# *Sall4-Gli3* system in early limb progenitors is essential for the development of limb skeletal elements

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**Limb skeletal elements originate from the limb progenitor cells, which undergo expansion and patterning to develop each skeletal element. Posterior-distal skeletal elements, such as the ulna/fibula and posterior digits develop in a Sonic hedgehog (*Shh*)-dependent manner. However, it is poorly understood how anterior-proximal elements, such as the humerus/femur, the radius/tibia and the anterior digits, are developed. Here we show that the zinc finger factors *Sall4* and *Gli3* cooperate for proper development of the anterior-proximal skeletal elements and also function upstream of *Shh*-dependent posterior skeletal element development. Conditional inactivation of *Sall4* in the mesoderm before limb outgrowth caused severe defects in the anterior-proximal skeletal elements in the hindlimb. We found that *Gli3* expression is reduced in *Sall4* mutant hindlimbs, but not in forelimbs. This reduction caused posteriorization of nascent hindlimb buds, which is correlated with a loss of anterior digits. In proximal development, *Sall4* integrates *Gli3* and the *Plzf-Hox* system, in addition to proliferative expansion of cells in the mesenchymal core of nascent hindlimb buds. Whereas forelimbs developed normally in *Sall4* mutants, further genetic analysis identified that the *Sall4-Gli3* system is a common regulator of the early limb progenitor cells in both forelimbs and hindlimbs. The *Sall4-Gli3* system also functions upstream of the *Shh*-expressing ZPA and the *Fgf8*-expressing AER in fore- and hindlimbs. Therefore, our study identified a critical role of the *Sall4-Gli3* system at the early steps of limb development for proper development of the appendicular skeletal elements.**

Sall4 | Gli3 | limb progenitors | appendicular skeletal elements | Plzf-Hox

How progenitor cells are spatially and temporarily organized to construct an organ is a central question in developmental biology. Limb skeletal elements develop from limb progenitors, which arise from the lateral plate mesoderm (LPM) that is originated from epithelial somatopleure (1). Limb progenitors initially form two paired protrusions, fore- and hindlimb buds, whose initiation occurs around embryonic day (E) 9.0 and E9.5, respectively, in mouse embryos. In the following steps, limb signaling centers, known as the zone of polarizing activity (ZPA) and apical ectodermal ridge (AER), are established. SHH (Sonic hedgehog) from the ZPA and FGF8 from the AER are major signal molecules that regulate proliferative expansion and patterning of early limb progenitor cells (reviewed in ref. 2). These processes lead to development of functional limbs with each skeletal element adopting a unique shape at a distinct location.

Several studies suggest that limb progenitors consist of two distinct pools, an anterior progenitor pool and a posterior progenitor pool. The posterior progenitor pool consists of cells that once expressed *Shh* and cells that received paracrine effects of SHH, which contribute to digit 2 (d2), d3, d4, and d5 and the posterior zeugopod (ulna, fibula) (3–6). Contrary to this, the anterior progenitors are not well characterized. However, a recent report suggested that the putative anterior progenitor pool contributes to d1 and anterior-proximal skeletal elements (tibia, femur) in hindlimbs. Inactivating *Irx3* and *Irx5* (*Irx3/5*), two homeodomain encoding genes expressed in the LPM and the anterior-proximal domain of developing fore- and hindlimb buds, resulted in failure to develop these anterior-proximal skeletons in hindlimbs, whereas forelimbs developed normally (7). The lack of the anterior-proximal hindlimb skeleton was observed only when *Irx3/5* were inactivated before hindlimb initiation, suggesting that the putative anterior-progenitor pool is specified before hindlimb outgrowth.

In addition, limb anterior genes appear to have a critical role in establishing limb signaling centers. One of factors involved in this process is a zinc finger factor GLI3 (8). *Gli3* functions for specifying anterior-posterior polarity in the nascent limb buds (9), and *Gli3* null embryos develop polydactyly without polarity (10). Furthermore, simultaneous inactivation of *Gli3* and *Irx3/5* caused failure to express *Shh* and significantly reduced levels of *Fgf8*, which resulted in severe limb truncation (11). This result suggests that cooperation of anterior genes is upstream of establishing limb signaling centers, and therefore expansion of SHH-dependent posterior progenitors. This phenotype was observed only in the hindlimbs, although *Irx3/5* and *Gli3* are similarly expressed in both fore- and hindlimb buds. Despite these recent reports, factors that regulate the putative anterior progenitors are not well understood. Moreover, it is unknown whether similar or distinct mechanisms involving anterior genes regulate limb signaling center establishment in fore- and hindlimbs.

*Sall* genes encode zinc finger transcription factors that are vertebrate homologs of the *Drosophila* homeotic gene, *spalt*. They play diverse roles in embryonic development, including the development of limbs (12, 13). Among four *Sall* genes in mammals, *Sall4* is a key regulator of stemness in stem cells and progenitor cells, such as embryonic stem cells, induced pluripotent stem cells, spermatogenial progenitor cells and cancer cells (14–19). Despite its robust expression during limb development, functions of *Sall4*

## Significance

The limb skeletal elements that have unique morphology and distinct locations are developed from limb progenitors, derived from the lateral plate mesoderm. These skeletal elements arise during limb development. In this study, we show genetic evidence that function of *Sall4* is essential prior to limb outgrowth for development of the anterior-proximal skeletal elements. Furthermore, genetic interaction between *Sall4* and *Gli3* is upstream of establishing *Shh* (Sonic hedgehog) expression, and therefore, *Shh*-dependent posterior skeletal elements. Our study identified early requirements of the *Sall4-Gli3* system for two putative progenitor pools that develop into distinct sets of limb skeletal elements.

