RESEARCH ARTICLE

Smad2 and Smad3 Regulate Chondrocyte Proliferation and Differentiation in the Growth Plate

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Abstract

TGFβs act through canonical and non-canonical pathways, and canonical signals are transduced via Smad2 and Smad3. However, the contribution of canonical vs. non-canonical pathways in cartilage is unknown because the role of Smad2 in chondrogenesis has not been investigated in vivo. Therefore, we analyzed mice in which Smad2 is deleted in cartilage (Smad2 CKO), global Smad3 -/- mutants, and crosses of these strains. Growth plates at birth from all mutant strains exhibited expanded columnar and hypertrophic zones, linked to increased proliferation in resting chondrocytes. Defects were more severe in Smad2 CKO and Smad2 CKO;Smad3 -/- (Smad2/3) mutant mice than in Smad3 -/- mice, demonstrating that Smad2 plays a role in chondrogenesis. Increased levels of Ihh RNA, a key regulator of chondrocyte proliferation and differentiation, were seen in prehypertrophic chondrocytes in the three mutant strains at birth. In accordance, TGFβ treatment decreased Ihh RNA levels in primary chondrocytes from control (Smad2 fx/fx) mice, but inhibition was impaired in cells from mutants. Consistent with the skeletal phenotype, the impact on TGFβ-mediated inhibition of Ihh RNA expression was more severe in Smad2 CKO and Smad2 CKO;Smad3 -/- (Smad2/3) mutant mice than in Smad3 -/- mice, demonstrating that Smad2 plays a role in chondrogenesis. Increased levels of Ihh RNA, a key regulator of chondrocyte proliferation and differentiation, were seen in prehypertrophic chondrocytes in the three mutant strains at birth. In accordance, TGFβ treatment decreased Ihh RNA levels in primary chondrocytes from control (Smad2 fx/fx) mice, but inhibition was impaired in cells from mutants. Consistent with the skeletal phenotype, the impact on TGFβ-mediated inhibition of Ihh RNA expression was more severe in Smad2 CKO than in Smad3 -/- cells. Putative Smad2/3 binding elements (SBEs) were identified in the proximal Ihh promoter. Mutagenesis demonstrated a role for three of them. ChiP analysis suggested that Smad2 and Smad3 have different affinities for these SBEs, and that the repressors SnoN and Ski were differentially recruited by Smad2 and Smad3, respectively. Furthermore, nuclear localization of the repressor Hdac4 was decreased in growth plates of Smad3 CKO and double mutant mice. TGFβ induced association of Hdac4 with Smad2, but not with Smad3, on the Ihh promoter. Overall, these studies revealed that Smad2 plays an essential role in the development of the growth plate, that both Smads 2 and 3 inhibit Ihh expression in the neonatal growth plate, and suggested they accomplish this by binding to distinct SBEs, mediating assembly of distinct repressive complexes.
Author Summary

The cartilage growth plate regulates the size and shape of nearly every skeletal element in the body. TGFβs are potent inducers of cartilage formation, but the mechanisms by which they transduce their signals in cartilage during development are poorly understood. Similarly, there is strong evidence that dysregulation of the TGFβ pathway increases the risk for osteoarthritis (OA) in humans, but the underlying mechanisms are unknown. TGFβs transduce their signals through a canonical pathway involving Smad2 and Smad3, and through several non-canonical pathways. However, the roles of canonical vs. noncanonical signaling are unknown in cartilage because the combined roles of Smad2 and Smad3 have not been determined. We generated mice lacking both Smad2 and Smad3 in cartilage in order to determine the role of canonical TGFβ signaling during embryonic development. We determined that Smad2 has a more prominent role than Smad3 in non-hypertrophic chondrocytes in the growth plate, and identified elevated levels of Ihh RNA in neonatal cartilage in Smad2 and Smad3 mutants. These findings may be important because Ihh is a vital regulator of cartilage proliferation and differentiation during cartilage development. More generally, the studies identify how Smad2 and Smad3 can regulate a common target gene through distinct mechanisms.

Introduction

The cartilage growth plate is the primary driver of endochondral bone growth. In the growth plate, resting, columnar, prehypertrophic and hypertrophic chondrocytes are arrayed in discrete zones. Resting chondrocytes, located at the top of the growth plate, are small and relatively quiescent. Upon stimulation by extracellular signals, cells near the bottom of the resting zone transition to columnar chondrocytes, which exhibit a higher rate of proliferation and a flatter morphology. These cells form stacks along the long axis of the developing skeletal element. Columnar cells at the bottom of this zone eventually exit the mitotic phase and become prehypertrophic chondrocytes. Prehypertrophic cells further differentiate into enlarged hypertrophic cells, comprising a zone adjacent to the site of replacement of cartilage by bone. Chondrocyte proliferation and differentiation in the growth plate is tightly regulated by Indian hedgehog (Ihh) and parathyroid hormone-related peptide (Pthrp). Ihh, a secreted protein expressed in prehypertrophic chondrocytes, stimulates cell proliferation and differentiation. Its role in proliferation is mediated in part by inducing Pthrp expression in epiphyseal resting chondrocytes. Secreted Pthrp maintains columnar cells in a mitotic state, preventing their transition to the pre-hypertrophic phase, and hence negatively regulating Ihh expression. Once cells escape the zone of influence of Pthrp, they exit the cell cycle, become prehypertrophic, and upregulate Ihh expression, which promotes hypertrophy and matrix mineralization. This feedback loop thus controls the transition of chondrocytes through each zone of the growth plate.

Transforming growth factor βs (TGFβs) and activins are secreted proteins that are members of the TGFβ superfamily of growth factors. TGFβs and activins bind to distinct receptor complexes, but activate similar signal transduction pathways. Binding of TGFβs or activins to their receptors leads to activation of the kinase activity of the receptor. The activated receptor complexes then transduce signals through multiple pathways. These pathways can be broadly divided into Smad-dependent and Smad-independent pathways [1–3]. In the canonical Smad-dependent pathway, activated receptor complexes phosphorylate the receptor-activated Smads.
Smad2 and Smad3 Function in Growth Plate Cartilage

(R-Smads), Smad2 and Smad3. Smads 2 and 3 are transcription factors; once phosphorylated, they form hetero-oligomeric complexes with the transcription factor Smad4. These complexes enter the nucleus, bind promoters, and regulate target gene expression. In addition, there exist numerous non-canonical Smad-independent pathways for transduction of TGFβ signals, such as MAP kinases, RhoA and mTOR [4–8].

TGFβs play critical roles in growth plate chondrocyte proliferation and differentiation, and in the maintenance of articular chondrocytes [9–17]. There are three TGFβ isoforms in mammals: TGFβ1, 2, 3. Only Tgfβ2−/− mice exhibit obvious skeletal dysplasia. Tgfβ2−/− mice exhibit embryonic lethality, accompanied by skeletal defects that include shortened limbs, axial, and craniofacial defects [18]. The role of the TGFβ signaling pathway in cartilage has been studied most extensively using mice lacking the type II TGFβ receptor TGFβRII. This receptor is required for transduction of the TGFβ pathway by TGFβs 1–3, but is not used by activin ligands. Tgfb2;Prrx1Cre mice, in which TGFβRII is ablated in limb bud mesenchyme, exhibit growth plates with decreased proliferation and accelerated onset of hypertrophy, but delayed terminal maturation [19, 20]. In contrast, conditional deletion of the TGFβRII in committed Col2a1-expressing chondrocytes did not lead to obvious defects in appendicular elements [21]. These findings suggest that TGFβRII transduces TGFβ signals at prechondrogenic stages and/or in perichondrium, but may not have a substantial role in cartilage once a growth plate forms.

