintained on a C57Bl6j background throughout the study. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Weill Cornell Medical College subcommittee on animal care (IACUC) under protocol number 2012-0005. Animals were euthanized by CO2 for primary cell isolation and analyses or morphological studies. Male and female Ctsk-Cre;R26R;mTmG, Floxed Twist1;Ctsk-Cre, ROSA-iDT;Ctsk-Cre, and Floxed Igf1;Ctsk-Cre mice between postnatal day 2 and 21 were used for isolation of primary skeletal cells. Male and female ROSA-iDT;Ctsk-Cre mice were injected with Diphtheria toxin, starting at postnatal day 5 and the skulls and femurs were analyzed by postnatal day 21 and 35. Male and female Ddr2-CreER;R26R;mTmG mice were used for lineage tracing experiments. CreER lines were induced with Tamoxifen or oil at postnatal day 2 and were analyzed day 1, 10, 30, 90, 180, 365, 545, and 730 after administration. No adverse effects were observed during this study. Male and female Ddr2 sile mice were used at postnatal day 3, 7, and 14 for this study. Male and female Floxed Osterix;Ddr2-CreER, ROSA-DTA;Ddr2-CreER were used for morphological studies. Tamoxifen was delivered subcutaneously into postnatal day 7 pups. Male and female H2B-GFP mice at 8 and 14 months of age were used for long-term label retention analysis. Male MIP-GFP and immunodeficient NSG-EGFP mice between 6 to 8 weeks were used for renal capsule transplantsations. 4-week-old male MIP-GFP mice were used for suture ablation and orthotopic transplantsations, which were analyzed 8 to 16 weeks post-transplantation. Female MIP-GFP and NSG-EGFP mice between 6 to 8 weeks were used for intramuscular transplantsation.

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve field-collected samples.

Ethics oversight

All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled...
Human research participants

Policy information about studies involving human research participants

Population characteristics

37 patients had parents consent for study inclusion. Participants had an age range of less than 1 year old and were 70% male. Detailed annotation of the clinical specimens including age, sex, and the primary diagnosis is provided in Supplementary Table 1.

Recruitment

Parents of patients already scheduled for cranial surgery were requested to sign a consent form prior to surgery for the use of surgical tissue specimens for the study.

Ethics oversight

This study was carried out under the Institutional Review Board (IRB) protocol (no. 1402014802), with all patients providing informed consent through the Center for Neurogenetics at Weill Cornell Medicine. All human tissue experiments followed all relevant guidelines and regulations. Written consent was provided by the parents or guardians of all study participants.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Detailed information is described in methods section of the manuscript. In brief, murine and human skeletal tissues were subjected to both mechanical and enzymatic digestion. Microdissection of mouse calvarial sutures was performed under a dissecting microscope. After digestion, medium was added to the tube and the digested tissue was re-suspended thoroughly by pipetting and then filtered through 70 μm nylon mesh. Tubes were centrifuged and the resulting cell pellet was subjected to FACS. Prepared single-cell suspensions were washed twice with ice-cold FACS buffer (2% serum + 1 mM EDTA in PBS) and incubated with Fc blocking buffer (1:100 dilution in FACS buffer; BD biosciences, cat. 553142 for mouse and cat. 564765 for human) for 15 min at 4 °C. Primary antibody dilutions were prepared in Brilliant Stain Buffer (BD biosciences, cat. 563794).

Cells were incubated in the dark for 40 min at 4 °C with the primary antibody solution, washed 2 times with FACS buffer, and incubated with secondary antibody solution for 20 min, if needed. Cells were then washed several times and re-suspended in FACS buffer with DAPI (BD biosciences, cat. 564907, lot. 0339103).

Instrument

FACS was performed using a BD Aria II and LSRFortessa equipped with 5 lasers.

Software

FACS data was collected with BD FACSAria II and LSRFortessa. BD FACSDiva Software version 9.0.1 (build 2020 02 06 11 31) UDI: (01)00382906642868(10)901 Firmware Version 1.87 (BD FACSAriaII). BD FACSDiva Software version 9.0 (build 2019 09 17 11 11) UDI: (01)00382906641175(10)90 Firmware Version 1.87 (BD LSRFortessa). Typically, 1 million and 10 million events were recorded for mouse samples and human samples, respectively, and the data were analyzed using FlowJo (v10.6.1).

Cell population abundance

Sorting was performed using a 4-way collection and it was validated to result in >95% purity of the intended population in post-sort fractions.

Gating strategy

Compensation beads (Invitrogen, cat. 01-3333-41 and 01-2222-42) and cells derived from fluorescent reporter animals (membrane-localized EGFP and membrane-localized tdTomato) were used to set internal FMO controls. For murine samples, mesenchymal/skeletal cell populations negative for lineage markers (CD31, CD45, CD235a) were analyzed according to the approach described in Extended Data Fig. 4l. For human specimens, mesenchymal/skeletal cell populations negative for lineage markers were analyzed according to the approach described in Extended Data Fig. 10d and 11c. Gating strategies and FACS profile showing parent/daughter plots for each experiment are provided in Figure 2f, Figure 3g, Figure 6e, Extended Data Fig. 1g, 3i, 4l, 5n, 10d, 11b, 11c, and 11e.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.