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in limb development are not understood well. A previous report suggested a role of *Sall4-Tbx5* interaction in most-anterior digit (d1) development in forelimbs (20). However, this study used embryos heterozygous for the *Sall4* gene trap allele (*Sall4GT*), which exhibited a different phenotype from $Sall4^{+/-}$ mice (15), indicating that *Sall4GT* allele wound not be a simple loss-of-function model. Moreover, *Sall4* null embryos die at peri-implantation stages (15), excluding the possibility of identifying *Sall4* functions in postimplantation stages. We show that inactivation of *Sall4* in the mesoendoderm before limb outgrowth causes defects of proximal-anterior skeletal elements specifically in hindlimbs. We provide genetic evidence that *Sall4* and *Gli3* interact and regulate establishment of *Shh*-expression, and this regulation is common in fore- and hindlimb buds. Therefore, the *Sall4-Gli3* system is an early and critical regulator acting on limb progenitor cells.

## Results

***Sall4* Is Required Before Outgrowth for Development of the Anterior-Proximal Skeletal Elements in Hindlimbs.** *Sall4* is broadly expressed before limb outgrowth and is expressed in the developing limb bud (Fig. S1 *A–C*). To investigate the role of *Sall4* in limb development, we conditionally inactivated *Sall4* using three *Cre* deleters, with which recombination occurs at different timings (Fig. S2). Recombination by *Tcre* occurs in mesoendoderm lineages at E7.5, much earlier than limb outgrowth (21), and *Sall4* transcripts were undetectable by E8.5 (Fig. S1*D*). Neonatal mutants (*Tcre; Sall4*$^{flox/flox}$) exhibited severe skeletal defects specifically in hindlimbs (Fig. 1 *A–F*). In the stylopod, the femur was not formed, instead, a small cartilage aggregate was present (Fig. 1 *D* and *F*). In the zeugopod the tibia was missing, whereas the fibula developed with variable length. In the autopod, two to three digits were present in most of mutants (Table S1, 60.5% and 28.9%, respectively). Based on the morphology, the missing digits include the anterior d1, which is consistent with the expression of *Hoxd12* and *Hoxd13* in the entire autopod region of *Tcre; Sall4* CKO hindlimbs (Fig. S3), whereas the remaining digits include d5 and the identity of other digits was difficult to determine. These skeletal defects were observed in both left and right hindlimbs.

A second *Cre* line, *Hoxb6Cre,* recombines in the LPM around E9.0 (22). Approximately half of the mutants (neonatal and >E16.5) exhibited milder hindlimb skeletal defects (Fig. 1 *G* and *H* and Table S1). The *Hoxb6Cre; Sall4*$^{flox/flox}$ mutants exhibited four (50%) or three (7.7%) digits. All mutants with reduced digit numbers developed the tibia, except for two hindlimbs (Fig. S4 *A–C* and Table S2). The expression pattern of *Hoxd12* and *Hoxd13* is consistent with the loss of d1 (Fig. S3). The loss of d1 has been reported in *Hoxa13* mutants (23); however, *Hoxa13* was detected in both *Tcre; Sall4* and *Hoxb6Cre; Sall4* CKO hindlimbs (Fig. S3), indicating that the loss of d1 is not caused by loss of *Hoxa13* expression. A third deleter, *Prx1Cre* recombines at the time of limb initiation in the forelimbs and slightly after the onset of outgrowth in hindlimbs (24). All neonatal mutants exhibited normal skeletons in fore- and hindlimbs (Fig. 1 *I* and *J*).

These results indicate that *Sall4* is required specifically for the development of the proximal and anterior hindlimb skeletons. Although forelimbs develop largely normal in the absence of *Sall4*, some severely affected mutants with tail truncation exhibited reduced forelimb size (Fig. 1*E* and Fig. S4 *D* and *E*). *Tcre*-dependent recombination occurs much earlier than that of *Hoxb6Cre* in the LPM (21, 22). Thus, the difference between the skeletal phenotype by inactivating *Sall4* using *Tcre* and *Hoxb6Cre* suggests a temporal difference in *Sall4* requirement before and after hindlimb initiation. *Sall4* seems to act before hindlimb outgrowth for subsequent development of the femur and tibia, and at the early hindlimb outgrowth stage *Sall4* seems to be required for anterior digit development. Moreover, development of normal hindlimbs in *Prx1Cre; Sall4* mutant suggests that *Sall4* is dispensable around E9.75 and later on. A recent study suggested that the anterior-proximal skeletons in hindlimbs are derived from the putative anterior progenitors that express *Irx3/5* (7). Therefore, our data suggest that *Sall4* functions in the putative anterior progenitors in hindlimbs.