The extent to which Smad2/3-dependent signaling mediated by TGFβ and activins is required in developing cartilage is unknown. Smad2, Smad3 and Smad4 are co-expressed throughout the growth plate [12, 22–24]. Smad2, 3 and 4 are all present in articular cartilage [12, 25]. Smad3−/− mice are born with a normal skeletal phenotype, but develop postnatal dwarfism and osteoarthritis-like pathologies in adulthood [12, 26–28].

The function of Smad2 in cartilage during embryogenesis has not been characterized. Smad2 and Smad3 have some distinct roles in mediating TGFβ/activin signaling. Smad3 can bind DNA directly, whereas Smad2 regulates gene expression by interacting with Smad3 or other transcription factors [29]. Mice lacking Smad2 die before 8.5 days of development (E8.5) [30], precluding a genetic analysis of its function in chondrogenesis. It is not known whether Smad2 partially compensates for the loss of Smad3 in the growth plates of Smad3−/− mice. Studies in Smad2−/− vs. Smad3−/− epiblast, epithelial cells and fibroblasts show that Smads 2 and 3 regulate some common and some distinct target genes [31, 32]. Overexpression of Smad2 or Smad3 can block the spontaneous maturation observed in Smad3−/− deficient chondrocytes [33], providing support for the hypothesis that Smad2 and Smad3 may have some overlapping functions in the growth plate.

To define the role of Smad2/3-mediated signaling in cartilage, we generated mice with conditional deletion of Smad2 using Col2a1-Cre (Smad2cko;Col2Cre, hereafter referred to as Smad2cko), mice with Smad3 globally deleted (Smad3−/−), and the corresponding double mutants (Smad2/3). Loss of Smad2 leads to a growth plate phenotype in neonates that is more severe than that seen in Smad3−/− mice. At the neonatal stage, Smad2/3 (Smad2cko;Smad3−/−) double mutants exhibit more severe defects in the hypertrophic zone than do Smad2cko or Smad3−/− mice. Defects include elevated levels of proliferation in resting zone chondrocytes at neonatal stages, leading to enlarged columnar and hypertrophic zones. This may be a consequence of depletion of the resting zone due to the accelerated entry of resting zone chondrocytes into the columnar zone. Overall, our results suggest that Smad2 inhibits proliferation of resting zone chondrocytes during embryogenesis, and acts as a negative regulator of Ihh expression, and that Smad2 and Smad3 have some overlapping functions in cartilage. Our results show that both of these Smads are required at neonatal stages for normal chondrocyte proliferation and differentiation in the growth plates.
Results

Smad2 and Smad3 are activated throughout the growth plate

We generated mice lacking Smad2 and Smad3 in cartilage in order to study the role of canonical Smad2/3-mediated signaling in chondrogenesis. Smad2\(^{fx/fx}\) mice were intercrossed with Col2a1Cre mice to generate Smad2\(^{fx/fx}\);Col2a1Cre (Smad2\(^{CKO}\)) mice. Smad2\(^{CKO}\) mice are viable and fertile. The Smad3\(^{-/-}\) allele we used has a LacZ cassette and internal ribosomal entry site (IRES) inserted in the second exon, leading to a loss-of-function allele [27]. We crossed Smad3\(^{-/-}\) mice with Smad2\(^{CKO}\) mice to generate Smad2\(^{fx/fx}\);Col2a1Cre;Smad3\(^{-/-}\) (Smad2/3 double mutant) mice.

To confirm efficient Cre-mediated excision, levels of activated C-terminal phosphorylated Smad2 and Smad3 were examined by IHC using an antibody that recognizes both pSmad2 and pSmad3 (S1 Fig). No pSmad2/3 was detected in E16.5 or P0 double mutant growth plates, verifying efficient loss of Smads 2 and 3 (S1D and S1H Fig). Consistent with previous studies [34], at E16.5 and P0, pSmad2/3 is present throughout the resting, columnar, and prehypertrophic zones of the E16.5 Smad2\(^{fx/fx}\) control growth plate, and this pattern of expression persists at least until birth (S1A and S1E Fig). No obvious differences were noted in the distribution of pSmad3 in Smad2\(^{CKO}\) or pSmad2 in Smad3\(^{-/-}\) mice compared with control Smad2\(^{fx/fx}\) littermates (S1B, S1C, S1F and S1G Fig). These findings confirm previous reports that pSmads 2 and 3 have extensively overlapping distributions in the growth plate [12, 22–24].

Smad2\(^{CKO}\);Smad3\(^{-/-}\) mutant mice exhibit subtle defects at birth followed by postnatal dwarfism

Consistent with previous studies [12, 26, 27], skeletal preparations revealed no apparent skeletal defects in Smad3\(^{-/-}\) mice at P0. Analysis of P0 Smad2\(^{CKO}\) mice also revealed no obvious defects (Fig 1A–1C). However, Smad2\(^{CKO}\);Smad3\(^{-/-}\) (Smad2/3) double mutant mice exhibited subtle defects in axial and craniofacial elements (Fig 1A and 1D). Lateral views of P0 skulls revealed reduced ossification of the bones of the inner ear in double mutants (Fig 1E–1H). The length of the body (from top of the skull to the proximal end of the tail) of double mutants is about 9.7% shorter than that of control Smad2\(^{fx/fx}\) littermates (n = 4, P = 0.04) (Figs 1A, 1D and S2A). Ventral views revealed that double mutants have slightly smaller skulls, with a shorter nasal to occipital length (94% of control (Smad2\(^{fx/fx}\)), n = 3, p = 0.05), narrower cranial base (93% of control, n = 3, p = 0.04), and reduced ossification of the occipital condyle (arrow, S2B Fig).

Dorsal views of the rib cage revealed no obvious differences between Smad2\(^{CKO}\) or Smad3\(^{-/-}\) mice compared with control Smad2\(^{fx/fx}\) littermates (S2C Fig). However, double mutants have a shorter sternum and a bifurcated xiphoid process (S2C Fig). Moreover, localized defects were seen in the vertebral columns of Smad2/3 mutants. Ventral views of the lumbar spine revealed shorter vertebrae and smaller ossified vertebral bodies in double mutants; reduced ossification was not observed in Smad2\(^{CKO}\) or Smad3\(^{-/-}\) mice (S2D Fig). No differences were evident in the cervical vertebrae in Smad3\(^{-/-}\) mice compared to control Smad2\(^{fx/fx}\) mice, but the ossification center of the axis (C2) was reduced in Smad2\(^{CKO}\) mice and in double mutants (S2E Fig). Cleared skeletal preparations revealed no clear differences in appendicular elements in Smad2\(^{CKO}\), Smad3\(^{-/-}\) mice, or Smad2/3 double mutants (Figs 1I–1L and S2F).

