Snail/Slug binding interactions with YAP/TAZ control skeletal stem cell self-renewal and differentiation

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Bone-marrow-derived skeletal stem/stromal cell (SSC) self-renewal and function are critical to skeletal development, homeostasis and repair. Nevertheless, the mechanisms controlling SSC behaviour, particularly bone formation, remain ill-defined. Using knockout mouse models that target the zinc-finger transcription factors Snail or Slug, or Snail and Slug combined, a regulatory axis has been uncovered wherein Snail and Slug cooperatively control SSC self-renewal, osteoblastogenesis and bone formation. Mechanistically, Snail/Slug regulate SSC function by forming complexes with the transcriptional co-activators YAP and TAZ in tandem with the inhibition of the Hippo-pathway-dependent regulation of YAP/TAZ signalling cascades. In turn, the Snail/Slug–YAP/TAZ axis activates a series of YAP/TAZ/TEAD and Runx2 downstream targets that control SSC homeostasis and osteogenesis. Together, these results demonstrate that SSCs mobilize Snail/Slug–YAP/TAZ complexes to control stem cell function.

Skeletal stem/stromal cells (SSCs) are found throughout tissues during embryogenesis and postnatal life1–2. Although the multipotent potential of these cells is subject to debate, bone-marrow-derived SSCs differentiate into osteoblasts, chondrocytes or adipocytes as well as haematopoietic stem cell-supportive stroma3. Interestingly, recent studies raise the possibility that SSCs express the transcriptional repressors Snail and Slug (alternatively termed Snail2)3. Although best known for their roles in orchestrating epithelial–mesenchymal transition programs associated with development4,6, recent studies suggest that Snail and Slug impact stem cell functions via largely undefined mechanisms7–12. While screening mouse tissues for Snail and Slug expression, we unexpectedly discovered that both transcription factors are expressed in SSCs during pre- and postnatal states. Interestingly, targeting either Snail or Slug alone exerts only subtle effects on developmental programs. By contrast, dual knockout of both transcription factors markedly impairs SSC self-renewal, differentiation and bone formation. As such, we set out to define the means by which Snail and Slug co-regulate SSC function.

RESULTS
Snail/Slug co-dependent regulation of SSC proliferation and differentiation

Using Snail/LacZ and Slug/LacZ knock-in mice11,13, β-galactosidase activity is associated with cranial sutures, calvaria, long bones and cartilage (Fig. 1a,c, Supplementary Fig. 1a–e). Furthermore, in bone marrow, Snail/LacZ as well as Slug/LacZ knock-in mice display β-galactosidase activity in fibroblast-like cells (Fig. 1b,d). As such, SSCs were isolated by fluorescence-activated cell sorting, and both Snail and Slug messenger RNA expression was confirmed in freshly sorted cells (Supplementary Fig. 1f). Furthermore, following the in vitro expansion of SSCs recovered from Snail/LacZ or Slug/LacZ knock-in mice, virtually all cells express β-galactosidase activity (Fig. 1e).

Next, bone-marrow-derived SSCs (herein termed SSCs) were isolated from Snail1/2/Slug+/+ or Snail1/2/Slug−/− mice and transduced with adenoviral Cre or a GFP control expression vector to yield Snail1/2/Slug+/+, Snail1/2/Slug−/+, Snail1/2/Slug+/− or Snail1/2/Slug+/− progenitors. Consistent with studies demonstrating that Snail and Slug transcriptionally compensate for each other’s loss14,15, deleting Snail upregulates Slug expression while the loss of Slug expression induces Snail expression (Fig. 1f and Supplementary Fig. 1f). Under these conditions, deleting Slug or Snail alone yields only subtle effects on SSC proliferation (Fig. 1g). By contrast, in the absence of both Snail and Slug, SSC proliferation is decreased by ~75% with attendant losses in KI67 expression (Fig. 1g and Supplementary Fig. 1g,h). Changes in stem cell-associated transcription factors, for example, Nanog, Sox2 or Oct4, are not observed in double-null SSCs relative to controls. Further, while Snail and Slug can mediate anti-apoptotic effects16, Snail/Slug-deleted SSCs display no changes in apoptosis (Supplementary Fig. 1i).

To assess the roles of Snail and Slug in SSC differentiation, Snail/Slug-expressing cells (that is, Snail1/2/Slug+/+/GFP), Snail-deleted cells (that is, Snail1/2/Slug+/+/Cre), Slug-null cells (that is, Snail1/2/Slug−/−/GFP) or double-null cells (that is,
Figure 1  Snail/Slug regulate SSC proliferation and differentiation. (a) LacZ expression in E17.5 Snail+/−/Slug+/− neonatal sagittal suture and parietal bone. Scale bar, 100 μm. Results are representative of three experiments performed. (b) LacZ expression in a subpopulation of bone marrow cells in the femur of a 2-week-old Snail+/−/Slug−/− mouse. Scale bar, 100 μm. Results are representative of three experiments performed. (c) LacZ expression in Snail+/−/Slug+/− 7-d-old sagittal suture and parietal bone. Scale bar, 100 μm. Results are representative of three experiments performed. (d) LacZ expression in a subpopulation of bone marrow cells in the femur of a 2-week-old Slug+/−/− mouse. Scale bar, 100 μm. Results are representative of three experiments performed. (e) LacZ expression in bone-marrow-derived SSCs isolated from 4-week-old Snail+/−/− or Slug+/−/− mice. Scale bar, 100 μm. Results are representative of three experiments performed. (f) Western blot of Snail and Slug in SSCs isolated from Snail+/+ or Slug+/+ mice transduced with adenoviral GFP or Cre expression vectors. Results are representative of three experiments performed. (g) Growth curve of SSCs isolated from Snail+/+ or Slug−/− mice, and transduced with adenoviral GFP or Cre expression vectors (mean ± s.d., n=3 independent experiments). **P < 0.01; one-way ANOVA. (h) SSCs were isolated from Snail+/+ or Slug−/− mice and transduced with adenoviral GFP or Cre. Cells were cultured under osteogenic conditions for 14 d, and stained with Alizarin Red S. The lower panels are magnified images of the upper panels. Scale bar, 100 μm. Results are representative of three experiments performed. (i) Relative mRNA expression of osteogenic markers in cultures from h (mean ± s.d., n=3 independent experiments). **P < 0.01; one-way ANOVA. (j) SSCs were isolated from Snail−/− or Slug−/− mice and transduced with adenoviral GFP or Cre. After culture with or without osteogenic medium for 7 d, cell lysates were immunoblotted. Results are representative of three experiments performed. (k) SSCs were isolated from Snail−/− or Slug−/− mice and transduced with adenoviral GFP or Cre. After culture under adipogenic conditions for 7 d, cultures were stained with Oil Red O. Scale bar, 100 μm. Results are representative of three experiments performed. (l) Relative mRNA expression of adipogenic markers in cultures from k (mean ± s.d., n=3 independent experiments). **P < 0.01; one-way ANOVA. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−} (Cre) were established as confluent cultures and induced to undergo osteogenesis. Over a 14 d culture period, the control Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+} SSCs differentiate into osteoblasts as assessed either by Alizarin Red S staining, the induction of the osteoblast markers Runx2, Osterix, alkaline phosphatase (ALP) and Bglap2 at the mRNA level, or Runx2 and Osterix at the protein level (Fig. 1h–j). When compared with control SSCs, Snail- or Slug-deleted SSCs display only modest defects in mineralization or osteoblast commitment while maintaining their ability to upregulate the master osteoblast transcription factor, Runx2 (Fig. 1h–j). By contrast, Snail/Slug-deficient SSCs display a complete defect in mineralization with commensurate maintaining their ability to upregulate the master osteoblast transcription factor, runx2 (Fig. 1h–j). Consequently, embryos from each of the crosses were harvested at Cre or Slug-targeted mice. Whereas Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−} (Cre) mice display a mild calvarial phenotype with retarded ossification and ossification are also undermined in the skulls of Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−}/Dermo1–Cre mice (Fig. 2b), with cross-sections of E17.5 parietal plates highlighting an almost complete absence of calcified bone (Supplementary Fig. 2f). Further, the condensed mesenchymal layer, dominated by SSCs and osteoprogenitors, is significantly thinner, consistent with a 60% decrease in K167 staining without changes in progenitor cell apoptosis (Figs. 2c–f and Supplementary Fig. 2h). Calvarial extracts recovered from Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−}/Dermo1–Cre mice also display normal Runx2 expression in tandem with marked defects in Osterix, ALP and Bglap2 expression (Fig. 2g).

To define the in vivo role of Snail in SSC homeostasis, colony-forming unit fibroblast (CFU-Fs) assays were performed\textsuperscript{22,26}. Of note, dual deletion of Snail and Slug induces a severe loss of CFU-Fs in the embryonic bone of Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−}/Dermo1–Cre mice relative to that found in the Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+}/Dermo1–Cre, Snail\textsuperscript{−/−}/Slug\textsuperscript{+/−} or Snail\textsuperscript{−/−}/Slug\textsuperscript{+/+} mice (Fig. 2h,i). The cell autonomous nature of the dual requirement for Snail and Slug is further confirmed following transplant of isolated Snail-deleted/Slug-null SSCs into nude mouse recipients. Whereas Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+}/Cre SSCs or Snail\textsuperscript{+/+}/Slug\textsuperscript{+/−}/GFP SSCs generate bone-rich osteoids comparably to control Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+}/GFP SSCs, the combined loss of Snail and Slug dramatically reduces bone formation (Fig. 2j,k).