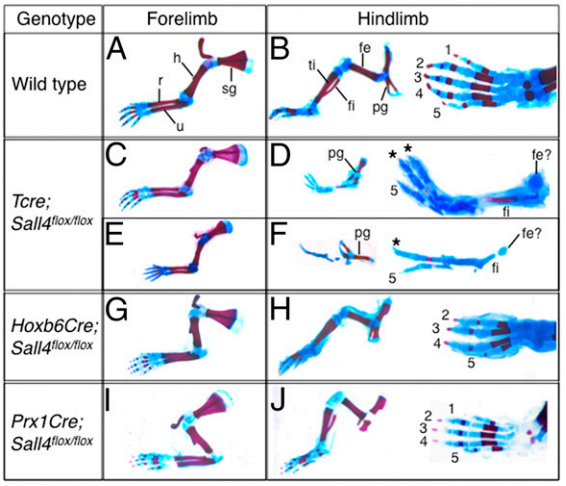


**Fig. 1.** *Sall4* is required for development of the anterior-proximal skeletal elements in a temporally restricted manner. (*A–J*) Appendicular skeletal preparations of control (*A* and *B*) and *Tcre; Sall4* CKO (*C–F*), *Hoxb6Cre; Sall4* CKO (*G* and *H*) and *Prx1Cre; Sall4* CKO (*I* and *J*) neonatal mice. (*A*, *C*, *E*, *G*, and *I*) Forelimbs develop without significant defects in mutants with size reduction in severely affected *Tcre; Sall4* mutants. (*D* and *F*) In *Tcre; Sall4* hindlimbs a small cartilage aggregate was present instead of the femur (fe). The fibula (fi) was present, but the tibia (ti) was missing. The pelvic girdle (pg) was small, but patterned normally. (*H*) In *Hoxb6Cre; Sall4* CKO hindlimbs, d1 was missing in the autopod. (*J*) Hindlimbs of *Prx1Cre; Sall4* CKO developed normally. fe, femur; fi, fibula; h, humerus; pg, pelvic girdle; r, radius; ti, tibia; u, ulna. Digits are numbered as 1–5. Asterisks mark digits whose identity is not completely determined.

***Sall4* Regulates Anterior Genetic Programs for Proper Anterior–Posterior Patterning.** We focused our analyses on *Tcre; Sall4*$^{flox/flox}$ conditional knockout mutants (hereafter referred to as *Sall4* CKO), given that all mutants exhibited strong phenotypes. The *Sall4* CKO neonates were obtained at a significantly lower than expected ratio (Table S3), suggesting that a large portion of mutants died in utero. Due to the early requirement of *Sall4*, we first examined expression of genes that mark hindlimb progenitors at the onset of hindlimb outgrowth (E9.5–9.75). We detected weak expression of *Tbx4* and *Fgf10* (25, 26), and, in particular, their expression in the anterior portion exhibited significant down-regulation (Fig. 2*A*). Expression of *Irx3/5*, whose functions are required for development of the anterior-proximal skeletal elements (7), seemed not to be significantly affected in *Sall4* CKO embryos (Fig. 2*B*). Although quantitative analysis indicated moderate reduction of *Irx5* levels, we did not detect alteration of *Irx3* levels (Fig. 2*D*). IRX3 immunoreactivity was also detected similarly in control and *Sall4* CKO hindlimb progenitor cells. Costaining with an anti-SALL4 antibody showed that immunoreactive IRX3 signals were similar in wild-type cells, relative to cells that lost SALL4 or cells that escaped from *Sall4* inactivation in *Sall4* CKO embryos. (Fig. 2*C*). These results suggest that *Sall4* and *Irx3/5* might function independently to regulate development of anterior-proximal skeletal elements.

We detected down-regulation of *Gli3*, a critical anterior factor, in nascent hindlimb buds at E10. Consistent with mutual antagonism between *Gli3* and *Hand2*, ectopic expression of *Hand2*, a posterior factor, was detected in the anterior portion of nascent hindlimb buds (Fig. 2*E*). HAND2 acts in concert with GLI3 to polarize the nascent limb mesenchyme and control transcriptional

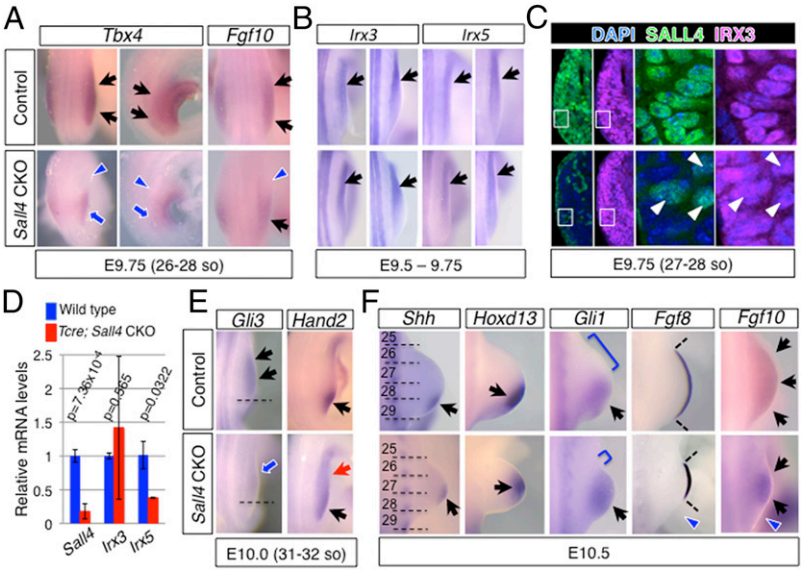


**Fig. 2.** Loss of *Sall4* caused defects of gene expression in hindlimb progenitors independent of *Irx3/5*. In situ hybridization (*A*, *B*, *E*, and *F*), immunofluorescence (*C*), and quantitative RT-PCR (*D*) of marker genes/proteins in control and *Sall4* CKO hindlimb buds. (*A*) Dorsal views of *Tbx4* and *Fgf10*, and lateral views of *Tbx4* at E9.75. (*B*) Dorsal views of *Irx3* and *Irx5* at E9.5-E9.75. (*C*) Immunofluorescence of SALL4 (green) and IRX3 (magenta) on transverse sections of E9.75 anterior hindlimb buds. White arrowheads point to cells that escaped from *Sall4* deletion in *Tcre*; *Sall4* CKO hindlimb buds. (*Right*) Close up of the boxed areas in *Left*. (*D*) Quantitative analysis of *Irx3* and *Irx5* transcript levels. Relative mRNA levels of indicated genes are shown as average ± SD. Asterisks indicate statistical significance. *P* values for expression of each gene are included. (*E*) Dorsal views of *Gli3* and *Hand2* at E10.0. (*F*) Dorsal views of *Shh*, *Hoxd13*, *Gli1*, *Fgf8* and *Fgf10* at E10.5. (*A*, *B*, *E*, and *F*) Black arrows and blue arrows point to normal and reduced expression, respectively. Red arrows point to ectopic expression. Blue arrowheads denote loss of expression. In *D*, broken lines mark normal expression border of *Gli3*. In *E*, dotted lines mark somite boundaries in *Shh* expression panels. Brackets in *Gli1* expression panels denote anterior domains that are free of *Gli1* signals.

