Intracellular biosynthesis of lipids and cholesterol by Scap and Insig in mesenchymal cells regulates long bone growth and chondrocyte homeostasis

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Summary statement: Conditional deletion of genes that regulate intracellular cholesterol biosynthesis in mesenchymal cells or chondrocytes shows that precise regulation of biosynthesis is required for chondrocyte homeostasis and long bone growth.

Abstract

During enchondral ossification, mesenchymal cells express genes regulating the intracellular biosynthesis of cholesterol and lipids. Here we investigated conditional deletion of Scap or Insig1 and Insig2 (inhibits or activates intracellular biosynthesis respectively). Mesenchymal condensation and chondrogenesis was disrupted in mice lacking Scap in mesenchymal progenitors, while mice lacking the Insig genes in mesenchymal progenitors had short limbs, but normal chondrogenesis. Mice lacking Scap in chondrocytes showed severe dwarfism, with ectopic hypertrophic cells, while deletion of Insig genes in chondrocytes caused a mild dwarfism and shorting of the hypertrophic zone. In-vitro studies showed that intracellular cholesterol in chondrocytes can derive from exogenous and endogenous sources, but that exogenous sources cannot completely overcome the phenotypic effect of Scap deficiency. Genes encoding cholesterol biosynthetic proteins are regulated by Hedgehog (Hh) signaling, and Hh signaling is also regulated by intracellular cholesterol in chondrocytes, suggesting a feedback loop in chondrocyte differentiation. Precise regulation of intracellular biosynthesis is required for chondrocyte homeostasis and long bone growth, and this data supports pharmacologic modulation of cholesterol biosynthesis as a therapy for select cartilage pathologies.
Introduction:

Long bones grow through endochondral ossification, a coordinated process in which mesenchymal progenitor cells condense and differentiate into chondrocytes (Kronenberg, 2003). The chondrocytes proliferate, generate columnar structures, and undergo hypertrophy, ultimately resulting in new bone formation and longitudinal growth. Cholesterol plays an important role in skeletal development, as inborn errors of metabolism inhibiting cholesterol synthesis are associated with skeletal dysplasias (Rossi et al., 2015). Experimental treatment with inhibitors of cholesterol biosynthesis also induce patterning defects including pre-axial syndactyly or post-axial polydactyly (Gofflot et al., 2003), and reduced tibial growth plate height (Wu and De Luca, 2004). However, inborn errors of metabolism, and pharmacologic inhibitors of cholesterol biosynthesis, alter cholesterol systemically in multiple cell types, confounding an understanding of the role of cholesterol synthesis in specific cell types. In addition to the skeletal manifestations of low cholesterol, high levels are associated with osteoarthritis, a form of cartilage degradation (Farnaghi et al., 2017). Hedgehog (Hh) signaling plays an important role in chondrocyte development and homeostasis (Lin et al., 2009). High levels of Hh signaling are associated with articular cartilage degeneration, and Hh signaling in these chondrocytes regulates intracellular cholesterol biosynthesis (Ali et al., 2015).

Intracellular cholesterol biosynthesis is regulated by proteins in the endoplasmic reticulum (ER) including sterol regulatory element-binding proteins (SREBPs), SREBP cleavage-activating protein (SCAP), and Insulin-induced gene protein (INSIG). SCAP forms a complex with SREBP and functions as a sterol sensor (Brown and Goldstein, 1999). When cholesterol levels are low, SCAP escorts SREBP to the Golgi where proteases cleave SREBP to release the N-terminal domain of SREBP to traffic to the nucleus. In the nucleus, SREBP activates target genes for the biosynthesis of cholesterol. Conversely, when intracellular cholesterol levels are high, INSIG proteins prevent cholesterol biosynthesis by tethering SREBP and SCAP complex to the ER membrane. The deletion of
Scap inhibits cholesterol production in the involved cells. There are two INSIG proteins with functional redundancy. Deletion of both Insig1 and Insig2 increases intracellular cholesterol biosynthesis. The relationship between systemic cholesterol levels and intracellular biosynthesis is complex. Plasma levels may not be related to intracellular levels, or intracellular levels to intracellular biosynthesis activity (August et al., 2007; Dietschy, 1998; Liscum and Underwood, 1995).

To elucidate the role of intracellular cholesterol biosynthesis within mesenchymal cells and chondrocytes in skeletal development, we focused on intracellular regulators of cholesterol biosynthesis using transgenic mice. Here we analyzed Scap deficient mice and Insig1 and 2 deficient mice to study the role of intracellular cholesterol and lipid production in mesenchymal progenitor cells and chondrocytes. We found that cholesterol and lipid intracellular biosynthesis is a critical process in chondrocyte development and homeostasis.