**Bone-marrow-directed deletion of Snail/Slug retards postnatal osteogenesis**

The lethal phenotype or Snail\textsuperscript{−/−}/Slug\textsuperscript{−/−}/Dermo1–Cre-targeted mice precludes our ability to assess the role of Snail and Slug in regulating postnatal osteogenesis. Recently, however, Osterix–Cre has been shown to target bone-marrow-associated stromal cells displaying SSC- as well as osteoprogenitor-like characteristics\textsuperscript{27–30}. Hence, Osterix–Cre mice were crossed with Snail\textsuperscript{−/−}/Slug\textsuperscript{−/−} (Cre) mice yielding Snail\textsuperscript{+/+}/Slug\textsuperscript{+/−} (that is, control) mice, Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+}/Osterix–Cre (that is, Snail-deleted) mice, Snail\textsuperscript{−/−}/Slug\textsuperscript{−/−} (Slug-null) mice and Snail\textsuperscript{−/−}/Slug\textsuperscript{−/−}/Osterix–Cre (that is, Snail/Slug-targeted) mice. Mice from all four groups are born in the expected Mendelian rations and display normal lifespans. Whereas Snail-deleted and Slug-null mice are slightly smaller in stature than controls, the Snail/Slug-targeted mice reach only 2/3 the size/weight of the controls (Fig. 3a). Further, although the Snail-deleted or Slug-null mice display a mild calvarial phenotype with retarded ossification and delayed suture fusion, bone development is strikingly compromised in the Snail/Slug-targeted mice (Fig. 3b). Femur cross-sections of Snail/Slug-targeted Osterix–Cre mice exhibit the most dramatic loss in bone mass, thickness and trabeculation (Fig. 3c–e). Consistent with our in vitro analyses, we also note a decrease in the proliferative potential, but not apoptotic rate, of bone surface-associated SSC/osteoprogenitors that is accompanied by a marked decrease in Osterix, ALP and Bglap2, but not Runx2, mRNA expression (Fig. 3f–h and Supplementary Fig. 3a,b). Likewise, a severe depletion of CFU-Fs is found in adult bone marrow of Snail\textsuperscript{+/+}/Slug\textsuperscript{−/−}/Osterix–Cre mice relative to the Snail\textsuperscript{+/+}/Osterix–Cre, Snail\textsuperscript{−/−}/Slug\textsuperscript{−/−} or Snail\textsuperscript{+/+}/Slug\textsuperscript{+/+} mice (Supplementary Fig. 3c,d). Although...
bone loss can also be caused by unbalanced bone resorption\(^2\), differences in osteoclast counts are not detected (Supplementary Fig. 3e,f).

Given the complex expression pattern of Osterix-Cre in transgenic mice\(^{27,28,30}\), we confirmed defects in Osterix-Cre-targeted SSC/osteoprogenitor function in vitro. As the Osterix-Cre
transgene is tet-off regulated, SCCs were isolated from Snail/Leg mice maintained on doxycycline in a Slug heterozygous background (that is, Slug+/– mice are indistinguishable from wild-type littermates while both Snail/Leg/Slug+/–/Dermo1–Cre and Osterix–Cre mice display bone defects, albeit less severe than those observed in the Slug-null background). As expected, SCCs cultured in the presence
Snail/Slug co-express Snail and Slug comparably to controls (that is, Snail\(^{+/+}\) / Slug\(^{+/+}\) SSCs). When doxycycline is removed from the media and osteogenesis induced (day 1), Snail protein levels fall to undetectable levels by culture day 3 while Slug proteins are induced relative to the heterozygous controls (Fig. 3i). Under these conditions, marked defects in osteoblastogenesis are again confirmed while early osteoblast commitment, reflected in Runx2 expression, is unaltered (Fig. 3j,k). Similar results are obtained when calvarial osteoprogenitors are recovered from Snail\(^{+/+}\)/Slug\(^{+/+}\) mice and transduced with adenoviral-Cre in vitro (Supplementary Fig. 3g,h). Thus, Snail and Slug are co-required during SSC as well as osteoprogenitor commitment to the bone lineage in vitro and in vivo.

Snail/Slug promote SSC proliferation and osteogenesis via YAP/TAZ activation

To date, a dual requirement for Snail and Slug has not been linked to the control of SSC proliferative responses or differentiation. Interestingly, the Hippo pathway components, YAP or TAZ, regulate stem cell self-renewal and differentiation programs\(^{32-35}\). Indeed, whereas control SSCs express YAP as well as TAZ at the protein level that each localize primarily to the nuclear compartment, Snail/Slug-deleted SSCs display significant decreases in YAP and TAZ protein levels with no changes in their relative mRNA levels (Fig. 4a,b and Supplementary Fig. 4a). Importantly, decreases in YAP/TAZ protein levels also correlate with lower expression of the YAP/TAZ targets Ctgf and Ankrd1\(^{32-35}\) (Supplementary Fig. 4b). Furthermore, while YAP and TAZ protein levels increase during SSC osteogenesis in tandem with increases in Snail and Slug levels, Snail/Slug-deleted SSCs continue to express lower protein levels of YAP and TAZ during differentiation (Fig. 4c). Likewise, osteoprogenitors undergoing osteoblast commitment with BMP-2 also increase YAP and TAZ protein levels in tandem with Snail and Slug (Fig. 4d,e and Supplementary Fig. 4c,d). By contrast, when both Snail and Slug are targeted, YAP and TAZ protein levels decrease (Fig. 4c,e). Similarly, SSCs/osteoprogenitors isolated from calvarial extracts of Snail\(^{+/+}\)/Slug\(^{+/+}\) / Dermo1-Cre mice or emurs of Snail\(^{+/+}\)/Slug\(^{+/+}\) / Osterix-Cre mice also exhibit decreases in YAP/TAZ protein levels (Fig. 4f,g and Supplementary Fig. 4e,f).

To determine whether decreases in YAP/TAZ protein levels affect SSC self-renewal and differentiation in a manner that phenocopies the Snail/Slug-deleted state, SSCs were isolated from YAP\(^{+/+}\)/TAZ\(^{+/+}\) mice and transduced with adenoviral GFP or Cre expression vectors. As predicted, YAP and TAZ protein levels decrease in hemizygous SSCs (without affecting either Snail/Slug protein levels or their subcellular localization) with coordinate decreases in SSC proliferation and CFU-F formation (Fig. 4h–i) and Supplementary Fig. 4g,h). Likewise, when YAP/TAZ-hemizygote SSCs are cultured under osteogenic conditions, mineralization is blocked (Fig. 4k). As observed in Snail/Slug double-null mice, Runx2 expression is upregulated normally in YAP\(^{+/+}\)/TAZ\(^{+/+}\) SSCs, while Osterix, ALP and Bglap2 are repressed (Fig. 4i). Similar changes are not observed in YAP\(^{+/+}\) SSCs or TAZ\(^{+/+}\) SSCs. Interestingly, transient expression of Snail in YAP\(^{+/+}\)/TAZ\(^{+/+}\) osteoprogenitors partially rescues both proliferative and osteogenic activities (Supplementary Fig. 4i–k).

Snail/Slug–YAP/TAZ complexes regulate the Hippo pathway

YAP/TAZ stability is regulated by the Hippo cascade-associated kinases, MST1 and MST2, as well as the Lats kinases, LAT1 and LAT2, that ultimately control YAP/TAZ phosphorylation, subcellular localization and proteasomal degradation\(^{34-37}\). While Snail/Slug did not affect either MST1 or LAT1 protein levels or phosphorylation-dependent activation states, the combined absence of Snail and Slug increases phospho-YAP and phospho-TAZ levels with consequent losses in total YAP/TAZ levels—both before, and during, osteogenesis (Fig. 5a). Consistent with increased YAP/TAZ phosphorylation levels, binding interactions between Lats1/2 and YAP/TAZ are increased in Snail/Slug-deleted SSCs (Supplementary Fig. 5a). The half-lives of YAP/TAZ proteins also decrease in Snail/Slug-deleted osteoprogenitors, while, by contrast, YAP/TAZ protein levels increase when proteasome activity is inhibited (Supplementary Fig. 5b–d). As such, we considered a model wherein Snail/Slug form complexes with YAP/TAZ that directly or indirectly protect the transcriptional co-activators from proteasomal degradation. Indeed, following co-expression of epitope-tagged YAP or TAZ with epitope-tagged Snail or Slug, interacting complexes between Snail and YAP, Snail and TAZ, Slug and YAP and Slug and TAZ are detected by co-immunoprecipitation in COS-1 cells as well as by in situ proximity ligation assay in the C3H10T1/2 progenitor cell line (Fig. 5b,c and Supplementary Fig. 5e). Further, endogenous complexes between Snail/Slug and YAP/TAZ are found in SSCs as well as calvarial osteoprogenitors undergoing osteogenesis (Fig. 5d–f and Supplementary Fig. 5f–h). Complementing these findings, formation of nuclear YAP/TAZ–Snail/Slug complexes occurs independently of the expression of TEAD, the dominant co-transcriptional activator of YAP/TAZ function\(^{34-36}\) (Fig. 5d–f and Supplementary Fig. 5f–h).