networks (27). Thus, the altered *Hand2-Gli3* balance in nascent *Sall4* CKO hindlimb buds might modify gene network and proliferation rates, which would contribute to *Shh* expression being shifted to the middle-distal region in hindlimb buds at E10.5 (Fig. 2*F*). A similar shift was also detected with the expression of *Hoxd13*, a target of SHH signaling (28) (Fig. 2*F*). This shift is correlated with anterior expansion of the expression domain of *Gli1*, a transcriptional target of SHH signaling (8), and with a reduction in size of the *Gli1*-free anterior domain. *Fgf8* expressing AER was narrower and its posterior domain was missing in *Sall4* CKO hindlimb buds, which is consistent with the positive feedback loop between the ZPA and AER (29, 30). Accordingly, expression of *Fgf10*, whose expression in developing limb buds is maintained by FGF8 signaling (26), exhibited a smaller expression domain (Fig. 2*F*). A similar but smaller degree of reduction of the SHH signaling-free domain was also observed in *Sall4* CKO forelimb buds (Fig. S5*C*). Changes in the balance between *Gli3* and *Hand2* were subtle in forelimb buds compared with hindlimb buds (Fig. S5 *A* and *B*). We also examined whether *Sall4* expression was altered in *Gli3* mutants, but detected only mild up-regulation in forelimb buds (Fig. S6). The alterations of gene expression in the nascent and developing hindlimb buds in *Sall4* CKO embryos support the idea that cells in the anterior portion, which are free of SHH signaling, are reduced in the absence of *Sall4*. A compatible interpretation is that limb progenitor cells might respond to the new geometry of ZPA and AER, which would cause fewer cells to be specified as anterior digit precursors. Such a reduction of the anterior cells would result in the lack of anterior digits in hindlimbs (Fig. 1) (7).

***Sall4* Regulates Proximal Development Through the *Plzf-Hox* System.** One of the notable phenotypes of *Sall4* CKO hindlimbs is the failure to develop the femur. Despite the early defects in the proximal region, proximal marker genes, such as *Meis2* and *Efnb2*, as well as *Irx3* and *Irx5* (7), were expressed similarly in both control and *Sall4* CKO hindlimb buds (Fig. 3*A* and Fig. S7*B*). Instead, we observed an alteration in expression of *Hox* genes, which are regulators of skeletal patterning. More specifically, a genetic study has demonstrated that mice lacking all *Hox10* genes (*Hoxa10*, *c10*, *d10*) exhibit a small cartilage condensation in the stylopod, specifically in the hindlimbs, whereas other skeletal elements in fore- and hindlimbs develop (31). In *Sall4* CKO hindlimb buds, expression of *Hoxa10* and *Hoxd10* was undetectable in the proximal-anterior region, and was reduced in other regions (Fig. 3*B*). Expression of *Hoxc10* was down-regulated in the anterior half of the hindlimb bud. These results indicate that *Sall4* is an upstream regulator of *Hox10* genes, and suggest that the proximal defects in *Sall4* CKO hindlimbs involve reduced function of *Hox10* genes in the anterior hindlimb bud.

*Hox* expression is known to be regulated by the *Plzf* zinc finger factor (32). We found that *Plzf* expression was also down-regulated in the anterior-proximal part of the hindlimb buds at E10.5 (Fig. 3*C*). This down-regulation was more significant in the nascent hindlimb bud at E10.0. A previous study showed that mice lacking both *Gli3* and *Plzf* developed a small cartilage condensation in the stylopod only in the hindlimbs (33), similar to *Sall4* CKO mice. Our results indicate that *Sall4* acts upstream of *Gli3* and the *Plzf-Hox10* system and suggest that the proximal defects in *Sall4* CKO hindlimbs are derived from, at least in part, reduced function of *Gli3* and the *Plzf-Hox10* system in the putative anterior progenitors.

*Sall4* is known to regulate proliferation of stem cells (15), thus, we examined whether the proliferation of hindlimb progenitor cells is affected in *Sall4* CKO embryos. Through serial section analysis (34), we found that the mesenchymal core of nascent hindlimb buds exhibited a reduced proliferation index (Fig. 3 *D* and *E*). In contrast, we did not detect alteration of cell death at E9.75 and E10.5 (Fig. 3 *D* and *E* and Fig. S7*A*). The reduced proliferation was detected only in the mesenchymal core, and cells in the anterior and posterior portions did not exhibit significant difference in cell proliferation. Reduced proliferation is associated with down-regulation of cell cycle progression related

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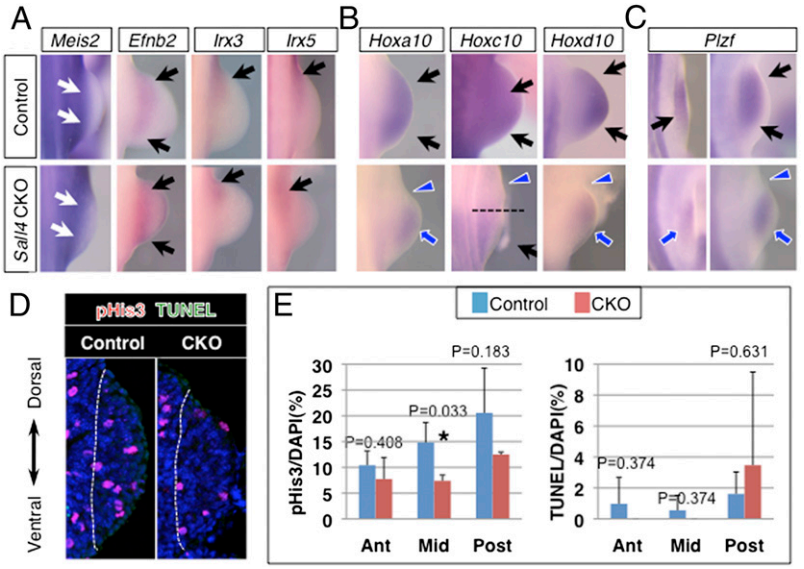