As discussed above, Smad2/3 double mutants had only subtle skeletal alterations at birth. However, these mice developed progressive postnatal dwarfism, which was not seen in Smad2\(^{CKO}\) and Smad3\(^{-/-}\) mice (S3 Fig). At 1 month, double mutants were 12.5% (n = 3, p = 0.05) shorter than control Smad2\(^{fx/fx}\) littermates (S3C Fig). These data indicate that Smad2 and Smad3 have compensatory roles in the regulation of axial skeletal growth after birth.
Increased neonatal chondrocyte proliferation and hypertrophy in *Smad2* and *Smad3* mutant growth plates

The above analysis revealed no clear impact of loss of *Smad2* or *Smad3* on appendicular length at P0. A previous study demonstrated that loss of *Smad3* led to appendicular defects beginning in the early postnatal period [12]. However, we used a different *Smad3* allele. We therefore performed a histological analysis of P0 appendicular cartilage to investigate whether the *Smad3* mutant allele we used [27] exhibits a similar phenotype. We also examined whether loss of *Smad2* exerts any effect on growth plate architecture. This analysis revealed that the lengths of the resting zones in *Smad2*^{CKO} and *Smad2/3* double mutants were shorter than in control *Smad2^{fx/fx}* mice. In contrast, the columnar and hypertrophic zones of *Smad2*^{CKO}, *Smad3^{−/−}* and *Smad2/3* double mutant mice were longer than those of control *Smad2^{fx/fx}* littermates (Figs 2A and S4). Although both *Smad2*^{CKO} and *Smad3^{−/−}* mice exhibited elongated columnar zones, the effect was greater in *Smad2*^{CKO} mice than in *Smad3^{−/−}* mice, and the degree of elongation did not differ between *Smad2/3* double mutants and *Smad2*^{CKO} mice, suggesting that *Smad2* has a more prominent role than *Smad3* in elongation of the columnar zone (S4 Fig). Both *Smad2*^{CKO} and *Smad3^{−/−}* mice exhibited elongated hypertrophic zones compared to *Smad2^{fx/fx}* controls, and there was a significant elongation of the hypertrophic zone in *Smad2/3* double mutants compared to either *Smad2*^{CKO} or *Smad3^{−/−}* mice. Immunohistochemistry for PCNA was performed to test whether the increases in lengths of the columnar zones were correlated with increased proliferation. A 2 to 3-fold increase in PCNA-positive cells was seen in the resting zones of *Smad2*^{CKO}, *Smad3^{−/−}* and *Smad2/3* double mutant mice compared with control.
Fig 2. Elevated proliferation in Smad2\(^{\text{cko}}\) and Smad3\(^{-/-}\) growth plates. All images are P0 proximal tibias. (A) Sections stained with safranin O. Heights of distinct growth plate zones are indicated by double arrows and demarcated by dashed lines. White dashed line is the demarcation between the columnar and prehypertrophic zones. All images are aligned to this boundary. Blue dashed lines demarcate the approximate boundary between the resting and columnar zones. Green dashed lines demarcate the boundary between the hypertrophic zone and zone of ossification. Blue double-headed arrows indicate the extent of the proliferative zone in the control Smad2\(^{fx/fx}\) growth plate. Green double-headed arrows demarcate the extent of the control Smad2\(^{fx/fx}\) hypertrophic zone. The double-headed arrows are superimposed on the images of Smad2\(^{\text{cko}}\), Smad3\(^{-/-}\) and Smad2\(^{\text{cko}}\); Smad3\(^{-/-}\) mutants to clarify the differences in lengths of these zones compared with control Smad2\(^{fx/fx}\). 3 litters containing mice of all of the genotypes shown in the figures were examined. At least 5 mice per genotype were examined. The images shown are from littermates. (B) PCNA immunostaining. All mice are littermates. (C) Quantitation of the percentage of PCNA-positive cells in distinct zones. The percentage of PCNA-positive cells in the resting zone (blue bars) and columnar zone (red bars) were quantified. Data are expressed as percent PCNA-positive + SE (n = 5). Asterisk, p < 0.05 compared with control Smad2\(^{fx/fx}\). (D) Quantitation of the percentage of
Smad2$^{fx/fx}$ littermates; the degree of PCNA staining in the resting zone was similar in Smad2/3 double mutants and Smad2$^{CKO}$ mice (Fig 2C). No differences were detected in the columnar zone (Fig 2B and 2C). TUNNEL assays were performed to evaluate whether differences in cell survival contribute to the longer hypertrophic zones in mutants. No differences were detected (Fig 2D). These results indicate that loss of Smad2 and/or Smad3 promotes the entry of resting chondrocytes into the highly proliferative columnar phase, and that Smad2 appears to have a more prominent role than does Smad3. The increased lengths of the columnar and hypertrophic zones in mutants is consistent with an increased pool of chondrocytes transiting out of the resting zone and eventually undergoing hypertrophy. These findings suggest that Smad2 and Smad3 function to maintain the pool of resting chondrocytes in a quiescent state in neonatal growth plates.

Increased Ihh RNA and protein levels in Smad2, Smad3, and Smad2/3 mutant chondrocytes

Ihh is expressed in prehypertrophic chondrocytes and is a critical regulator of chondrocyte proliferation and differentiation. Since pSmads 2 and 3 are present in prehypertrophic chondrocytes (S1 Fig), RNA in situ hybridization was performed to assess Ihh RNA levels. The zone of Ihh expression is increased (Fig 3A), and qPCR quantification of RNA isolated from P0 growth plate cartilage showed that the level of Ihh RNA is increased in all three mutant strains compared with control Smad2$^{fx/fx}$ littermates (S5 Fig), suggesting that Smad2 and Smad3 normally inhibit Ihh expression in the growth plate. Smad2/3 double mutants had elevated levels of Ihh RNA and protein compared to both Smad2$^{CKO}$ and Smad3$^{-/-}$ mice, suggesting that both Smad2 and Smad3 contribute to elevated Ihh expression. As shown previously [34], Ihh protein was found in the control Smad2$^{fx/fx}$ growth plate, with highest levels in the prehypertrophic and hypertrophic zones (Fig 3B). Ihh protein was detected in these regions in Smad2 and Smad3 single and double mutants, but unlike control Smad2$^{fx/fx}$ littermates, was also detected in the resting zones (Figs 3B, 4C and S5). This suggests that the elevated Ihh RNA level in the prehypertrophic zone leads to increased diffusion of Ihh protein to the resting zone in mutants. Patched1 (Ptch1) is a direct transcriptional target of Ihh signaling. Consistent with elevated levels of Ihh RNA and protein, immunostaining for Ptch1 demonstrated increased levels throughout the growth plates in mutants; this was most evident in the resting zones of Smad2$^{CKO}$ and Smad2/3 double mutant mice (Figs 3D and S5). Ihh is a downstream target of BMP signaling [35, 36], raising the possibility that the increased Ihh RNA expression is a consequence of increased BMP signaling in the growth plates of Smad2$^{CKO}$, Smad3$^{-/-}$ and Smad2/3 double mutant mice. However, immunohistochemical examination in P0 growth plates revealed no obvious change of pSmad1/5/8 in mutant mice compared with control Smad2$^{fx/fx}$ mice (Figs 3E and S5). In summary, the elevated level of Ihh RNA in the prehypertrophic zone, and increased domain of Ihh protein localization to the resting zone is correlated with an increase in the level and distribution of Ptch1. As Ihh promotes chondrocyte proliferation in the growth plate [37], the increased Ihh level and activity seen in Smad2$^{CKO}$, Smad3$^{-/-}$, and double mutants may contribute to the increased rate of proliferation in the resting zone in mutants.
Fig 3. Increased Ihh mRNA and protein levels and activity in Smad2 and Smad3 mutant growth plates. All images are P0 proximal tibias (A) RNA in situ hybridization for Ihh showing increased expression in Smad2<sup>CKO</sup>, Smad3<sup>−/−</sup>, and double mutant growth plates. (B) Immunostaining for Ihh protein showing diffusion into the resting zones of Smad2<sup>CKO</sup>, Smad3<sup>−/−</sup>, and double mutant growth plates. (C) Magnifications of the boxed regions in (B). (D) Immunostaining for Ptc1, showing increased protein levels throughout the growth plate in mutants, particularly in the resting zone. (E) Immunostaining for phospho-Smad1/5/8 (PS158) protein showing no significant difference in levels of activated BMP signaling comparing Smad2<sup>CKO</sup>, Smad3<sup>−/−</sup>, or double mutant to control Smad2<sup>fx/fx</sup> growth plates. Smad2<sup>CKO</sup> = Smad2<sup>fx/fx</sup>;Col2a1Cre.