Snail/Slug–YAP/TAZ complexes regulate transcriptional activity

YAP and TAZ can regulate transcriptional events by forming complexes with TEAD transcription factors (that is, TEAD1–4)\(^{34-36}\). Given the ability of Snail and Slug to interact with YAP/TAZ, we sought to determine whether YAP/TAZ-dependent TEAD transcriptional activity is modulated by Snail/Slug complex formation. As such, TEAD–luciferase reporter activity (that is, 8X GtiIC–Luc)\(^{38}\) was assessed in cells transfected with wild-type YAP or TAZ in the absence or presence of Snail/Slug. As expected, YAP as well as TAZ increase the activity of the TEAD reporter with only subtle effects exerted by Snail or Slug alone (presumably due to interactions with endogenous YAP/TAZ) (Fig. 6a). When YAP or TAZ is expressed in tandem with Snail or Slug, however, reporter activity is increased significantly with binding interactions between YAP/Snail or YAP/Slug and TEAD confirmed by co-immunoprecipitation (Fig. 6a,b). As YAP or TAZ can also associate with Runx2, and Runx2/TAZ complexes play key roles in controlling downstream Runx2 signalling cascades\(^{33,37,39}\), the modulation of transcriptional activity was assessed with a Runx2-responsive luciferase reporter construct (that is, 6X OSE–Luc)\(^{40}\). YAP, a previously described Runx2 transcriptional inhibitor\(^{39}\), is unable to support 6X OSE–Luc reporter activity in the presence of either Snail or Slug. By contrast, although Runx2 reporter activity is moderately upregulated by TAZ alone, the combination of TAZ and either Snail or Slug induces a synergistic effect where both Snail and Slug are found in association with TAZ in Runx2 complexes (Fig. 6c,d).
Figure 4  Snail/Slug regulate YAP/TAZ levels in SSCs. (a) YAP and TAZ expression levels in SSCs isolated from Snail+/+ and Snail–/– mice and transduced with adenoviral GFP or Cre. Results are representative of three experiments performed. (b) YAP and TAZ localization in Snail+/+ and Snail–/– SSCs following transduction with adenoviral-LacZ or Cre. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. Results are representative of three experiments performed. (c) YAP and TAZ expression levels in Snail+/+ and Snail–/– SSCs cultured under osteogenic conditions for 14 d. Results are representative of three experiments performed. (d) BMP2 induces Snail/Slug and YAP/TAZ levels in osteoblast progenitors. Calvarial osteoblast progenitors were isolated from E18 mice embryos, treated with 100 ng·ml⁻¹ BMP2 and protein expression monitored by western blot. Results are representative of three experiments performed. (e) Deletion of Snail/Slug in osteoblast progenitors blunts the BMP2-induced upregulation of YAP/TAZ. Calvarial osteoblast progenitors were isolated from Snail+/+ and Snail–/– mice and transduced with adenoviral GFP or Cre. Cells were then treated with 100 ng·ml⁻¹ BMP2 for 2 h, and protein expression monitored by western blot. Results are representative of three experiments performed. (f) YAP and TAZ expression levels in E17.5 Snail+/+ and Snail–/–/Dermo1–Cre calvarial lysates. Results are representative of five experiments performed. (g) YAP and TAZ expression levels in E17.5 Snail+/+ and Snail–/–/Osterix–Cre femur lysates. Results are representative of five experiments performed. (h) CFU-F colony count from bone marrow cells isolated from YAP+/+ or YAP–/– mice following transduction with lentiviral GFP or Cre expression vectors (mean ± s.d., n = 5 independent experiments). **P < 0.01; unpaired t-test. (i) Quantification of Ki67-positive cells from i (mean ± s.d., n = 3 independent experiments). **P < 0.01; unpaired t-test. (k) Alizarin Red S staining of SSCs isolated from YAP+/+ or YAP–/– mice following transduction with adenoviral GFP or Cre. Scale bar, 50 μm. Results are representative of three experiments performed. (j) Relative mRNA expression of osteogenic markers in cultures from k (mean ± s.d., n = 3 independent experiments). **P < 0.01; unpaired t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
As neither the TEAD nor OSE reporter constructs contain definable Snail- or Slug-binding sites, we mapped the Snail/Slug–YAP/TAZ protein domains that mediate complex formation. YAP and TAZ can be divided into TEAD binding (TB), WW, SH3 binding (SH3), transcriptional activation (TA) and PDZ binding (PB) domains (Supplementary Fig. 6a,d)\(^3\),\(^4\). Following expression of a series of deletion mutants of YAP and TAZ in combination with either epitope-tagged, wild-type Snail or Slug, the dominant YAP/TAZ domains responsible for Snail/Slug binding can be localized to the WW domains of YAP and TAZ (Supplementary Fig. 6a–f). Conversely, deletion mutants of Snail and Slug identify the conserved amino-terminal repressor motifs (termed the extended SNAG domain)\(^4\), as the key binding elements mediating interactions with the WW domains (Fig. 6e–g and Supplementary Fig. 6g–i). Confirming the importance of these interactions, SNAG domain-deletion mutants of Snail or Slug no longer support the YAP/TAZ-dependent cooperative effects on TEAD or Runx2 transcriptional activity (Fig. 6h–k).
Given the characterization of Snail/Slug–TAZ complexes, we examined the ability of Snail/Slug to control the expression of YAP/TAZ/TEAD- and Runx2-downstream gene targets.

Co-deletion of Snail/Slug significantly decreases the expression of the YAP/TAZ/TEAD targets Ctgf, Ankrd1, Axl, Dkk1 and Cyr61, while simultaneously inhibiting expression of the Runx2 target

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**Figure 6** Mapping of Snail/Slug–YAP/TAZ binding interactions and regulation of transcriptional activity. (a) 8xGTIIC luciferase activity was determined in Cos-1 cells co-transfected with YAP, TAZ, Snail or Slug (mean ± s.d., n=3 independent experiments). (b) Snail/Slug binding to YAP/Tead4 complexes. Epitope tagged Snail, Slug, YAP and Tead4 were co-transfected into Cos-1 cells, lysates immunoprecipitated with anti-Myc antibody (Tead4 tagged with Myc) and immunoblotted. Results are representative of three experiments performed. (c) 6xOSE luciferase activity was monitored in Cos-1 cells co-transfected with Runx2, Snail, Slug, YAP or TAZ (mean ± s.d., n=3 independent experiments). (d) Snail/Slug incorporation into TAZ/Runx2 complexes. Epitope tagged Snail, Slug, YAP and Runx2 were co-transfected into Cos-1 cells, lysates immunoprecipitated with anti-HA antibody (Runx2 tagged with HA) and samples immunoblotted. Results are representative of three experiments performed. (e) Schematic of HA-tagged Snail mutants. (f) YAP interacts with the SNAG domain of Snail. Cos-1 cells were co-transfected with the indicated Snail–HA or mutant constructs in tandem with Flag–YAP. Cell lysates were subjected to anti-HA immunoprecipitation, and YAP in the complexes determined by anti-Flag immunoblotting. Results are representative of three experiments performed. (g) TAZ interacts with the SNAG domain of Snail. Cos-1 cells were co-transfected with the indicated Snail–HA or mutant constructs in tandem with Flag–TAZ. Cell lysates were subjected to anti-Flag immunoprecipitation, and wild-type Snail or Snail mutants in the complexes were assessed following anti-HA immunoblotting. Results are representative of three experiments performed. (h) 8xGTIIC luciferase activity was measured in Cos-1 cells following co-transfection with YAP, TAZ, Snail or Snail mutants (mean ± s.d., n=3 independent experiments). (i) 8xGTIIC luciferase activity was determined in Cos-1 cells co-transfected with YAP, TAZ, Slug or Slug mutants (mean ± s.d., n=3 independent experiments). (j) 6xOSE luciferase activity was determined in Cos-1 cells co-transfected with Runx2, TAZ, Snail or Snail mutants (mean ± s.d., n=3 independent experiments). (k) 6xOSE luciferase activity was monitored in Cos-1 cells co-transfected with Runx2, TAZ, Slug or Slug mutants (mean ± s.d., n=3 independent experiments). Unprocessed original scans of blots are shown in Supplementary Fig. 9.
Figure 7 Snail/Slug-YAP/TAZ complexes regulate gene expression. (a) Snail/Slug regulates YAP/TAZ/TEAD targeted genes. mRNA expression of YAP/TAZ/TEAD-direct targets in calvarial osteoblast progenitors isolated from Snail1−/−/Slug−/+/GFP or Snail1−/−/Slug−/−/Cre mice and transduced with adenoviral GFP or Cre expression vectors (mean ± s.d., n = 3 independent experiments). **P < 0.01, unpaired t-test. (b) Relative mRNA expression levels of Runx2 and its downstream genes were assessed in calvarial osteoblast progenitors re-expressed with Flag–Snail under osteogenic culture conditions and transduced with adenoviral GFP or Cre expression vectors, cultured under osteogenic conditions (mean ± s.d., n = 3 independent experiments). **P < 0.01, unpaired t-test. (c) Co-occupation of YAP and Snail in the YAP/TAZ/TEAD targeted promoters. ChIP experiments using anti-YAP antibody were performed in Snail/Slug double-null osteoblast progenitors re-expressed with Flag–Snail and then re-ChIPed with anti-Flag antibody. Results are shown as the fold-enrichment relative to IgG IP controls (mean ± s.d., n = 3 independent experiments). (d) Snail and Slug increase YAP/TAZ/TEADs binding to the Ctgf promoter. ChIP experiments using anti-YAP or anti-TEAD were performed in SSC lysates isolated from Snail1−/−/Slug−/+/GFP and Snail1−/−/Slug−/−/Cre mice that were transduced with adenoviral GFP or Cre, and then transfected with GFP siRNA or Teads(1–4) siRNA. Results are presented as fold-enrichment relative to IgG IP controls (mean ± s.d., n = 3 independent experiments). (e) Snail and Slug increase YAP/TAZ/TEADs binding to the Ankrd1 promoter. ChIP experiments using anti-YAP or anti-TEAD were performed in SSCs isolated from Snail1−/−/Slug−/+/GFP and Snail1−/−/Slug−/−/Cre mice and transduced with adenoviral GFP or Cre followed by transfection with GFP siRNA or Teads(1–4) siRNA. Results are presented as fold-enrichment relative to IgG IP controls (mean ± s.d., n = 3 independent experiments). (f) Snail and Slug increase Runx2 binding to the Bglap2 promoter. ChIP experiments using anti-Runx2 or anti-TEAD were performed in lysates of calvarial osteoblast progenitors isolated from Snail1−/−/Slug−/+/GFP and Snail1−/−/Slug−/−/Cre mice and transduced with adenoviral GFP or Cre followed by transfection with GFP siRNA or Teads(1–4) siRNA. Results are presented as fold-enrichment relative to IgG IP controls (mean ± s.d., n = 3 independent experiments).