**Fig. 3.** *Sall4* integrates *Plzf-Hox10* system and proliferative expansion of limb progenitors. (*A*) Expression pattern of *Meis2*, *Efnb2*, *Irx3*, and *Irx5* in control and *Sall4* CKO hindlimbs at E10.5. (*B*) Expression pattern of *Hoxa10*, *Hoxc10*, and *Hoxd10* in control and *Sall4* CKO hindlimbs at E10.5. (*C*) Expression pattern of *Plzf* in control and *Sall4* CKO hindlimbs at E9.75 and E10.5. Black arrows and blue arrows point to normal and reduced expression, respectively. In *A*, *Meis2* expression is indicated by white arrows. Blue arrowheads denote loss of expression. In *B*, a broken line indicates the anterior boundary of *Hoxc10* expression in *Sall4* CKO hindlimb buds. (*D*) Representative images of pHis3 and TUNEL double staining of transverse sections of nascent hindlimb bud at E9.75 in control and *Sall4* CKO embryos. Dotted lines indicate boundary between the nascent hindlimb bud and trunk. (*E*) Quantitative evaluation of cell proliferation and cell death in transverse sections of nascent hindlimb buds at E9.75 in control and *Sall4* CKO embryos. *P* values are included.

genes, such as *Ccnd1*, *Ccne1*, and *Mycn* (Fig. S8 *A–L*). These results suggest that loss of *Sall4* caused failure to expand cells in a discrete region in the nascent hindlimb buds.

**Interaction Between *Sall4* and *Gli3* Regulates Development of the Limb Signaling Centers in both Fore- and Hindlimbs.** A major difference between fore- and hindlimbs of *Sall4* CKO mutants is *Sall4* regulation of *Gli3*, which was evident in hindlimb buds but was subtle in forelimb buds (Fig. 2*E* and Fig. S5). To test whether hindlimb-specific skeletal defects in *Sall4* CKO limbs involve reduced *Gli3* rather than loss of *Sall4* alone, we genetically removed *Gli3* from the *Sall4* CKO background. Because *Sall4* CKO; $Gli3^{+/-}$ embryos did not survive beyond E10.5, we focused our analysis on *Shh,* whose expression is significantly affected in *Sall4* CKO hindlimb buds (Fig. 2*F*). We also monitored *Fgf8* expression, given a recent genetic study, involving *Irx3/5* and *Gli3*, indicated that cooperation of anterior genes is necessary for establishing limb signaling centers, such as *Shh*-expressing ZPA and *Fgf8*-expressing AER (11).

In hindlimbs, removing one allele of *Gli3* from the *Sall4* CKO background caused a failure to express both *Shh* and *Fgf8* (Fig. 4*A*), which was also observed in *Sall4* CKO; $Gli3^{-/-}$ double KO hindlimbs. Although *Sall4* CKO; *Gli3* double mutants exhibited smaller hindlimb buds, the lack of *Shh* and *Fgf8* is unlikely simply due to small size of hindlimb buds, because wild-type hindlimbs with similar size (earlier stages) showed evident expression of both genes. Given that *Gli3* is down-regulated in *Sall4* CKO hindlimb buds, this result suggests that the *Sall4* CKO; $Gli3^{+/-}$ genotype caused a condition in the hindlimb similar to the *Sall4* CKO; $Gli3^{-/-}$ genotype.

In forelimb buds of *Sall4* CKO; $Gli3^{+/-}$ mutants, *Shh* expression became weak, and its expression domain shifted to the middle-distal region (Fig. 4*B*). In addition, the *Fgf8* expression domain was short. These expression patterns in forelimb buds resembled those of *Sall4* CKO hindlimb buds. In *Sall4* CKO; $Gli3^{-/-}$ double KO forelimbs, expression of *Shh* and *Fgf8* was further down-regulated and only trace levels of signals were detected. The residual *Shh* and *Fgf8* in *Sall4* CKO; $Gli3^{-/-}$ forelimb buds implies that additional anterior factor(s) might participate in signaling center establishment in forelimb buds. Comparison of the expression pattern of *Shh* and *Fgf8* between forelimb and hindlimb buds supports the idea that the hindlimb-specific phenotype in *Sall4* CKO mutants involves reduced *Gli3* levels. These results demonstrate that genetic interaction between two anterior factors, *Sall4* and *Gli3*, is necessary to establish limb signaling centers in both fore- and hindlimbs. The results also suggest that the difference of *Sall4* CKO skeletal phenotypes in fore- and hindlimbs would be derived from differences in *Gli3* regulation by *Sall4* (Fig. 2*E* and Fig. S5).

Because SALL4 and GLI3 are both nuclear proteins, they might physically interact. Indeed, we observed both SALL4a (long form) and SALL4b (short form) could interact with GLI3, when transfected in cultured cells (Fig. S8 *M* and *N*). This result suggests that SALL4 and GLI3 might directly interact, in addition to genetically, to regulate downstream developmental programs.