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A

Relative levels of Ihh mRNA (normalized to Gapdh)

control
TGF\(\beta\)1
TGF\(\beta\)2

Smad2\(^{lox/lox}\)
Smad2\(^{lo}\)
Smad3\(^{-}\)
Smad2\(^{lo}\);Smad3\(^{-}\)

B

Relative Ihh 742-Luc activity (arbitrary units)

control
TGF\(\beta\)1

Scr
siSmad2
siSmad3
siSmad2/3

C

Relative P3TP-Luc activity (arbitrary units)

control
TGF\(\beta\)1

Scr
siSmad2
siSmad3
siSmad2/3
Direct effect of Smads 2 and 3 on Ihh promoter activity

The above findings indicate that Smads 2 and 3 act to decrease Ihh RNA levels in the neonatal growth plate. To investigate whether this effect is direct, primary rib chondrocytes were isolated, matured to the prehypertrophic phase by maintenance in chondrogenic differentiation medium, and then treated with TGFβ1 or TGFβ2. TGFβ1 and TGFβ2 decreased Ihh RNA levels in control Smad2fx/fx chondrocytes (Fig 4A). However, the ability of TGFβ to inhibit Ihh expression was impaired in Smad3-/- mutant chondrocytes, and abolished in Smad2 CKO and Smad2/3 double mutant chondrocytes. However, a caveat of these findings is that although levels of Ihh RNA in primary chondrocytes from Smad2 CKO and Smad3-/- mice were not reduced compared to control Smad2fx/fx chondrocytes under basal conditions (no serum and no growth factor addition), levels of Ihh RNA were reduced in prehypertrophic chondrocytes from Smad2/3 double mutants under basal conditions (Fig 4A). It is unclear whether this reflects a defect in the ability of Smad2/3 double mutant primary chondrocytes to undergo timely differentiation in vitro. Alternatively, Smads2/3 may play a role in maintaining basal levels of Ihh RNA. Overall however, the in vivo (Fig 3A) and in vitro (Fig 4A) results indicate that Smad2 and Smad3 are required to inhibit Ihh expression in prehypertrophic chondrocytes in the neonatal growth plate.

To test whether Smads 2/3 play a direct role in regulating Ihh promoter activity, a luciferase reporter containing the proximal 742 bp of the mouse Ihh promoter [38] was transfected into ATDC5 chondrocytic cells. After culture in differentiation medium to induce prehypertrophy, the cells were treated with TGFβ1 for 24 hours. To test whether Smad2 and/or Smad3 mediate Ihh inhibition, Smad2 and/or Smad3 levels were knocked down by transfection of verified siRNAs. Reporter assays showed that TGFβ1 inhibits Ihh promoter activity in control chondrocytes by > 50% (Fig 4B). p3TP-Luc activity was used as a positive control for TGFβ activity, and showed robust activation under the same conditions (Fig 4C). Consistent with the analysis in vivo (Figs 3A and S5), knockdown of Smad2 and/or Smad3 blocked the inhibitory effect of TGFβ on Ihh promoter activity.

Smad2 and Smad3 binding elements are required for inhibition of Ihh promoter by TGFβ

Activated Smads 2 and 3 bind to Smad binding elements (SBEs) that contain (C)AGAC motifs [39–42]. ChIP-chip/ChIP-seq studies have confirmed that the SBE is enriched in Smad2/3 binding regions. [43–47]. In silico examination of the 742bp proximal Ihh promoter identified 5 putative SBEs, designated S1 to S5 (Fig 5A). ChIP analysis performed in ATDC5 cells for Smad2 and Smad3 binding showed differential occupation of S1-S3 in the presence of TGFβ; neither Smad2 nor Smad3 associated with S4 or S5 (Fig 5B). In addition, comparative Genomic Analysis using the UCSC Genome Browser (http://genome.ucsc.edu) [48] showed that S1, S2 and S3 are 100% conserved in the mouse, rat, human and dog genomes (S6 Fig). At S1, TGFβ...
Fig 5. Smads 2 and 3 bind to distinct sites on the Ihh promoter and recruit different repressors. (A) Schematic diagram of the proximal mouse 5' Ihh promoter and enhancer. The sequences and approximate locations of 5 putative canonical SBEs sites (1)–(5) are indicated below the promoter and enhancer. 1 is defined as the transcription start site. The primer locations used to analyze the SBEs are indicated above the promoter. See Materials and Methods for primer sequences. (B) ChIP analysis of Smad2 and Smad3 binding to
increased binding of Smad2 but not Smad3 (Fig 5B). In contrast, at S2, TGFβ increased the association of Smad3, but had no effect on Smad2 binding. Association of both Smad2 and Smad3 is increased by TGFβ at S3. These results reveal 3 binding elements for pSmad2/3 within the 742 bp Ihh promoter region, and demonstrate that Smad2 and Smad3 have both common and distinct binding patterns.

To examine whether S1-S3 mediate the inhibitory effect of TGFβ on Ihh, six nucleotides covering the conserved SBE regions in S1, S2 and S3 were replaced with PsiI recognition sites in pIhh742-Luc to generate mutated constructs M1, M2 and M3, respectively. Reporter assays revealed no significant inhibitory effect of TGFβ on activity of the M1 and M3 constructs, and the inhibitory effect of TGFβ on activity of the M2 construct was decreased compared with of the control construct (Fig 5C). These results indicated that S1 and S3 exert more inhibitory function than S2.

Similar to the results in Fig 4B showing lower basal activity of the Ihh promoter in Smad2/3 double mutant primary chondrocytes, M1 and M3 exhibited decreased basal activity compared to the control Ihh promoter (Fig 5C), indicating that S1 and S3 also play a role in mediating basal activity of the Ihh promoter. The basis for the lower basal activity of the Ihh promoter is unclear. However, BMP signaling enhances Ihh promoter activity and association of Smad4 with SBEs in mouse teratocarcinoma P19 cells [35]. This raised the possibility that S1-S3 might recruit Smad4 to the Ihh promoter in response to BMPs, and that this recruitment is required for basal Ihh promoter activity. We therefore compared the activities of M1, M2 and M3 in response to BMP treatment in ATDC5 cells. We observed slightly lower basal levels of activity as in Fig 5C, but found no significant differences in BMP-mediated induction between the control and mutant promoters (S7 Fig). Together, our data indicate that S1 and S3 are important for Smad2 and Smad3-mediated inhibition of Ihh expression. We also find that S1 and S3 are important for maintaining basal levels of Ihh expression, but that this activity is not due to a role for these SBEs in mediating BMP responsiveness in chondrocytes.

### Smad2 and Smad3 associate with different transcriptional repressors on the Ihh promoter

Smad2 and Smad3 interact with a variety of DNA-binding proteins in different contexts. Hdac4 is expressed in prehypertrophic and hypertrophic zones of the growth plate and represses hypertrophy by binding to and blocking Runx2 activity [49]. Although Runx2 is a potent inducer of Ihh expression [50], whether or not Hdac4 directly regulates Ihh expression is unknown. Immunostaining confirmed that Hdac4 is present in the lower proliferative, prehypertrophic and hypertrophic zones of control Smad2fx/fx P0 growth plates, and is localized in the nucleus, as reported previously [49] (Fig 6A). The level of Hdac4 protein was greatly diminished in the growth plates of Smad2CKO and double mutant mice (Figs 6A and 8A). The percentage of cells expressing nuclear Hdac4 was significantly decreased at the border of the prehypertrophic and lower columnar zones of Smad2/3 double mutants compared to either Smad2CKO or Smad3−/− mice (8A Fig), indicating that both Smad2 and Smad3 contribute to decreased Hdac4 nuclear localization. To test whether the effect on Hdac4 expression is transcriptional, RNA levels were examined in growth plate cartilage from control Smad2fx/fx, Smad2CKO, Smad3−/−, and double
mutant neonatal mice. No significant differences were observed (S8B Fig). These results suggest that both Smad2 and Smad3 regulate Hdac4 localization and/or stability at the border of the lower columnar and prehypertrophic zones, but Smad2 has a greater impact than Smad3.