Further, when Snail is re-expressed at physiologic levels in Snail/Slug-null osteoblast progenitors, chromatin immunoprecipitation (ChIP) analysis confirms the presence of both YAP and Snail in the promoter regions of Ctgf, Ankrd1, Axl and Dkk1 (Fig. 7c and Supplementary Fig. 7a). Similarly, a sequential ChIP for Runx2
A Snail/Slug–YAP/TAZ axis regulates SSC function. (a) CFU-F generation from Snail+/+Slug+/+/GFP or Snail−/−Slug−/−/Cre SSCs that were transduced with lenti-GFP, -YAP or -TAZ constructs. Results are representative of five experiments performed. (b) CFU-F colony counts from a (mean ± s.d., n=5 independent experiments). **P<0.01; one-way ANOVA. (c) Alizarin Red S staining of Snail+/+Slug−/−/Cre or Snail−/−Slug−/−/GFP SSCs transduced with lenti-GFP, -YAP or -TAZ, and cultured under osteogenic conditions for 14 d. Results are representative of three experiments performed. (d) Relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp or Bglap2) in cultures from c (mean ± s.d., n=3 independent experiments). **P<0.01; one-way ANOVA. (e) Immunoblotting of YAP/TAZ, Snail/Slug and mutant Snail (N150). C3H10T1/2 cells were transfected with mutant Snail (N150) or empty vector (EV). Results are representative of three experiments performed. (f) Mutant Snail (N150) inhibits proliferation of C3H10T1/2 cells. Proliferative response in cells from e were assayed by XTT assay (mean ± s.d., n=3 independent experiments). **P<0.01; unpaired t-test. (g) Relative mRNA expression of YAP/TAZ/TEAD targets was assessed in cells from e (mean ± s.d., n=3 independent experiments). **P<0.01; unpaired t-test. (h) Mutant Snail (N150) inhibits osteogenesis of C3H10T1/2 cells. Cells from e were cultured under osteogenic conditions for 7 d. Osteogenesis was monitored by ALP staining. (i) Relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp and Bglap2) was assessed in cells from h (mean ± s.d., n=3 independent experiments). **P<0.01; unpaired t-test. (j) Schematic model of Snail/Slug in regulating SSC proliferation and osteogenic differentiation. Unprocessed original scans of blots are shown in Supplementary Fig. 9.

followed by anti-Flag–Snail (or Flag–Slug) re-ChIP documents the ability of Runx2 and Snail/Slug to co-occupy the Bglap2 promoter (Fig. 7d and Supplementary Fig. 7bc). Finally, endogenous Snail/Slug increases YAP/TAZ binding to the promoters of the YAP/TAZ/TEAD-target genes, Ankrd1 and Ctgf, in a TEAD-dependent manner (Fig. 7c,f and Supplementary Fig. 7d), while simultaneously increasing TAZ binding to Runx2 in the Bglap promoter in a TEAD-independent fashion (Fig. 7g).
Snail/Slug–YAP/TAZ complexes control SSC function

The ability of Snail/Slug to promote YAP/TAZ protein stability and transcriptional activity led us to postulate that overexpressing wild-type YAP or TAZ should only partially rescue defects in Snail/Slug-deleted SSCs, and a dominant-negative mutant that prevents binding interactions between endogenous Snail/Slug and YAP/TAZ would inhibit SSC function. Indeed, whereas Snail/Slug-deleted SSCs display defects in CFU-F formation, cells overexpressing wild-type YAP or TAZ display only a partially rescued phenotype (Fig. 8a,b and Supplementary Fig. 8a). Likewise, overexpressing YAP and TAZ only partially reverses defects in the osteogenic potential of Snail/Slug-deleted SSCs (Fig. 8c,d). To next block binding interactions between YAP/TAZ and Snail/Slug, we constructed a Snail-deletion mutant that retains the Snail domain, but deletes the Snail zinc-finger domains that support its nuclear import and DNA-binding abilities74,75 (Supplementary Fig. 8b). As expected, the Snail mutant, termed N150, binds YAP/TAZ (Supplementary Fig. 8c), blocks the formation of YAP/TAZ–Snail/Slug complexes and inhibits the cooperative effect of wild-type Snail/Slug and YAP or TAZ on either TEAD- or OSE-reporter activities, as well as the ability of YAP to occupy the Ctgf promoter or Rnx2 to bind to the Bglyap2 promoter (Supplementary Fig. 8d–h). When the Snail N150 mutant is transfected into C3H10T1/2 cells, YAP and TAZ levels decrease in the predicted fashion with no effect on Snail/Slug levels (Fig. 8e). More importantly, SSC proliferation and the expression of YAP/TAZ/TEAD target genes are inhibited while osteogenesis, despite normal Runx2 induction, is blocked in tandem with decreased expression of Runx2 downstream targets (Fig. 8f–i). Hence, inhibiting Snail/Slug–YAP/TAZ complex formation alone recapitulates the phenotypic defects observed in Snail/Slug-null or YAP/TAZ hemizygous cells, thereby supporting a model wherein the formation of Snail/Slug–YAP/TAZ complexes controls SSC proliferation and differentiation (Fig. 8j).

DISCUSSION

Skeletal SSCs comprise a heterogeneous assortment of mesoderm- and neural crest-derived progenitors that play distinct and age-specific roles in osteoblastogenesis8,9,12,18,19,25,49–51. Although effects of Snail/Slug on stem cell function are normally assigned to epithelial cell populations, we demonstrate herein that SSCs—despite their mesenchymal origin—co-express these factors, emphasizing their ability to function outside of classic epithelial–mesenchymal transition-related programs. While recent reports have proposed major roles for Snail alone in osteoblastogenesis under in vitro conditions52,53, neither Snail- nor Slug-targeted mice display remarkable stem cell phenotypes.

In considering complementary pathways that intersect with the Snail/Slug-dependent transcriptional programs operative in SSCs, YAP/TAZ, like Snail/Slug, undergo cytosolic–nuclear translocation, are regulated by phosphorylation-dependent β-TRCP-mediated ubiquitination, and interface with canonical Wnt as well as TGFβ signalling5,34,35,37. Extending these regulatory interactions, we find Snail/Slug forms binary complexes with YAP/TAZ that not only modulate YAP/TAZ protein levels, but also the expression of YAP/TAZ-targeted genes. Further studies demonstrated that the extended SNAG domains of Snail/Slug (that recruit chromatin-modifying enzymes critical for transcriptional repression) mediate binding interactions with the YAP/TAZ WW domains5,35,37. WW domains classically interact with protein partners via embedded PPPxY motifs35,37,54, but the extended SNAG domains of neither Snail nor Slug include this sequence. Interestingly, the Snail and Slug extended SNAG domains contain a conserved PxY motif that potentially participates in YAP/TAZ binding4.

Recent studies demonstrate that genome-wide, YAP/TAZ DNA-binding regions include a small subset of promoters with most YAP/TAZ/TEAD sites localized to distal enhancers that associate with target promoters through chromatin looping in a context-specific fashion41–43. As such, the regulatory effects mediated by Snail/Slug–YAP/TAZ complexes are likely to extend beyond the targets that we have characterized, although it is noteworthy that several of the affected genes identified herein regulate osteogenesis55–57. Given the widespread expression of Snail/Slug as well as YAP/TAZ in both epithelial as well as mesenchymal cell populations, we posit that the interacting transcriptional networks outlined here may prove equally important to stem cell function in a variety of normal and neoplastic states.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.T. designed and performed experiments, analysed data and wrote the paper. T.F. and X.-Y.L. designed and performed experiments. E.T.K. and X.-Y.L. designed and wrote the paper. S.J.W. oversaw the project, designed experiments, analysed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Klouy, Y. & Scadden, D. T. Mesenchymal cell contributions to the stem cell niche. Cell Stem Cell 16, 239–253 (2015).
2. Bianco, P. & Robey, P. G. Skeletal stem cells. Development 142, 1023–1027 (2015).
3. Battie, R. et al. Snail1 controls TGFβ responsiveness and differentiation of mesenchymal stem cells. Oncogene 32, 3381–3389 (2013).
4. Bansal-Gimeno, A. & Nieto, M. A. Evolutionary history of the Snail/Scratch superfamily. Trends Genet. 25, 248–252 (2009).
5. Nieto, M. A. Epithelial plasticity: a common theme in embryonic and cancer cells. Science 342, 1234850 (2013).
6. Puisieux, A., Brabletz, T. & Caramel, J. Oncogenic roles of EMT-inducing transcription factors. Nat. Cell Biol. 16, 488–494 (2014).
7. Desgrosellier, J. S. et al. Integrin alphavbeta3 drives slug activation and stemness in the pregnant and neoplastic mammary gland. Dev. Cell 30, 295–308 (2014).
8. Guo, W. et al. Slug and Sox9 cooperatively determine the mammary stem cell state. Cell 148, 1015–1028 (2012).
9. Horvay, K. et al. Snail1 regulates cell lineage allocation and stem cell maintenance in the mouse intestinal epithelium. EMBO J. 34, 1319–1335 (2015).
10. Hwang, W. L. et al. MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells. Nat. Cell Biol. 16, 268–280 (2014).
11. Lin, Y. et al. Snail1-dependent control of embryonic stem cell pluripotency and lineage commitment. Nat. Commun. 5, 3070 (2014).
12. Ye, X. et al. Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. Nature 525, 256–260 (2015).
13. Drom, K. F., Carter, E. A. & Gridley, T. Slug expression during organogenesis in mice. Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 271, 189–191 (2003).
et al. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89, 747–754 (1997).
45. Ducy, P. & Karsenty, G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol. Cell Biol. 15, 1858–1869 (1995).
46. Harada, H. et al. Cbf1 isoforms exert functional differences in osteoblast differentiation. J. Biol. Chem. 274, 6972–6978 (1999).
47. Mingot, J. M., Vega, S., Maestro, B., Sáiz, J. M. & Nieto, M. A. Characterization of Snai nuclear import pathways as representatives of C2H2 zinc finger transcription factors. J. Cell Sci. 122, 1452–1460 (2009).
48. Choi, S. et al. Structural basis for the selective nuclear import of the C2H2 zinc-finger protein Snail by importin beta. Acta Crystallogr. D Biol. Crystallogr. 70, 1050–1060 (2014).
49. Chan, C. K. et al. Identification and specification of the mouse skeletal stem cell. Cell 160, 285–298 (2015).
50. Isern, J. et al. The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. eLife 3, e03696 (2014).
51. Ouyang, N., Ono, W., Nagasawa, T. & Kronenberg, H. M. A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Nat. Cell Biol. 16, 1157–1167 (2014).
52. de Frutos, C. A. et al. Snai1 controls bone mass by regulating Runx2 and VDR expression during osteoblast differentiation. EMBO J. 28, 686–696 (2009).
53. Park, S. J. et al. The transcription factor snail regulates osteogenic differentiation by repressing Runx2 expression. Bone 46, 1498–1507 (2010).
54. Wang, W. et al. Defining the protein–protein interaction network of the human hippo pathway. Mol. Cell Proteomics 13, 119–131 (2014).
55. Itoh, S. et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130, 2779–2791 (2003).
56. MacDonald, B. T. et al. Bone mass is inversely proportional to Dkk1 levels in mice. Bone 41, 331–339 (2007).
57. Nakashima, K. et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108, 17–29 (2002).
METHODOLOGY