## Discussion

Proper development of skeletal elements depends on specification, expansion and patterning of limb progenitor cells (35). Genetic experiments have shown that posterior skeletal elements are developed in a *Shh*-dependent manner (3, 4). However, how anterior skeletal elements are developed has been poorly understood. A recent report demonstrated that anterior progenitor cells, marked by *Irx3/5*, give rise to the femur, tibia, and d1 in the hindlimb (7). These skeletal elements are missing (d1, tibia) or are present as a small cartilage condensation (femur) in *Sall4* CKO hindlimbs, suggesting that *Sall4* function is required for the putative anterior progenitors. This phenotype was observed only when *Tcre* was used to inactivate *Sall4*, and *Hoxb6Cre*-dependent *Sall4* inactivation only resulted in the lack of d1 in approximately half of the mutants. These different skeletal phenotypes from using *Tcre* and *Hoxb6Cre* suggest that the progenitors for the femur and tibia are specified before digits, and this specification occurs before hindlimb outgrowth.

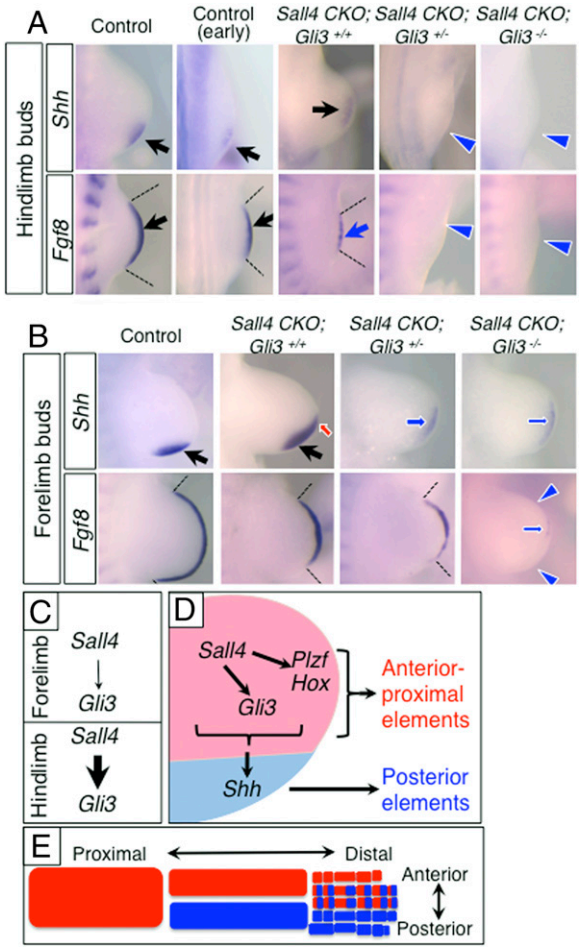


**Fig. 4.** Genetic interaction between *Sall4* and *Gli3* and a model of the role of *Sall4-Gli3* system in limb progenitor cells. (*A*) Expression pattern of *Shh* and *Fgf8* in hindlimbs of indicated genotypes. Due to reduced size of hindlimb buds in *Sall4* CKO; *Gli3* mutants, wild-type hindlimb buds at an earlier stage with similar size were included. (*B*) Expression pattern of *Fgf8* and *Shh* in forelimbs of indicated genotypes. Black arrows and blue arrows point to normal and reduced expression, respectively. Red arrows point to ectopic expression. Blue arrowheads denote loss of expression. (*C–E*) A model of the role of *Sall4* in the two-population model for the development of limb skeletal elements. (*C*) *Sall4* regulation of *Gli3* differs in fore- and hindlimb progenitor cells. (*D*) Model of *Sall4* regulation of gene network in the putative anterior progenitors (red) and posterior progenitors (blue). (*E*) Schematic drawing of skeletal elements derived from the putative anterior progenitors (red) and posterior progenitors (blue).

The skeletal phenotype of *Tcre; Sall4* CKO mutants are different from a previous study using $Sall4^{+/GT}$ mice, which exhibited elongated d1 in the forelimb autopod (20). The *Sall4GT* allele would generate N-terminal truncated SALL4, fused with βGeo protein, which could interact with other SALL proteins and interfere with their functions (36). Therefore, the *Sall4GT* allele would generate complex functional interference of all SALL proteins. In contrast, our approach could address functions specific to *Sall4* during limb development.

Our data and a recent report (7) indicate that *Sall4* CKO and *Irx3/5* mutants share similar skeletal defects and altered gene expression in hindlimbs. We observed comparable expression patterns of *Irx3/5* and immunoreactivities of IRX3 in *Sall4* CKO embryos at E9.5–9.75, although *Irx5* levels showed moderate reduction by qRT-PCR analysis. Given that one allele of *Irx3/5* is sufficient for development of the anterior-proximal skeletons in hindlimbs (7), our data suggest that *Sall4* and *Irx3/5* function independently to regulate development of anterior-proximal skeletal elements. If so, it is conceivable that *Sall4* and *Irx3/5* share common downstream targets in the putative anterior progenitors. Alternatively, *Irx3/5* might function upstream of *Sall4* in the anterior progenitors.

Proximal skeletal defects are one of the notable phenotypes of *Sall4* CKO hindlimbs. Our analysis suggests that *Sall4* integrates *Gli3* and the *Plzf-Hox* system for the development of the proximal skeletal elements. Because these genes are not completely down-regulated, additional mechanism(s) might be involved in the proximal defects. It has been suggested that development of a skeletal element requires expansion of precursors to sufficient levels, otherwise the element fails to form (37, 38). The reduced proliferation of the mesenchymal core of nascent hindlimb buds of *Sall4* CKO embryos suggests that, in addition to regulation of *Gli3* and the *Plzf-Hox* system, *Sall4* regulation of proliferation of discrete populations of limb progenitors also contributes to the skeletal phenotype.