Next, we tested whether Smad2 and/or Smad3 associate directly with Hdac4 in chondrocytes. Immunoprecipitation assays revealed weak association between Hdac4 and Smad2 without TGFβ, and this association was increased by TGFβ; no association of Hdac4 with Smad3 was detected (S8C Fig). Because this analysis revealed that Hdac4 associates with Smad2, we used ChIP to test whether Smad2 and Hdac4 interact on the \textit{Ihh} promoter. TGFβ increased the level of Hdac4 associated with SBE1, the site exhibiting the highest level of pSmad2 binding; we did not detect Hdac4 binding to SBE3, in spite of the fact that Smad2 binds to this site (Fig 6B). These findings suggest that Smad2 regulates Hdac4 protein expression or stabilization, and also recruits Hdac4 to a repressive complex at SBE1 in the \textit{Ihh} promoter.

SnoN is a repressor that can be induced by TGFβ [51, 52]. It is expressed in prehypertrophic chondrocytes, and inhibits chondrocyte hypertrophy [53]. Immunostaining of P0 growth plates showed that SnoN was expressed and localized within the nucleus in prehypertrophic and hypertrophic zones in control \textit{Smad2}f/fx mice (Fig 6C). There was no obvious change in SnoN protein levels in prehypertrophic and hypertrophic chondrocytes in \textit{Smad2}CKO, \textit{Smad3}−/− and double mutant mice (Fig 6C). However, there was an increase in SnoN protein levels in the proliferative zones in all mutant strains (Fig 6C). This increase in protein levels was not due to increased levels of \textit{SnoN} RNA; in fact, \textit{SnoN} RNA levels were decreased in \textit{Smad3}−/− and \textit{Smad2}/3 mutant growth plates (S8D Fig). TGFβ signaling leads to rapid degradation of SnoN in a Smad2/Smad3-dependent manner [54]. Hence the elevated protein levels in mutants in spite of reduced mRNA levels may reflect increased SnoN stability in the absence of Smads 2 and 3.

Ski proteins can suppress TGFβ and BMP signaling by binding to R-Smads and Smad4 [55, 56]. Ski was localized to the prehypertrophic and hypertrophic zones in \textit{Smad2}f/fs mice (Fig 6D). There was no apparent change in protein levels in prehypertrophic and hypertrophic zones in any mutant strain, but there were increased levels of immunostaining in the proliferative zones in \textit{Smad3}−/− and \textit{Smad2}/3 double mutant strains. As is the case for SnoN, this effect appears to be posttranscriptional because loss of \textit{SnoN} led to reduced \textit{Ski} RNA levels in spite of the elevated protein levels (S8E Fig).

ChIP was performed to test whether SnoN and Ski might mediate the repressive effects of Smads 2 and 3 on \textit{Ihh} expression in chondrocytes. This analysis showed that TGFβ increased the association of SnoN and Ski with SBE1, SBE2, and SBE3 in the \textit{Ihh} promoter in ATDC5 cells (Fig 6E). SBE1 exhibited greater association with SnoN than with Ski, whereas more Ski than SnoN bound to SBE2; SnoN and Ski associated with SBE3 at similar levels (Fig 6E). These results parallel the differential binding of Smads 2 and 3 to these sites (Fig 5B), suggesting that SnoN and Ski may interact preferentially with Smad2 and Smad3, respectively. In addition, siRNA knockdown of \textit{SnoN} abolished the ability of TGFβ to repress \textit{Ihh}742-luc reporter activity; siRNA against \textit{Ski} and \textit{Hdac4} significantly reduced the ability of TGFβ to repress

Fig 6. Hdac4, SnoN, and Ski protein distribution in the neonatal growth plate and binding to S1-3 in the \textit{Ihh} promoter. (A) Immunohistochemical staining of Hdac4 in E18.5 proximal tibias. Bottom panel shows magnifications of the boxed regions in top panel, representing the lower columnar and prehypertrophic zones. (B) ChIP analysis of Hdac4 binding to SBE 1–3 in ATDC5 cells. (C and D) Immunohistochemical staining of SnoN and Ski in E18.5 proximal tibias. Asterisks in (C and D) indicated SnoN or Ski present in columnar zone cells. (E) ChIP analysis of SnoN and Ski binding to SBE 1–3 in ATDC5 cells. Cells in (B and E) were matured to prehypertrophy and subsequently treated with TGFβ1 (5 ng/ml) or non-treated (Control) for 24 hrs. Asterisk in (B and E), p = or < 0.05. All experiments were performed in triplicate and repeated twice. \textit{Smad2}CKO = \textit{Smad2}f/fs:Col2a1Cre.

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Ihh742-luc reporter activity (S9 Fig). Overall, the data suggest that both Smad2 and Smad3 mediate suppression of Ihh transcription by binding to distinct SBEs and associating with different repressors, but that Smad2 has a greater impact on Ihh RNA levels than Smad3.

Discussion

Canonical TGFβ and activins transduce signals through Smad2 and Smad3 [57, 58]. Smad2 and Smad3 interact with different transcriptional regulators on DNA and can bind to distinct sites [32, 58]. Although Smad2 and Smad3 have similar functions in a number of contexts [32, 59, 60], they exert distinct, and even opposing, effects in others [32, 61, 62]. Whether this is the case in cartilage was unknown. Analysis of Smad3−/− mice demonstrated previously that Smad3 suppresses chondrocyte hypertrophy [12]. The finding that overexpression of Smad2 can block the accelerated chondrocyte maturation seen in Smad3−/− chondrocytes suggested that Smad2 and Smad3 exert at least some similar functions in vitro [33]. A limitation of the previous study as well as ours is the use of global Smad3 mutants, raising the possibility that some aspects of the Smad3 mutant growth plate phenotype are due to effects on other cell types, such as the perichondrium. However, direct effects on chondrocytes seem plausible based on in vitro studies [63, 64] and our results, which document direct effects in Smad3-deficient neonatal chondrocytes. Nonetheless, loss of Smad2 appears to have a greater impact in the non-hypertrophic zone chondrocytes of the growth plate during embryogenesis than does loss of Smad3.

We found that both Smad2 and Smad3 are essential for chondrogenesis in vivo to inhibit proliferation and hypertrophy. Some of the alterations in proliferation, maturation, and gene expression were more pronounced in Smad2CKO;Smad3−/− double mutants than in single mutants, indicating that Smads 2 and 3 exert similar functions in the growth plate. This may be mediated in part by the ability of Smads 2 and 3 to repress Ihh expression. Elevated Ihh RNA levels in mutants were correlated with increased levels of Ihh protein and its direct target Ptc1 in the resting zone. Although altered matrix properties in Smad2/3 mutants may contribute to increased Ihh protein diffusion, it is likely that the elevated Ihh mRNA levels seen in these mutants are also responsible for the elevated Ihh protein levels in the resting zone and elevated Ptc1 levels throughout the growth plate. In accordance, the growth plates of mutants are lengthened at midgestation stages and P0. This is consistent with previous studies of Ihh function in the growth plate, where depletion of Ihh in prenatal cartilage caused a loss of columnar structure and dwarfism [65]. In spite of the longer growth plates at these stages, double mutants exhibit postnatal dwarfism. This can be explained if the accelerated rate of entry of resting chondrocytes into the proliferative columnar phase leads to premature depletion of the pool of resting chondrocytes and an inability to sustain growth plate elongation at postnatal stages. The precise role of Ihh in the postnatal dwarfism in Smad2/3 double mutants is unclear; postnatal loss of Ihh in cartilage leads to dwarfism [66]. On the other hand, Ihh can promote terminal hypertrophic maturation, which could lead to dwarfism, in cells outside the range of PTHrP [67, 68]. Additional studies at postnatal stages would be needed to identify the mechanism underlying the postnatal dwarfism phenotype in Smad2/3 mutants. We speculate that direct effects of Smad2 and Smad3 on genes regulating cell cycle progression may contribute.