Mice. Mice with Snail^{tm} and Snail^{tm} alleles have been generated in our laboratory and are maintained on a C57BL/6 genetic background. YAP^{TM}/TAZ^{TM} and Slug^{tm} mice have been previously developed. C3H/10T1/2 and MC3T3-E1 cells were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6 background. Dermito-1Cre knock-in mice and Osterix-GFP-Cre transgenic mice were obtained from the Jackson Laboratory. Nu/Nu mice were from Charles River Laboratories International, Inc. All mouse work was performed with IACUC approval and in accordance with a protocol approved by the University of Michigan Institutional Animal Care & Use Committee.

CFU-F assay. Fetal limb skeletal tissues were dissected from embryos, cut into small pieces and digested with 3 mg ml^{-1} collagenase (type I; Sigma) for 30 min at 37 °C with constant agitation. Postnatal femur andibia specimens were similarly treated, but digested with 3 mg ml^{-1} collagenase for 60 min at 37 °C with constant agitation. Following enzymatic digestion, the skeletal pellets were filtered through a 40 μm cell strainer, and the cell suspensions pelleted, washed and resuspended. For fibroblast colony-forming unit (CFU-F) assays, > 10^6 mouse bone marrow cells were plated into individual wells of 6-well plates, and 3 d later, the unattached cells were removed by washing, and the adherent cells were further cultured for 7–10 d. The total number of colonies formed was determined after the samples were fixed and stained with crystal violet. Colonies with more than 50 cells were scored as CFU-Fs.

Quantitative RT-PCR. RNA purified from embryonic, neonatal skull, femurs or cell cultures was used for cDNA synthesis and amplification by real-time PCR according to the manufacturer’s instructions (Power SYBR Green, Applied Biosystems). The primer information is provided in Supplementary Table 1.

Cell culture. Femur andibia specimens from 4–6-week-old mice were dissected and cleaned from surrounding tissue, cut into pieces and digested with 3 mg ml^{-1} collagenase for 60 min at 37 °C with constant agitation. Single-cell suspensions were collected after passing through a 40 μm cell strainer (BD Biosciences) and incubated in tissue culture-grade plastic dishes with DMEM supplemented with 10% fetal bovine serum. After 2 d of culture, cells were washed twice with PBS and the adherent cells were further cultured in DMEM supplemented with 10% fetal bovine serum. The adherent colonies were then sorted by flow cytometry with antibodies against Sca-1, CD29, CD45 and CD11b as described previously. To further deplete haematopoietic cell populations from the cultures, cell suspensions were purified with a mouse haematopoietic progenitor cell enrichment set (BD Biosciences), and haematopoietic cell lineages were captured with BD IMag Streptavidin Particles Plus-DM magnetic nanoparticles according to the manufacturer’s instructions. The purified cells isolated from CFU-Fs were used as SSCs.

For culture of calvarial mesenchymal progenitor cells, calvaria were harvested from embryonic skulls and digested in 0.1% collagenase and 0.2% dispase solution. Mesenchymal progenitor cells isolated from digested calvaria were cultured in alfa-Minimum Essential Medium supplemented with 10% FBS. Human mesenchymal stem cells (Lonza), C3H/10T1/2 and MC3T3-E1 cells (ATCC) were cultured according to the manufacturer’s instructions. Human mesenchymal stem cells were authenticated by cell surface markers and cell differentiation assay. C3H/10T1/2 and MC3T3-E1 cells were confirmed by cell differentiation assay. All cell lines were routinely tested for mycoplasma contamination and were negative. None of the cell lines used in this study is present in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Differentiation of SSCs in vitro. SSCs were induced with osteogenic medium (50 μM ascorbic acid, 10 nM dexamethasone, 10 mM β-glycerophosphate and 2% fetal bovine serum (FBS) in low-glucose DMEM) or adipogenic medium (1 μM dexamethasone, 50 μM indomethacin, 500 nM IBMX, 5 μg ml^{-1} insulin and 2% FBS in low-glucose DMEM). For chondrogenesis, micro-mass cultures were performed as described previously with SSCs induced with a STEMPRO Chondrogenesis Differentiation Kit (Invitrogen). Differentiated cells were stained with Aizlein Red S to detect calcium deposits in osteogenic cultures, Oil Red O for detecting lipid deposits during adipogenesis, and Safranin O (cartilage mucopolysaccharides staining)/Fast Green for monitoring chondrogenesis.

Histology. Embryonic, neonatal or adult skeletal tissues were dissected, and fixed (4 h at 4 °C for embryonic and neonatal bones, and overnight at 4 °C for adult bones) in 4% paraformaldehyde. Adult bones were decalcified in EDTA. The skeletal specimens were embedded in OCT compound or paraffin, and sectioned (10 μm for cryostat sections and 5 μm for paraffin sections). Sections were stained with haematoxylin/eosin (H&E) according to standard procedures while LacZ activity was analysed in frozen sections. Femur osteoclasts were identified by staining for tartrate resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase assay kit (Sigma-Aldrich). The number of TRAP-positive cells per millimetre of trabecular bone perimeter was quantified in the secondary spongiosa.

Microcomputed tomography. Tibias and femurs from adult mice were fixed in 4% paraformaldehyde overnight at 4 °C, and stored in 70% ethanol. Femurs were scanned in water at an 18 μm isotropic voxel resolution using Explore Locus SP (GE Healthcare Pre-Clinical Imaging), and calibrated three-dimensional images were reconstructed. Bone morphometric variables were analysed, including bone mineral density, bone volume/tissue volume, trabecular number, trabecular separation and trabecular thickness as described previously.

In vivo assay of SSCs osteogenic capacity. For in vivo osteogenic differentiation, 2 × 10^6 mouse SSCs were seeded into gelatin sponges that were implanted subcutaneously into Nu/Nu mice. The specimens were dissected from the mouse recipients and analysed 4 weeks later as described previously.

Skeleton analysis. Skulls collected at embryonic day 17.5 or postnatal day 0 were skinned, and fixed in 95% ethanol. Skull tissues were stained with Alizarin Red S and Alcian Blue as described previously.

Proliferation assay. Cells were seeded at 5 × 10^3 cells per well in 96-well plates and proliferation was determined using aXTT Cell Viability Kit (Cell Signaling Technology) according to the manufacturer’s instructions. Alternatively, cells were seeded on glass coverslips, incubated overnight, fixed and immunostained for the cell proliferation marker, Ki67. Total cell number was evaluated by DAPI staining. The ratio of Ki67-positive cells to the total cell number was determined.