In the autopod, it has been suggested that the time and concentration that cells are exposed to SHH pattern the digits (3, 4). In this model, posterior d4 and d5 are derived from cells that expressed *Shh*, and d3 is partially derived from cells that expressed *Shh* and cells that received paracrine SHH, whereas d2 is derived from cells that received only paracrine SHH. In *Sall4* CKO hindlimbs, ~90% of mutants exhibited an absence of d2 or d2+d3 phenotype, in addition to the absence of d1. This digit phenotype was correlated with reduced *Gli1*-free domain, which suggested that the putative anterior progenitor-derived cells are reduced. Thus, the failure to expand the putative anterior progenitors in the absence of *Sall4* would contribute to the loss of d2 and d3 through failure to provide enough digit precursor cells, even in the presence of precursors derived from the posterior progenitor cells. This idea suggests that development of d2 and d3 requires both the putative anterior progenitors and *Shh*-dependent posterior progenitors (Fig. 4*E*).

A recent report showed that cooperation of anterior genes at the beginning of limb development is necessary for establishing limb signaling centers. In particular, simultaneous inactivation of *Irx3/5* and *Gli3* caused failure to establish a *Shh*-expressing ZPA, and caused trace *Fgf8* expression in the AER. However, this phenotype was hindlimb specific and forelimbs developed polydactyly, similar to *Gli3* KO limbs (11). In significant contrast to this report, we found that *Sall4-Gli3* cooperation regulates limb signaling center development in both fore- and hindlimbs. As discussed above, *Sall4* CKO skeletal defects are specific to hindlimbs and forelimbs developed largely normal. However, this difference between two types of limbs is likely derived from difference in *Sall4* regulation of *Gli3* (Fig. 4 *C* and *D*). Due to reduction of *Gli3* expression in *Sall4* CKO hindlimb buds, the mutant hindlimb bud would be similar to *Sall4* CKO; $Gli3^{+/-}$ condition. This idea is supported by genetic removal of *Gli3* from *Sall4* CKO background. Due to severe defects to establish expression of *Fgf8* and *Shh*, we speculate that the *Sall4-Gli3* system would regulate early genetic systems, including *Fgf10* induction and/or its upstream mechanisms, such as expression of *Hoxa* and *Hoxd* cluster genes (39) in limb progenitor cells. Such mechanisms are to be investigated in the future to deepen our understanding of how limb progenitor cells are controlled to develop into functional limbs. Nonetheless, our genetic experiments indicate that the *Sall4-Gli3* system is a common mechanism to regulate early limb progenitors in fore- and hindlimb buds.

The data presented here and a recent report (11) support the two-population model, in which the anterior and posterior progenitor pools contribute to specific limb skeletal elements, located in the anterior-proximal region and posterior region, respectively (Fig. 4 *D* and *E*). In this model the *Sall4-Gli3* system is upstream of establishing *Shh*-expressing ZPA, which is a critical regulator of

proliferative expansion of the posterior progenitor pool. Moreover, the *Sall4*, together with *Gli3* and the *Plzf-Hox* system, regulates the putative anterior progenitor pool. Therefore, our data suggests that the *Sall4-Gli3* system is a critical upstream regulator of both progenitor pools, and thus, development of the limb skeletal elements.

## Materials and Methods

**Mouse Mutants.** The mouse lines for *Sall4* (15, 40), *Gli3* (41), *Tcre* (21), *Hoxb6Cre* (22), and *Prx1Cre* (24) were maintained on a mixed genetic background. Skeletal preparation was done as published (42). Animal breeding and procedures were performed according to the approval by the Institutional Animal Care and Use Committee of the University of Minnesota.

**Immunofluorescence and TUNEL Assay.** Immunofluorescence of IRX3 (43) and SALL4 (Santa Cruz, sc-101147, 1/300 dilution) was performed on cryo-sections according to standard procedures (42). Simultaneous cell proliferation and cell death analyses were performed by using anti-phospho histone H3 (Upstate, catalog no. 06–570) and the In Situ Cell Death Detection kit (Roche) as published (34). Cell death analysis at E10.5 was performed on coronal sections by using the In Situ Cell Death Detection kit. DAPI was used for nuclear staining. Fluorescent confocal images were obtained by using Zeiss LSM 710 laser scanning microscope system (Carl Zeiss Microscopy), and analyzed using ZEN2009 software (Carl Zeiss Microscopy).

**Skeletal Preparation and in Situ Hybridization.** Preparation of skeletal staining and in situ mRNA detection were performed according to a standard procedure (42).

**Quantitative RT-PCR for *Irx3/5*.** Relative expression of *Irx3/5* in wild-type and *Sall4* CKO was determined by isolating RNA from the hindlimb forming region/nascent hindlimb buds at E9.5–9.75 and performing quantitative PCR after reverse transcription. Details are provided in *SI Materials and Methods*.

**Coimmunoprecipitation Assay.** HEK293T cells were transfected with expression constructs by using Polyethylenimine MAX (Polysciences). Cell lysates were prepared after two days and coimmunoprecipitation assay were performed by using protein G-Sepharose (GE Healthcare) and anti-Flag antibody (M2, Sigma). Proteins were resolved by SDS/PAGE, transferred to PVDF membranes (Millipore) and detected by anti-HA antibodies (4B2, Wako), HRP goat anti-mouse IgG, and a chemiluminescence system.