Interestingly, the growth plate phenotype at the neonatal stage in Smad2/3 double mutants is distinct from that seen in mice lacking the type II TGFβ receptor TßRII (Tgfr2) in cartilage [21]. TßRII is required for responsiveness to all TGFßs. Tgfr2CKO mice, which were generated using the same Col2a1-Cre allele used here, exhibit defects in formation of intervertebral discs (IVD), but no apparent alterations in chondrocyte differentiation in axial or appendicular elements [21]. Obvious defects in IVD formation were not observed at birth in Smad2/3 double
mutants, but there were clear defects in the tibial growth plates. There are several possible explanations for these differences. Loss of Tgfb1r2 impacts both canonical Smad2/3 and non-canonical pathways. Hence, the axial defects seen in neonatal Tgfb1r2cko mice but not in neonatal Smad2/3 double mutants could reflect the actions of non-canonical TGFβ pathways. Furthermore, the growth plate defects at neonatal stages seen in Smad2/3 double mutants but not in Tgfb1r2cko mice could reflect a role for Smads 2 and 3 in signaling mediated by ligands other than TGFβs, such as activins [69].

Our analysis suggests that Smads 2 and 3 may regulate Ihh RNA levels by binding to distinct elements in the Ihh promoter. Candidate SBEs can be predicted in the promoter regions of many genes, but these motifs are common, and the majority of them are not occupied by R-Smad when examined using ChIP-chip/ChIP-seq [70]. We found three SBEs within the proximal Ihh promoter that bind Smads 2 and 3. These sites exhibit differential recruitment of Smads 2 and 3, and differential association with distinct co-repressors. S1 and S3 in the Ihh promoter mediate more of the repressive activity of TGFβ on Ihh expression than does S2. A caveat of this study is that the Ihh promoter sequences that regulate Ihh expression in the growth plate have not yet been identified; in vivo mutagenesis studies will be required to identify these. However, the proximal promoter and enhancer region we investigated is the most highly conserved region among 60 mammalian species (S6 Fig), and this region was shown previously to mediate Smad4 effects on Ihh expression [35] as well as the impact of multiple transcription factors on Ihh expression in chondrocytes [38, 39, 50].

Our studies revealed that Smad2 and Smad3 associate with transcriptional inhibitors Hdac4, SnoN and Ski after TGFβ stimulation. Hdac4 inhibits Runx2 activity and Ihh expression [49]; SnoN and Ski repress the transcriptional activation activities of Smad2 and Smad3 [51, 52]; SnoN and Ski also inhibit Smad1/5/8 mediated transcription activity in ATDC5 cells and W-20-17 osteoblasts [53, 55]. The studies indicate that Smads 2 and 3 act to repress Ihh promoter activity in vitro, consistent with the elevated Ihh RNA and protein levels seen in Smad2cko, Smad3−/− and Smad2/3 double mutant mice in vivo; however, they also showed that although chondrocytes isolated from Smad2/3 double mutant chondrocytes are impaired in their ability to decrease Ihh RNA levels in response to TGFβ, they also exhibit decreased basal levels of Ihh mRNA compared with control cells (Fig 4A). The reason for lower basal Ihh RNA levels in Smad2/3 chondrocytes in vitro is unclear. Combined loss of Smad2 and Smad3 may affect the ability of these cells to undergo differentiation in vitro. It is important to bear in mind that the isolated chondrocytes were maintained in the absence of added growth factors, a condition that does not mimic the intact growth plate, in order to study the direct role of TGFβ. Additional studies will be required to establish the role of Smad2 and Smad3 in basal Ihh expression. A low level of association of Smad2 and Smad3 with S1 and S3 may be required to maintain basal activity of the Ihh promoter. Evidence for this comes from the finding that basal levels of Ihh promoter activity are lower in M1 and M3 Ihh promoter constructs. However, it is also possible that these mutations have an impact on the binding of other factors that are required for basal promoter activity. Nonetheless, comparison of the effects of TGFβ vs. no growth factor reveals the importance of S1 and S3 for the inhibitory effects of TGFβ.

In summary, our studies reveal a role for Smad2 in the neonatal growth plate, and indicate that both Smad2 and Smad3 maintain the pool of resting chondrocytes in the growth plate. Future studies are warranted to investigate the function of Smad2 directly in postnatal articular cartilage.
Materials and Methods

Generation of Smad2^{fl/fl};Col2-cre and Smad2^{fl/fl};Smad3^{-/-};Col2-cre mice

Smad2^{fl/fl} mice [31] were intercrossed with the Col2a1-Cre deleter strain [71] to generate Smad2^{fl/fl};Col2a1Cre (Smad2^{CKO}) mice. These mice were intercrossed with Smad3^{-/-} mice [27] to generate Smad2^{fl/fl};Smad3^{-/-};Col2a1-Cre (Smad2/3 double mutant) mice. Primers for PCR genotyping include sense 5'-TGCTCTGTCCGTTTCCG-3' and anti-sense 5'-ACTGTGTCAGACCAGGC-3' for detecting the Col2-Cre allele, sense 5'-CCCGGTAAATCTCTACCTTAAG-3' and anti-sense 5'-TTCTAAAACATATTTGCCCAAG-3' for detecting the Smad2-floxed allele, sense 5'-GGATGGTCCGCTGGATGTC-3' and anti-sense 5'-TGTTGAAGGCAAACTCACAGAC-3' for detecting the Smad3 WT allele, sense 5'-GTTGAGATGAGCAGGGCAATACACTGCTGA-3' and anti-sense 5'-GCCACTGGTGGTGACCATAATTCAATTGC-3' for detecting the Smad3 mutant allele. Embryos and mice were on a mixed C57BL/6J/CD1 background and were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the institute's subcommittee on animal care (IACUC).

Ethics statement

This research was approved by the UCLA Animal Research Committee (ARC) under protocol 1995-018-71.

Skeletal preparation and histology

Whole mount skeletal preparations were performed as described [72]. For histological analyses, paraffin sections were produced from E16.5, P (postnatal day) 0, 2 weeks, 1 month, and 4 month-old mice. Limb tissues were dissected and fixed in 4% paraformaldehyde in PBS. They were then decalcified with Immunocal (Decal Chemical Corp., Tallman, NY, USA) for 3 days at 4°C, embedded in paraffin, and cut at a thickness of 7 μm. Sections were stained with alcian blue (Sigma-Aldrich, A5268) and nuclear fast red (Sigma-Aldrich, N8002). Safranin-O staining was performed as described (Rosenberg, 1971). Heights of proliferative and hypertrophic zones were measured directly from images (n = 5) taken from each of five mice per genotype and significance was evaluated using Student's t-test.