Immunostaining, in situ PLA and imaging. Cells cultured on glass surfaces were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized and incubated with primary antibodies at 4 °C. For frozen sections, permeabilization and antibody incubations were performed under identical conditions as described above. All primary antibodies were used at 1:200 dilution. For in situ PLA, protein–protein interactions between Snail/Slug and YAP/TAZ were detected with secondary proximity probes (Anti-Rabbit Plus and Anti-Mouse Minus) according to the Duolink In Situ Fluorescence User Guide (Sigma-Aldrich). Images were captured with a Leica confocal microscope. The antibody information is provided in Supplementary Table 2.

siRNAs, constructs, gene expression and reporter assays. Human or mouse TEF-1, TEF-3, TEF-4 and TEF-5 (TEAD1–4) siRNAs, and human SNAIL or SLUG siRNAs were obtained from Santa Cruz Technology. To silence gene expression, Pempute siRNA Transfection Reagent (SignaGen Laboratories) was used for all siRNA transfections. Flag-Snail, Snail-ΔHA, Slug-ΔMyc, Flag-YAP, Flag-TAZ and 8xGTTG were vectorized from Addgene, Runx2 and 6xOSE reporters were provided by G. Stein (University of Vermont, USA). Snail, Slug, YAP and TAZ mutants were constructed by PCR. Where indicated, Flag-YAP and Flag-TAZ were cloned into pLentioX-IRES-GFP lentiviral vectors and used to transduce SSCs, with the expression of the exogenous proteins confirmed by western blot. For gene transfection, COS-1 cells or osteoblast progenitors were transfected with Lipofectamine 2000 (Invitrogen). For reporter assays, COS-1 cells were transfected with expression vectors and either 8xGTTG or 6xOSE reporters. Luciferase activity was determined as previously described.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was carried out by using the Imprint Chromatin Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, cells were harvested and resuspended in fresh culture medium and then crosslinked with 1% formaldehyde (for 10 min), and quenched with 0.125 M glycine for 5 min at room temperature. Harvested cells were washed twice with ice-cold PBS and then resuspended in nucleic preparation buffer. The nuclear pellet was harvested and resuspended in shearing buffer and sonicated on ice until the sheared DNA was approximately 200–1,000 bp in size. The samples were then centrifuged at 16,000g for 10 min at 4 °C to remove debris, and the supernatants were diluted 1:1 in dilution buffer. The prepared material was then used for protein/DNA immunoprecipitation. Antibodies directed against YAP, TAZ, Runx2 or Flag, normal control mouse IgG or rabbit IgG (Cell Signaling) were pre-bound to the assay wells and immunoprecipitation reactions were carried out with chromatin extracts. Five percent of the chromatin extract was set aside for input. After the immunoprecipitation, crosslink reversal was carried out and the precipitated DNA was purified. DNA was quantitated by real-time PCR analysis. All ChIP signals were normalized to the input, and relative fold-enrichment was compared with IgG controls.

For ChIP–reChIP, chromatin isolated from cells expressing Flag–Snail or Slug was incubated with anti-YAP or anti-Runx2 antibody overnight at 4 °C. Following
Methods

Washing, the immunoprecipitated complexes were incubated with protein A/G magnetic beads (Thermo) and then eluted by incubating beads in lysis buffer with purified GST–YAP or HA–Runx2 proteins, respectively for 2 h at 4 °C. The GST–YAP fusion protein was purified by GST-pulldown assay by using the pGEX–KG–GST–YAP construct (Addgene). While HA–Runx2 was purified from HEK293 cells transfectected with a HA–Runx2 expression vector following incubation with anti-HA magnetic beads (ThermoFisher) and elution with HA peptide (Sigma). The eluted chromatin was diluted 1:3 with lysis buffer, supplemented with 1% Triton X-100 and incubated with normal mouse IgG, anti-Flag or anti-TAZ antibodies overnight at 4 °C. Following immunoprecipitation, crosslink reversal was carried out and the precipitated DNA was purified. DNA was quantitated by real-time PCR analysis. The antibody information is provided in Supplementary Table 2, and the primer information is provided in Supplementary Table 1.

Immunoprecipitation and immunoblotting. Embryonic, neonatal skeleton homogenates or cell lysates were prepared using RIPA buffer for western blotting. For immunoprecipitation, embryonic, neonatal skeleton homogenates or cell lysates were prepared using a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA. The lysates were incubated with antibody and Dynabeads Protein G (Life Technologies). Following immunoprecipitation, the immunocomplexes were analysed by western blotting. The antibody information is provided in Supplementary Table 2.

Statistics and reproducibility. Statistical analysis was performed with Student’s t-test or by one-way ANOVA. *P < 0.05; **P < 0.01. All tests are two-sided with all experiments performed three or more times. In each experiment, the sample size is determined on the basis of our prior knowledge of the variability of experimental output. In animal experiments, the gender, age of animals were matched and a sample size of 4–10 mice per group allows us to detect the difference in bone differentiation and proliferation markers, CFU-F counts, microCT and bone formation with a confidence interval of 90% and α = 0.05. No randomization was performed in all the experiments. The researchers were not blinded during experiments and outcome assessment. For in vitro experiments analyses, in most of the cases three or more independent experiments were performed and each with three or more replicates. The corresponding information has been included in the figure legends. The difference between means of different experimental groups was analysed by using two-tailed unpaired Student’s t-test as noted in the respective legends. F-tests were performed to compare variation within different groups. The statistical information is further provided in Supplementary Table 3.

Data availability. Statistics source data for Figs 1g,i,l; 2d,f,g,i,k; 3c,g,h,k; 4h,j,l; 7a,b and 8b,d,f,i; and Supplementary Figs 1h,j,k,l; 2f; 3b,d,f,h and 4a,b,d,j,k are provided in Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding authors on request.

58. Rowe, R. G. et al. Mesenchymal cells reactivate Snail1 expression to drive three-dimensional invasion programs. J. Cell Biol. 184, 399–408 (2009).
59. Xin, M. et al. Hippo pathway effector YAP promotes cardiac regeneration. Proc. Natl Acad. Sci. USA 110, 13839–13844 (2013).
60. Yu, K. et al. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development 130, 3063–3074 (2003).
61. Wu, X. et al. Inhibition of Sca-1-positive skeletal stem cell recruitment by alendronate blunts the anabolic effects of parathyroid hormone on bone remodeling. Cell Stem Cell 7, 571–580 (2010).
62. Anjos-Afonso, F. & Bonnet, D. Isolation, culture, and differentiation potential of mouse marrow stromal cells. Curr. Protoc. Stem Cell Biol. http://dx.doi.org/10.1002/9780470151808.sc02b03s7 (2008).
63. McLeod, M. J. Differential staining of cartilage and bone in whole mouse fetuses by alizarin red S. Teratology 22, 299–301 (1980).
64. McLeod, M. J. Differential staining of cartilage and bone in whole mouse fetuses by alizarin red S. Teratology 22, 299–301 (1980).
65. Tang, Y., Liu, Z., Zhao, L., Clemens, T. L. & Cao, X. Smad7 stabilizes beta-catenin binding to E-cadherin complex and promotes cell-cell adhesion. J. Biol. Chem. 283, 23956–23963 (2008).
Supplementary Figure 1 Expression and Function of Snail and Slug in SSCs. (a) LacZ expression in Snail\textsuperscript{fl/+}LacZ 30-day old mouse skull. Scale bar: 0.5 mm. Results are representative of 3 experiments performed. (b) LacZ expression in Snail\textsuperscript{fl/+}LacZ neonatal knee joint. Scale bar: 1 mm. Results are representative of 3 experiments performed. (c) LacZ expression in Snail\textsuperscript{fl/+}LacZ neonatal femur periosteum. Scale bar: 1 mm. Results are representative of 3 experiments performed. (d) LacZ expression in Slug\textsuperscript{fl/-}LacZ 7-d old femur. Scale bar: 1 mm. Results are representative of 3 experiments performed. (e) LacZ expression in Slug\textsuperscript{fl/-}LacZ 7-d old femur periosteum and on trabecular bone surface. Scale bar: 1 mm. Results are representative of 3 experiments performed. (f) Real-time PCR of Snail and Slug expression in SSCs isolated from Snail\textsuperscript{fl/+} Slug\textsuperscript{fl/+} or Snail\textsuperscript{fl/-} Slug\textsuperscript{fl/-} mice and transduced with adeno-GFP or Cre (mean ± s.d., n=3 independent experiments). **p<0.01; one-way ANOVA. (g) Ki67 immunostaining in SSCs isolated from Snail\textsuperscript{fl/+} Slug\textsuperscript{fl/+} and Snail\textsuperscript{fl/-} Slug\textsuperscript{fl/-} mice and transduced with adeno-GFP or Cre. Scale bar: 50 µm. Results are representative of 5 experiments performed. (h) Quantification of Ki67-positive cells from (g) (mean ± s.d., n=5 independent experiments). **p<0.01; unpaired t-test. (i) Cleaved Caspase-3 was monitored by immunohistochemistry in SSCs isolated from Snail\textsuperscript{fl/+} Slug\textsuperscript{fl/+} and Snail\textsuperscript{fl/-} Slug\textsuperscript{fl/-} mice and transduced with adeno-GFP or Cre. Scale bar: 50 µm. Results are representative of 5 experiments performed. (j) SSCs isolated from Snail\textsuperscript{fl/+} Slug\textsuperscript{fl/+} or Snail\textsuperscript{fl/-} Slug\textsuperscript{fl/-} mice were transduced with adeno-GFP or Cre. After culture under chondrogenic conditions for 14 d, cells were stained with Safranin O/ Fast Green (upper panel) and relative mRNA expression of chondrogenic markers (Sox9, Collagen II and Aggrecan) determined by RT-PCR (mean ± s.d., n=3 independent experiments). (k) SSCs were isolated from 3-month old Snail\textsuperscript{fl/+} Slug\textsuperscript{fl/+} and Snail\textsuperscript{fl/-} Slug\textsuperscript{fl/-} mice and transduced with adeno-GFP or Cre expression vectors. (l) Cell proliferation was determined by Ki67 immunohistochemistry (mean ± s.d., n=3 independent experiments). p<0.01; unpaired t-test. Cells from (k) were cultured under osteogenic conditions for 14 d, relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp and Bglap2) were examined by RT-PCR (mean ± s.d., n=3 independent experiments). **p<0.01; unpaired t-test.
Supplementary Figure 2 Phenotypic Characterization of Snail\(^{+/+}\)/Slug\(^{++}\), Snail\(^{+/+}\)/Slug\(^{++}\)/Dermo1-Cre, Snail\(^{+/+}\)/Slug\(^{-/-}\) and Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre Mice. (a) Alizarin Red/Alcian Blue staining of skulls isolated from neonatal Snail\(^{+/+}\)/Slug\(^{++}\) and Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre mice. Scale bar: 2 mm. Results are representative of 5 experiments performed. (b) Histology of femur recovered from 6-wk old Snail\(^{+/+}\) and Snail\(^{+/+}\)/Dermo1-Cre mice. Scale bar: 1 mm. Results are representative of 5 experiments performed. (c) Alizarin Red/Alcian Blue staining of skulls isolated from neonatal Slug\(^{++}\) and Slug\(^{-/-}\) mice. Scale bar: 2 mm. Results are representative of 5 experiments performed. (d) Histology of femurs recovered from 10-wk old Slug\(^{++}\) and Slug\(^{-/-}\) mice. Scale bar: 1 mm. Results are representative of 5 experiments performed. (e) Growth plate histology of E15 Snail\(^{+/+}\)/Slug\(^{++}\), Snail\(^{+/+}\)/Slug\(^{++}\)/Dermo1-Cre, Snail\(^{+/+}\)/Slug\(^{-/-}\) and Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre embryos. Scale bar: 100 µm. Results are representative of 5 experiments performed. (f) Ki67 expression in cartilage isolated from E15 Snail\(^{+/+}\)/Slug\(^{++}\) and Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre femurs. Scale bar: 50 µm. Results are representative of 5 experiments performed. Quantification of Ki67-positive cells are presented as mean ± s.d. (n=5 mice). **p<0.01; unpaired t-test. (g) Alizarin Red S/Fast Red staining of parietal bone isolated from E17.5 Snail\(^{+/+}\)/Slug\(^{++}\) or Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre embryonic skulls. Scale bar: 100 µm. Results are representative of 3 experiments performed. Arrow: calcified bone. (h) Cleaved Caspase-3 expression as assessed by immunohistochemistry in the parietal mesenchymal cell layers of E15 Snail\(^{+/+}\)/Slug\(^{++}\) and Snail\(^{+/+}\)/Slug\(^{-/-}\)/Dermo1-Cre skulls. Scale bar: 50 µm. Results are representative of 5 experiments performed.
Supplementary Figure 3  Phenotypic Characterization of Snail<sup>f/f</sup>, Snail<sup>f/f</sup>/Slug<sup>++</sup>, Snail<sup>f/f</sup>/Slug<sup>++</sup>/Osterix-Cre, Snail<sup>f/f</sup>/Slug<sup>+</sup> and Snail<sup>f/f</sup>/Slug<sup>−</sup>/Osterix-Cre mice (a) Cleaved Caspase-3 expression as assessed by immunohistochemistry in neonatal Snail<sup>f/f</sup>/Slug<sup>++</sup> and Snail<sup>f/f</sup>/Slug<sup>−</sup>/Osterix-Cre femurs. Scale bar: 50 µm. Results are representative of 5 experiments performed. (b) Quantification of cleaved Caspase-3-positive cells from (a). Data are presented as mean ± s.d. (n=5 mice). p>0.05; unpaired t-test. (c) CFU-F generation from bone marrow cells isolated from 6-wk old Snail<sup>f/f</sup>/Slug<sup>++</sup>, Snail<sup>f/f</sup>/Slug<sup>++</sup>/Osterix-Cre, Snail<sup>f/f</sup>/Slug<sup>+</sup>/ or Snail<sup>f/f</sup>/Slug<sup>−</sup>/Osterix-Cre mice. Results are representative of 5 experiments performed. (d) CFU-F colony counts from (c) (mean ± s.d., n=5 mice). **p<0.01; one-way ANOVA. (e) Calvarial osteoblast progenitors were isolated from Snail<sup>f/f</sup>/Slug<sup>++</sup> and Snail<sup>f/f</sup>/Slug<sup>−</sup> mice and transduced with adeno-GFP or Cre expression vectors. Following culture under osteogenic conditions for 14 d, the cells were stained with Alizarin Red S. Results are representative of 3 experiments performed. (f) Quantification of osteoclast number in sections captured from images shown in (e) (mean ± s.d., n=5 mice). p>0.05; unpaired t-test. (g) Relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp and Bglap) in cultures from (g) (mean ± s.d., n=3 independent experiments). **p<0.01; one-way ANOVA. 