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1. Gros J, Tabin CJ (2014) Vertebrate limb bud formation is initiated by localized epithelial-to-mesenchymal transition. *Science* 343(6176):1253–1256.
2. Zeller R, López-Ríos J, Zuniga A (2009) Vertebrate limb bud development: Moving towards integrative analysis of organogenesis. *Nat Rev Genet* 10(12):845–858.
3. Harfe BD, et al. (2004) Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* 118(4):517–528.
4. Ahn S, Joyner AL (2004) Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* 118(4):505–516.
5. Kraus P, Fraidenraich D, Loomis CA (2001) Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech Dev* 100(1):45–58.
6. Chiang C, et al. (2001) Manifestation of the limb prepattern: Limb development in the absence of sonic hedgehog function. *Dev Biol* 236(2):421–435.
7. Li D, et al. (2014) Formation of proximal and anterior limb skeleton requires early function of Irx3 and Irx5 and is negatively regulated by Shh signaling. *Dev Cell* 29(2): 233–240.
8. Hui CC, Angers S (2011) Gli proteins in development and disease. *Annu Rev Cell Dev Biol* 27:513–537.
9. te Welscher P, Fernandez-Teran M, Ros MA, Zeller R (2002) Mutual genetic antagonism involving GLI3 and dHAND prepatterns the vertebrate limb bud mesenchyme prior to SHH signaling. *Genes Dev* 16(4):421–426.
10. Hui CC, Joyner AL (1993) A mouse model of greig cephalopolysyndactyly syndrome: The extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat Genet* 3(3):241–246.
11. Zhulyn O, et al. (2014) A switch from low to high Shh activity regulates establishment of limb progenitors and signaling centers. *Dev Cell* 29(2):241–249.
12. de Celis JF, Barrio R (2009) Regulation and function of Spalt proteins during animal development. *Int J Dev Biol* 53(8-10):1385–1398.
13. Kawakami Y, et al. (2009) Sall genes regulate region-specific morphogenesis in the mouse limb by modulating Hox activities. *Development* 136(4):585–594.
14. Rao S, et al. (2010) Differential roles of Sall4 isoforms in embryonic stem cell pluripotency. *Mol Cell Biol* 30(22):5364–5380.
15. Sakaki-Yumoto M, et al. (2006) The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development. *Development* 133(15): 3005–3013.
16. Hobbs RM, et al. (2012) Functional antagonism between Sall4 and Plzf defines germline progenitors. *Cell Stem Cell* 10(3):284–298.
17. Yong KJ, et al. (2013) Oncofetal gene SALL4 in aggressive hepatocellular carcinoma. *N Engl J Med* 368(24):2266–2276.
18. Buganim Y, et al. (2014) The developmental potential of iPSCs is greatly influenced by reprogramming factor selection. *Cell Stem Cell* 15(3):295–309.
19. Lim CY, et al. (2008) Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages. *Cell Stem Cell* 3(5):543–554.
20. Koshiba-Takeuchi K, et al. (2006) Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart. *Nat Genet* 38(2):175–183.
21. Perantoni AO, et al. (2005) Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development* 132(17):3859–3871.
22. Lowe LA, Yamada S, Kuehn MR (2000) HoxB6-Cre transgenic mice express Cre recombinase in extra-embryonic mesoderm, in lateral plate and limb mesoderm and at the midbrain/hindbrain junction. *Genesis* 26(2):118–120.
23. Fromental-Ramain C, et al. (1996) Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development* 122(10):2997–3011.
24. Logan M, et al. (2002) Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis* 33(2):77–80.
25. Chapman DL, et al. (1996) Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev Dyn* 206(4):379–390.
26. Ohuchi H, et al. (1997) The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* 124(11):2235–2244.
27. Osterwalder M, et al. (2014) HAND2 targets define a network of transcriptional regulators that compartmentalize the early limb bud mesenchyme. *Dev Cell* 31(3): 345–357.
28. Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75(7):1401–1416.
29. Niswander L (2002) Interplay between the molecular signals that control vertebrate limb development. *Int J Dev Biol* 46(7):877–881.
30. Zúñiga A, Haramis AP, McMahon AP, Zeller R (1999) Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401(6753): 598–602.
31. Wellik DM, Capecchi MR (2003) Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. *Science* 301(5631):363–367.
32. Barna M, Hawe N, Niswander L, Pandolfi PP (2000) Plzf regulates limb and axial skeletal patterning. *Nat Genet* 25(2):166–172.
33. Barna M, Pandolfi PP, Niswander L (2005) Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development. *Nature* 436(7048):277–281.
34. Akiyama R, et al. (2014) Distinct populations within Isl1 lineages contribute to appendicular and facial skeletogenesis through the β-catenin pathway. *Dev Biol* 387(1): 37–48.
35. Mariani FV, Martin GR (2003) Deciphering skeletal patterning: Clues from the limb. *Nature* 423(6937):319–325.
36. Kiefer SM, et al. (2003) Expression of a truncated Sall1 transcriptional repressor is responsible for Townes-Brocks syndrome birth defects. *Hum Mol Genet* 12(17): 2221–2227.
37. Wolpert L, Tickle C, Sampford M (1979) The effect of cell killing by x-irradiation on pattern formation in the chick limb. *J Embryol Exp Morphol* 50:175–193.
38. Galloway JL, Delgado I, Ros MA, Tabin CJ (2009) A reevaluation of X-irradiation-induced phocomelia and proximodistal limb patterning. *Nature* 460(7253):400–404.
39. Sheth R, et al. (2013) Decoupling the function of Hox and Shh in developing limb reveals multiple inputs of Hox genes on limb growth. *Development* 140(10): 2130–2138.
40. Yuri S, et al. (2009) Sall4 is essential for stabilization, but not for pluripotency, of embryonic stem cells by repressing aberrant trophectoderm gene expression. *Stem Cells* 27(4):796–805.
41. Büscher D, Grotewold L, Rüther U (1998) The XtJ allele generates a Gli3 fusion transcript. *Mamm Genome* 9(8):676–678.
42. Itou J, et al. (2012) Islet1 regulates establishment of the posterior hindlimb field upstream of the Hand2-Shh morphoregulatory gene network in mouse embryos. *Development* 139(9):1620–1629.
43. Chen JA, et al. (2011) Mir-17-3p controls spinal neural progenitor patterning by regulating Olig2/Irx3 cross-repressive loop. *Neuron* 69(4):721–735.