Immunohistochemistry (IHC), RNA in situ hybridization and TUNEL assay

Paraffin sections were deparaffinized and rehydrated by passage through xylene and 100, 95, and 70% ethanol. Endogenous peroxidase activity was quenched by incubation for 15 min in 3% hydrogen peroxide. Samples were treated with 1mg/ml hyaluronidase for 30 min at 37°C. Sections were blocked with 5% goat serum, in TBS for 1 h at room temperature and incubated with 1:100 diluted primary antibody at 4°C overnight. Antibodies used were: phospho-Smad2 (Cell Signaling, Beverly, MA, USA, #3108), PCNA (Cell Signaling, #13110), Ihh (Abcam, ab52919), Patched1 (Novus Biologicals, NB200-118), Collagen X (Abcam, ab140230), SnoN (Santa Cruz, sc-9141), Ski (Santa Cruz, sc-9140) and Hdc4 (Cell Signaling, cs-2072). For colorimetric detection, sections were treated with secondary antibodies conjugated to HRP as per manufacturer's instructions, and HRP visualized with EnzMet-TM HRP Detection Kit (Nanoprobes, Yaphank NY, #6001). Nuclei were counter-stained with fast red. For fluorescence detection, sections were treated with secondary antibody (1:500) labeled with fluorescent dyes (Cell Signaling, Goat anti-Rabbit Red:R37117, Goat anti-Rabbit Green: R37116, Goat anti-Mouse Red: A11032) at room temperature for 1 h, and nuclei were counter-stained with DAPI. Alternate sections used for IHC
analysis were used for RNA in situ hybridization with 35S-labeled probes for Ihh [38, 65]. Apoptotic cells were detected by in situ terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Sigma, #1168795910) following the manufacturer’s instructions. All experiments were repeated on sections from at least three embryos of each genotype. All comparisons were between littermates.

RNA isolation and quantitative PCR
Primary rib chondrocytes isolated from P0 mice were maintained in αMEM (Gibco, #12571) plus 10% FBS for 3 days in order to mature the cells to the prehypertrophic stage. After 4 hours of cell starvation, recombinant TGFβ1 or TGFβ2 (R&D Systems, Minneapolis, MN, USA) was added to the chondrocyte cultures at a concentration of 5 ng/ml followed by incubation in αMEM without FBS for 24 hours. Cells were then fixed in TRIzol. Total RNA was isolated by the phenol-chloroform method and converted to cDNA. The cDNA was amplified and quantified using SYBR Green reagent (Sigma) in a Stratagen-TM Mx3005P qPCR System (Thermo Scientific, USA). Primer sequences were Ihh: sense 5’-GACTCATTGCTCCAGAACTG-3’ and antisense 5’-CCAGGTAGTAGGGTCATGC-3’, Gapdh sense 5’-ACCAGGTGTTCTCCTCTGACTTCAA-3’ and antisense 5’-TACTCCTTGGAGGCCATGTTGGG-3’.

siRNA, DNA transfection and reporter assay
ATDC5 cells were plated at 1.5 × 10⁵ cells/well in 24-well plates. After 18 h, the cells were transfected with Lipofectamine. For siRNA transfection, 30 μM siRNA was added per well. Silencer Select Smad2 (s69492) and Smad3 (s69494) siRNAs and non-targeting siRNA (1193893) were from Thermo Fisher Scientific (Life Sciences, USA). For DNA transfection, 0.25 μg of pIhh742-Luc [38] (Addgene), P3TP-Luc (Addgene), and 0.025 μg of Renilla plasmids (Addgene) were added per well. 24 h post-transfection, medium was replaced with chondrogenic differentiation medium: α-MEM containing 5%FBS, 200 μg/ml ascorbic acid, 60 nm Na₂SeO₃, 10 μg/ml transferrin, 1% antibiotic. After 4-days of culture, cells were treated with TGFβ1 at a concentration of 5 ng/ml. Luciferase assays were performed 24 h later. Dual-luciferase reporter assay was performed using the Promaga kit (E1910) in a FLUOstar Omega system (BMG Labtech, Ortenberg, Germany) following the manufacturer’s instructions. Data are presented as ratios of Luc/Renilla activity from at least three different experiments and each experiment was performed in triplicate for each DNA sample. The sequence information for the 742bp Ihh construct is available in Addgene. It includes the 264bp 5’ untranslated region and 478bp of proximal promoter sequence.

Chromatin immunoprecipitation (ChIP)
ChIP for Smad2 and Smad3 was performed in ATDC5 cells that were matured to prehypertrophy by 4-days of culture in chondrogenic differentiation medium followed by treatment with or without TGFβ1 (5ng/ml) for 4 hours. ChIP was performed using a ChIP kit (Cell Signaling, #9005S) according to the manufacturer’s instructions. In brief, after cross-linking and cell lysis, chromatin was sheared by sonication to yield DNA fragments in the range of 150 to 900 bp. 90% of the DNA fragments were in the range of 150–200 bp, confirmed by gel electrophoresis. 2% of the diluted cell supernatant was kept as input material to quantify DNA content of the samples. The supernatants were immunoprecipitated overnight at 4°C with antibodies against Smad2 (Cell Signaling, cs-5339s), Smad3 (Cell Signaling, cs-9523s), SnoN (Santa Cruz, sc-9141), Ski (Santa Cruz, sc-9140) or Hdac4 (Cell Signaling, cs-2072). For a negative control a rabbit IgG immunoprecipitation was performed in parallel using the same concentration as the ChIP antibody. DNA was isolated using phenol-chloroform followed by quantitative PCR analysis. ChIP-enriched DNA was quantitated using the Stratagen-TM Mx3005P qPCR System (Thermo.
Scientific, USA) with SYBR green PCR master mix (Sigma), using the absolute quantification method, in which ChIP DNA PCRs were run alongside a standard curve of genomic DNA. PCR signals were quantitated by normalization to the total input DNA reaction and the internal intergenic control primer pair (QIAGEN, #GPM100001C(-)01A). At least three independent samples were analyzed. Primer-amplified fragments between 100 and 200 bp were centered on the Smad2/Smad3 consensus binding sites. Primer sequences are as follows: SBE1, 5’-CTAACCGGGGTCCCTTC-3’, and 5’-GCCTCGACTCTGAC-3’; SBE2, 5’-CTATTTCCCCTCTCAC-3’, and 5’-GAAGGGACCCCGCTTAG-3’; SBE3, 5’-CTTGCACGAGTTCCGCTG-3’, and 5’-CGATGAGAGGGGAAATTGGA-3’; SBE4, 5’-GGCATCTCCTGTCAGGA-3’, and 5’-CTGCTGCTGATTGTCCTC-3’; SBE5, 5’-ACACCGTGGCCTGTGTG-3’, and 5’-TCCTGGACAGGAGATGCC-3’.

**Mutagenesis**

Six nucleotides within SBE1, SBE2 or SBE3 in a luciferase construct pHh742-Luc [38] were replaced by restriction endonuclease Psil recognition sites (TTATAA) to generate three mutant constructs M1-Luc, M2-Luc, M3-Luc, respectively, using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, #200519). The primers for mutagenesis are as follows: M1-Luc, 5’-GCTTTATAACGAGGCGCCAGGGGGA-3’ and 5’-TCGTATTAAGCGCTCGCCG-3’; M2-Luc, 5’-CCGTTATAAGCAGCAGCTCCCGCTCT-3’ and 5’-TGCATTATAACGCAGCCCGGGTC-3’; M3-Luc, 5’-TGCTTATAACGGCGTCGCGCC-3’ and 5’-GGGTGATGACCTTGCTCC-3’.