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Supplementary Figure 4 Snail/Slug Regulate YAP/TAZ levels during SSC Differentiation and Bone Development. (a) YAP and TAZ mRNA expression in SSCs isolated from Snail\(^{f/f}\)/Slug\(^{+/+}\) or Snail\(^{f/f}\)/Slug\(^{-/-}\) mice that were transduced with adeno-GFP or Cre expression vectors and cultured in the absence or presence of osteogenic medium (mean ± s.d., n=3), p>0.05; unpaired t-test. (b) Ctgf and Ankdr1 mRNA expression in SSCs isolated from Snail\(^{f/f}\)/Slug\(^{+/+}\) or Snail\(^{f/f}\)/Slug\(^{-/-}\) mice and transduced with adeno-GFP or Cre expression vectors (mean ± s.d., n=3 independent experiments). **p<0.01; unpaired t-test. (c) Western blotting of Snail, Slug, YAP and TAZ in SSCs cultured under osteogenic conditions for the indicated time periods. Results are representative of 3 experiments performed. (d) Deletion of Snail/Slug in osteoblast progenitors blunts BMP2-induced osteogenesis. Calvarial osteoblast progenitors isolated from Snail\(^{f/f}\)/Slug\(^{+/+}\) or Snail\(^{f/f}\)/Slug\(^{-/-}\) mice and transduced with adeno-GFP or Cre expression vectors were treated with 100 ng/ml BMP2 for 7 d. Relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp and Bglap2) were assessed (mean ± s.d., n=3 independent experiments). **p<0.01; unpaired t-test. (e) Immunostaining of YAP/TAZ in calvaria from E15 Snail\(^{f/f}\)/Slug\(^{+/+}\) or Snail\(^{f/f}\)/Slug\(^{-/-}\)/Dermo1-Cre mice. Scale bar: 100 µm Results are representative of 3 experiments performed. (f) Immunostaining of YAP/TAZ in femurs recovered from neonatal Snail\(^{f/f}\)/Slug\(^{+/+}\) or Snail\(^{f/f}\)/Slug\(^{-/-}\)/Osterix-Cre mice. Scale bar: 100 µm Results are representative of 3 experiments performed. (g) Western blot of YAP and TAZ levels in SSCs isolated from YAP\(^{f/+}\)/TAZ\(^{f/+}\) mice and transduced with adenovirus-GFP or Cre expression vectors. Results are representative of 3 experiments performed. (h) Immunofluorescence staining of Slug in SSCs isolated from YAP\(^{f/+}\)/TAZ\(^{f/+}\) mice and transduced with adenovirus-GFP or Cre expression vectors. Results are representative of 3 experiments performed. (i) Western blot of YAP/TAZ in SSCs isolated from YAP\(^{f/+}\)/TAZ\(^{f/+}\) mice and transduced with adenovirus-GFP or Cre expression vectors. Results are representative of 3 experiments performed. (j) Snail increases SSC proliferation. Proliferative response in cells from (i) were assayed by XTT assay (mean ± s.d., n=3 independent experiments). **p<0.01; unpaired t-test. (k) Relative mRNA expression of osteogenic markers (Runx2, Osterix, Alp and Bglap2) was assessed in cells from (i) cultured under osteogenic conditions for 7 d (mean ± s.d., n=3 independent experiments). **p<0.01; unpaired t-test.
Supplementary Figure 5 Snail/Slug Regulate YAP/TAZ Protein Levels. (a) Lats(1/2)-YAP/TAZ complex levels as detected following immunoprecipitation in SSCs isolated from Snail\(^{+/+}\)/Slug\(^{+/+}\) and Snail\(^{-/-}\)/Slug\(^{+/+}\) mice and transduced with adeno-GFP or Cre. Results are representative of 3 experiments performed. (b) Calvarial-derived osteoblast progenitors were isolated from Snail\(^{+/+}\)/Slug\(^{+/+}\) or Snai1\(^{-/-}\)/Slug\(^{+/+}\) mice and transduced with adeno- GFP or Cre expression vectors. These cells were then transfected with Flag-tagged YAP, and protein synthesis blocked by treatment of 50 \(\mu\)g/ml cycloheximide (CHX) for the indicated times. YAP protein levels were monitored by Western blot (upper panels), and the relative YAP levels quantified as the ratio between Flag-YAP and actin, which was arbitrarily set 1.0 at time point 0 (lower panels). T1/2 is the half-life of the protein. Results are representative of 3 experiments performed. (c) Calvarial-derived osteoblast progenitors were isolated from Snai1\(^{-/-}\)/Slug\(^{+/+}\) or Snai1\(^{-/-}\)/Slug\(^{-/-}\) mice and transduced with adeno- GFP or Cre expression vectors. The cells were then transfected with Flag-tagged TAZ, and protein synthesis blocked by treatment of 50 \(\mu\)g/ml CHX for the indicated times. TAZ protein levels were monitored by Western blot (upper panels), and the relative TAZ levels quantified as the ratio between Flag-TAZ and actin, which was arbitrarily set 1.0 at time point 0 (lower panels). T1/2 is the half-life of the protein. Results are representative of 3 experiments performed. (d) Western blot of YAP/TAZ levels in Snail\(^{-/-}\)/Slug\(^{+/+}\)/Cre SSCs treated with the proteasome inhibitor, MG132 (10\(\mu\)M), for 4 hours. Results are representative of 3 experiments performed. (f) Mesenchymal progenitor C3H10T1/2 cells were transfected with Flag-YAP, Flag-TAZ, Snail-HA or Slug-Myc, respectively. Protein-protein interactions between YAP/TAZ and Snail/Slug were assessed by proximity ligation assay. Scale bar: 10 \(\mu\)m. Results are representative of 3 experiments performed. (g) Western blot of SNAIL and TEADs(1-4) in human SSCs transfected with siGFP, siSnail and siTEADs(1-4). Results are representative of 3 experiments performed. (h) Western blot of SLUG in human SSCs transfected with siGFP and siSlug. Results are representative of 3 experiments performed. (h) Co-localization of endogenous SNAIL/SLUG and YAP/TAZ in human SSCs. Scale bar: 10 \(\mu\)m. Results are representative of 3 experiments performed.
Supplementary Figure 6 Snail/Slug-YAP/TAZ Binding Domains. (a) Schematic of Flag-tagged YAP mutants. (b) Snail complexes with the WW domain of YAP. Cos-1 cells were co-transfected with the indicated Flag-tagged wild-type YAP or YAP mutant constructs in tandem with HA-tagged Snail. Cell lysates were subjected to anti-Flag immunoprecipitation, and the presence of Snail in the complexes assessed by anti-HA immunoblotting. Results are representative of 3 experiments performed. (c) Snail interacts with the WW domain of YAP. Cos-1 cells were co-transfected with the indicated Flag-tagged YAP or YAP mutant constructs and Myc-tagged Slug. Cell lysates were immunoprecipitated with anti-Flag antibodies, and the presence of Slug in the complexes assessed by anti-Myc immunoblotting. Results are representative of 3 experiments performed. (d) Schematic of Flag-tagged TAZ mutants. (e) Snail binding interactions with the WW domain of TAZ. Cos-1 cells were co-transfected with the indicated Flag-tagged TAZ or TAZ mutant constructs with Snail-HA. Cell lysates were subjected to anti-Flag immunoprecipitation, and the presence of Snail in the complexes determined by anti-HA immunoblotting. Results are representative of 3 experiments performed. (f) Slug interacts with the WW domain of TAZ. Cos-1 cells were co-transfected with the indicated Flag-tagged TAZ or TAZ mutant constructs along with Myc-tagged Slug. Cell lysates were immunoprecipitated with anti-Flag antibodies and the presence of Slug in the complexes assessed by anti-Myc immunoblotting. Results are representative of 3 experiments performed. (g) Schematic of Myc-tagged Slug and Slug mutants. (h) YAP interacts with the SNAG domain of Slug. Cos-1 cells were co-transfected with the indicated Myc-tagged Slug or Slug mutant constructs and Flag-tagged YAP. Cell lysates were immunoprecipitated with anti-Myc antibodies, and the presence of YAP in the complexes assessed by anti-Flag immunoblotting. Results are representative of 3 experiments performed. (i) TAZ interacts with the SNAG domain of Slug. Cos-1 cells were co-transfected with the indicated Myc-tagged Slug or Slug mutant constructs and Flag-tagged TAZ. Cell lysates were immunoprecipitated with anti-Flag antibodies and the presence of Slug or Slug mutants in the complexes assessed by anti-Myc immunoblotting. Results are representative of 3 experiments performed.
Supplementary Figure 7 Snail/Slug-YAP/TAZ Complexes Regulate Gene Expression. (a) Western blot of the re-expressed Flag-Snail in calvarial osteoblast progenitors isolated from Snail$^{1+/}$Slug$^{-/-}$ mice and transduced with adeno-Cre, and then were transfected with empty vector (EV) or Flag-Snail expressing vector. The expression level of Flag-Snail was comparing to the Snail/Slug wild type cells. Results are representative of 3 experiments performed. (b) Western blot of Flag-Snail and Flag-Slug in calvarial osteoblast progenitors isolated from Snail$^{+/}$Slug$^{-/-}$ mice and transduced with adeno-Cre to generate. Snail$^{-/-}$/Slug$^{-/-}$ cells followed by transfection with an empty vector (EV), Flag-Snail or Flag-Slug expressing vectors. Results are representative of 3 experiments performed. (c) Co-occupation of Runx2 and Snail/Slug in the Bglap2 promoter. ChiP experiments using anti-Runx2 antibody were in osteoblast progenitors from (K) and then re-ChiPed with anti-Flag antibody. Results are shown as the fold-enrichment relative to IgG IP controls (mean ± s.d., n=3 independent experiments). (d) Western blot of pan-Teads(1-4) in mouse SSCs transfected with siGFP or siTeads siRNAs. Results are representative of 3 experiments performed.
Supplementary Figure 8 A Snail/Slug-YAP/TAZ Axis Directs SSC Function.
(a) Western blot of Snail^{f/f}/Slug^{-/-} SSCs transduced with lentiviral constructs carrying an empty vector (EV), Flag-YAP or Flag-TAZ. Results are representative of 3 experiments performed. (b) Schematic of Snail and Snail mutants. Snail (N150)-YAP/TAZ complex detected by immunoprecipitation in C_{3}H_{10}T1/2 cells transfected with Flag-tagged mutant (c) Snail (N150). Results are representative of 3 experiments performed. (d) Snail/Slug-YAP/TAZ complex levels detected by immunoprecipitation in C_{3}H_{10}T1/2 cells transfected with mutant Snail (N150) or empty vector (EV). Results are representative of 3 experiments performed. (e) 8XGTIIC luciferase activity was measured in Cos-1 cells co-transfected with YAP, Snail or the Snail (N150) mutant (mean ± S.D., n=3 independent experiments). (f) 6XOSE luciferase activity was determined in Cos-1 cells co-transfected with Runx2, TAZ, Snail and the Snail (N150) mutant (mean ± S.D., n=3 independent experiments). (g) Snail (N150) mutant inhibits YAP binding to Ctgf promoter. ChIP experiments using anti-YAP antibody were performed on lysates prepared from C_{3}H_{10}T1/2 cells transfected with mutant Snail (N150) or empty vector (EV). Results are expressed as the fold-enrichment relative to IgG IP controls (mean ± S.D., n=3 independent experiments). (h) Snail (N150) mutant inhibits Runx2 binding to Bglap2 promoter. ChIP experiments using anti-Runx2 antibody were performed on lysates prepared from MC3T3-E1 osteoblasts transfected with mutant Snail (N150) or empty vector (EV). Results are expressed as the fold-enrichment relative to IgG IP controls (mean ± S.D., n=3 independent experiments).
Supplementary Figure 9 Unprocessed Blots 1. (A) Western blot corresponding to Figure 1f. (B) Western blot corresponding to Figure 1j. (C) Western blot corresponding to Figure 3i. (D) Western blot corresponding to Figure 4a. (E) Western blot corresponding to Figure 4c. (F) Western blot corresponding to Figure 4d. (G) Western blot corresponding to Figure 4e. (H) Western blot corresponding to Figure 4f. (I) Western blot corresponding to Figure 4g. (J) Western blot corresponding to Figure 5a. (K) Western blot corresponding to Figure 5b. (L) Western blot corresponding to Figure 5c. (M) Western blot corresponding to Figure 5d. (N) Western blot corresponding to Figure 5e. (O) Western blot corresponding to Figure 6b. (P) Western blot corresponding to Figure 6d. (Q) Western blot corresponding to Figure 6f. (R) Western blot corresponding to Figure 6g. (S) Western blot corresponding to Figure 8e.
Supplementary Figure 9 continued. Unprocessed Blots 2. (a) Western blot corresponding to Supplementary Figure 4c. (b) Western blot corresponding to Supplementary Figure 4g. (c) Western blot corresponding to Supplementary Figure 4i. (d) Western blot corresponding to Supplementary Figure 5a. (e) Western blot corresponding to Supplementary Figure 5b. (f) Western blot corresponding to Supplementary Figure 5c. (g) Western blot corresponding to Supplementary Figure 5d. (h) Western blot corresponding to Supplementary Figure 5f. (i) Western blot corresponding to Supplementary Figure 5g. (j) Western blot corresponding to Supplementary Figure 6b. (k) Western blot corresponding to Supplementary Figure 6c. (l) Western blot corresponding to Supplementary Figure 6e. (m) Western blot corresponding to Supplementary Figure 6f. (n) Western blot corresponding to Supplementary Figure 6h. (o) Western blot corresponding to Supplementary Figure 6i. (p) Western blot corresponding to Supplementary Figure 7a. (q) Western blot corresponding to Supplementary Figure 7b. (r) Western blot corresponding to Supplementary Figure 7d. (s) Western blot corresponding to Supplementary Figure 8a. (t) Western blot corresponding to Supplementary Figure 8c. (u) Western blot corresponding to Supplementary Figure 8d.
Supplementary Table 1 Primer Information
The primers used for detecting gene expression and ChIP are listed.

Supplementary Table 2 Antibody Information
Antibody name, catalogue number, company and application are listed.

Supplementary Table 3 Statistical Information
All data are organized into different sheets tagged by the corresponding figure numbers.

Sheet 1 corresponding to Figure 1g,i,l
Sheet 2 corresponding to Figure 2d,f,g,i,k
Sheet 3 corresponding to Figure 3e,g,h,k
Sheet 4 corresponding to Figure 4h,j,l
Sheet 5 corresponding to Figure 7a,b
Sheet 6 corresponding to Figure 8b,d,f,g,i
Sheet 7 corresponding to Supplementary Figure 1f,h,j,k,l
Sheet 8 corresponding to Supplementary Figure 2f
Sheet 9 corresponding to Supplementary Figure 3b,d,f,h
Sheet 10 corresponding to Supplementary Figure 4a,b,d,j,k