**Co-Immunoprecipitation (Co-IP) and western blot**

Co-IP was performed in ATDC5 cells that were matured to prehypertrophy and treated with TGFβ1 (5ng/ml) or not, as described above. The Pierce-TM Co-Immunoprecipitation Kit (ThermoFisher, #26149) was used according to the manufacturer’s instructions. Protein concentrations were determined using the Coomassie Plus Protein Assay kit (Pierce, Rockford, IL, USA). SDS-PAGE was used to separate the protein extracts (30 μg). After transfer to a polyvinylidene fluoride (PVDF) membrane (NEN Life Science Products, Boston, MA, USA), and blocking with 5% milk, the blots were probed with rabbit anti-Hdac4 (1 μg/ml). After washing, the membrane was incubated with appropriate HRP–conjugated secondary antibody (Sigma) for 2 h at room temperature. The immune complexes were detected using ECL substrate (Pierce, Rockford, IL, USA). The blot was repeated using two independent cell preparations.

**Statistical analysis**

Statistical comparisons were made between the groups using either ANOVA or Student’s t-test as appropriate. P values of <0.05 were considered significant and are denoted in each of the figures.

**Supporting Information**

**S1 Fig. Localization of pSmad2/3 in growth plate cartilage.** (A-D) Immunohistochemical staining of E16.5 proximal tibias. (E-H) Immunofluorescent staining of P0 proximal tibias. Approximate locations of the resting, proliferative, prehypertrophic, and hypertrophic zones are indicated by brackets. Smad2CKO = Col2a1-::Cre;Smad2lox/lox. (TIF)

**S2 Fig. Subtle skeletal defects in P0 Smad2 and Smad3 mutants.** (A) Lateral views of P0 littermates demonstrating reduced crown-rump length in Smad2/3 (Smad2CKO;Smad3−/−) double mutants compared with Smad2lox/lox littermates (90.3%, p < 0.05; n = 4). (B) Ventral views of skulls.
Arrowhead, delayed ossification of the occipital condyle in double mutants. (C) Frontal (ventral) views of ribcages. Arrow highlights the shortened sternum and bifurcated xiphoid process (xp) in double mutants. (D) Ventral views of lumbar vertebrae, showing delayed ossification of the centra in double mutants (arrow). (E) C1 (atlas) and C2 (axis). Arrows show delayed ossification of the centra in Smad2$^{CKO}$ and Smad2/3 (Smad2$^{CKO}$;Smad3$^{-/-}$) double mutants. (F) Measurements of P0 tibial lengths (n = 5 per genotype) showing no significant differences. Smad2$^{CKO}$ = (Smad2$^{fx/fx}$;Col2a1Cre), S2$^{fx/fx}$ = Smad2$^{fx/fx}$, S2$^{CKO}$ = (Smad2$^{fx/fx}$;Col2a1Cre), S3$^{-/-}$ = Smad3$^{-/-}$, S2$^{CKO}$;S3$^{-/-}$ = (Smad2$^{fx/fx}$;Col2a1Cre;Smad3$^{-/-}$).

S3 Fig. Postnatal dwarfism in Smad2$^{CKO}$;Smad3$^{-/-}$ mice. (A) Cleared skeletal preparations of P7 mice showing decreased axial length in Smad2/3 (Smad2$^{CKO}$;Smad3$^{-/-}$) double mutants. (B and C) P14 and P30 littermates, showing that Smad2$^{CKO}$ and Smad3$^{-/-}$ mice are not obviously smaller than control Smad2$^{fx/fx}$ littermates, but Smad2/3 double mutants are smaller (N = 3, P < 0.05). Smad2$^{CKO}$ = Smad2$^{fx/fx}$;Col2a1Cre.

S4 Fig. Lengths of resting zones, columnar zones and hypertrophic zones in growth plates. Lengths of zones were measured directly from images (n = 5) through P0 proximal tibial growth plates of littermate mice from each of 5 mice per genotype. RZ = resting chondrocyte zone, CZ = columnar chondrocyte zone, and HZ = hypertrophic chondrocyte zone. Measurements were performed by individuals blinded to genotype. Significance was established using Student’s t-test. S2$^{fx/fx}$ = Smad2$^{fx/fx}$, S2$^{CKO}$ = (Smad2$^{fx/fx}$;Col2a1Cre), S3$^{-/-}$ = Smad3$^{-/-}$, S2$^{CKO}$;S3$^{-/-}$ = (Smad2$^{fx/fx}$;Col2a1Cre;Smad3$^{-/-}$).

S5 Fig. Increased Ihh RNA and protein levels in Smad2 and Smad3 mutant growth plate cartilage. Levels of Ihh RNA were determined by quantitative real time PCR on RNA isolated from P0 growth plate cartilage. Values were normalized using Gapdh and are plotted relative to control (Smad2$^{fx/fx}$) + SE (n = 3). Levels of Ihh, Ptc1 and pSmad1/5/8 proteins in resting chondrocyte zone were determined by counting the percentage of immunohistochemistry (IHC) staining positive cells over total cells (n = 3). Significance was established using Student’s t-test. S2$^{fx/fx}$ = Smad2$^{fx/fx}$, S2$^{CKO}$ = (Smad2$^{fx/fx}$;Col2a1Cre), S3$^{-/-}$ = Smad3$^{-/-}$, S2$^{CKO}$;S3$^{-/-}$ = (Smad2$^{fx/fx}$;Col2a1Cre;Smad3$^{-/-}$).

S6 Fig. Conservation of the SBEs in Ihh promoter. Comparative Genomic Analysis in the 5’ proximal Ihh promoter and transcription enhancer region using UCSC Genome Browser (http://genome.ucsc.edu) showed that Smad Binding Elements SBE1(CAGAGT), SBE2 (CAGAC) and SBE3(GTCTG) are 100% conserved in mouse, rat, human and dog genomes.

S7 Fig. No difference in BMP induction between control and mutant Ihh promoters. ATDC5 chondrocytes were transfected with control or mutant Ihh-promoter constructs and then treated with BMP2 (200 ng/ml) for 4 hrs. BMP2 significantly increased luciferase activities of all control and mutant constructs. There is no significant difference of BMP induction between control and mutant constructs. All experiments were performed in triplicate and repeated twice. Asterisks, p < 0.05.

S8 Fig. Smad2 and Smad3 regulate RNA and protein levels of Hdac4, SnoN and Ski in growth plate cartilage. (A) Levels of Hdac4 proteins in the nucleus of prehypertrophic
chondrocyte were determined by counting the percentage of immunohistochemistry (IHC) staining-positive cells with nuclear signal over total cell numbers in the prehypertrophic zone (n = 3). (B) Quantitative real time PCR showed Hdac4 RNA level in growth plate cartilage (n = 3). (C) Co-IP analysis of Hdac4 associating with Smad2 and Smad3 in ATDC5 cells. The western blot images of Hdac4 associated with Smad2 were quantified using Adobe Photoshop CS3. The value for TGFβ1 treatment is expressed relative to control. (D-E) Quantitative real time PCR showed SnoN and Ski RNA level in growth plate cartilages. Values in B, D-E were normalized using Gapdh and are plotted relative to control (Smad2fx/fx) + SE (n = 3). Significance was established using Student’s t-test. Asterisk in A, D-E, p < 0.05. S2fx/fx = Smad2fx/fx, S2CKO = (Smad2fx/fx;Col2a1Cre), S3−/− = Smad3−/−, S2CKO,S3−/− = (Smad2fx/fx;Col2a1Cre;Smad3−/−).

Fig. SnoN, Ski, Hdac4 are required for the inhibitory effect of TGFβ on Ihh promoter activity. 742 bp Ihh-Luc activity in ATDC5 chondrocytes matured to prehypertrophy. Cells were treated with siRNAs against SnoN, Ski and Hdac4 expression, and then treated with TGFβ1 (2 ng/ml) for 24 hrs. Scr, scrambled siRNA control. All experiments were performed in triplicate and repeated twice. Significance was established using Student’s t-test.

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