Results:

**Scap is required for normal mesenchymal condensation.**

We first examined the expression of Scap in in mesenchymal precursors. Studies of microdissected limb buds showed that Scap is expressed throughout embryonic development (Fig 1A). To determine the effect of intracellular cholesterol and lipid biosynthesis in mesenchymal precursors, Scap was depleted in early limb bud mesenchyme by crossing Scapoi mice with Prx1-Cre mice. Prx1 is expressed in limb bud cells that give rise to mesenchymal cells (Logan et al., 2002). The limb buds of mice lacking Scap were shorter and contained smaller mesenchymal condensations than controls (Fig. 1B, C, G). A hematoma was observed in many of the forelimbs. Later in development, there was severe forelimb shortening, without normal digit separation (Fig 1 D, E, F). At E18.5, the differences became more apparent with arrested forelimb development. While the hind limbs were not as severely affected,
they were also shorter than controls (Fig 1 F). At P0, both the fore limb and hind limb showed a very small area of mineralization. Histologic analysis confirmed the changes observed in the skeletal preparations (Fig. 1G).

**Scap regulates mesenchymal cell proliferation and differentiation to chondrocytes.**

Embryonic limbs from *Scap* deficient mice contained fewer mesenchymal cells and there was decreased chondrogenesis. To determine if this observed phenotype was due to intrinsic changes in cells differentiating to chondrocytes, to the chondrocytes themselves, or to both we undertook micromass cultures. These cultures from *Scap* deficient cells showed less alcian blue staining (Fig. 2A), consistent with a differentiation defect. Expression analysis showed that *Acan*, *Col2a1*, and *Sox9* were strongly down regulated in the mutant limbs (Fig. 2B). Decreased cell proliferation, increased apoptosis, or both might also explain the observed limb phenotype. BrdU staining in the limbs showed a reduction in the number of positively stained cells in mutant mice (Fig. 2C). TUNEL positive and cleaved caspase 3 positive cells existed in interdigital spaces at E12.5 in wild type mice, but TUNEL positive and cleaved caspase 3 positive cells were noted throughout the limb in mutant animals.

Western analysis also showed Cyclin D1 was strongly down regulated in the mutant limbs and cleaved caspase-3 and Bax was upregulated in in mutant limb (Fig. 2D and E). Thus, *Scap* is required for multiple processes necessary for enchondral growth including differentiation to chondrocytes, the maintenance of cell proliferation, and the prevention of ectopic apoptosis.

**Loss of Scap in chondrocytes results in a disordered growth plate.**

We next examined *Scap* expression in growth plate chondrocytes. Immunofluorescent staining and *in situ* hybridization from E16.5 embryo distal femurs showed that Scap protein was expressed in round/resting cell zone (RZ) and proliferation zone (PZ), but its level of expression was lower in the hypertrophic zone (HZ) (Fig. 3A). To confirm the changes in *Scap* expression during chondrocyte
hypertrophy, we microdissected growth plate cells into the PZ and HZ and extracted mRNA. Scap expression, as well as multiple other genes involved in cholesterol biosynthesis, were decreased in HZ chondrocytes (Fig. 3B). Cholesterol levels were also lower in the HZ cells (Fig. 3C). Micromass cultures showed that Scap expression was decreased as chondrocytes differentiated as well (Fig. 3D). The results of Western analysis were consistent with that of QPCR (Fig. 3E).

To determine the chondrocyte autonomous role of Scap in-vivo, we generated mice in which Scap\textsuperscript{0/0} conditional deletion was driven by the regulatory elements of type II collagen (Fig. 4A). Scap deficient fetal mice were shorter than control littermates, had a foreshortened snout, a rounded skull, a short tail, severe dwarfism of the limbs, and protruding abdomens. The shortening of limbs was not observed at E12.5, but was obvious at E15.5 (Fig. 4B and C). Whole mount staining showed that the Scap deficient mice had a primitive ribcage, a shorter sternum and shorter ribs, with delayed mineralization of the vertebrae. Mice lacking both Scap alleles within chondrocytes died at birth. Examination of the growth plates at E16.5, showed less primary ossification, with a disorganized round cell zone (Fig. 4D and E). There was a disrupted columnar structure in proliferation zone, and a reduced number of hypertrophic chondrocytes (Fig. 4E). The hypertrophic zone was small and disordered, and there was decreased expression of the hypertrophic marker, Type X Collagen (Fig. 4F and G). The proportion of BrdU incorporating cells was lower (Fig. 4H). There was also decreased expression of genes expressed by chondrocytes, such as Acan, Col2a1, and Sox9 (Fig. 4I).

The phenotype of Scap deficient chondrocytes cannot be fully rescued by exogenous cholesterol

To investigate the relative contribution of intracellular cholesterol synthesis and extracellular cholesterol, we first analyzed serum and intracellular cholesterol levels in the mice lacking Scap in Col2a1 expressing cells. There was not a difference in serum cholesterol level between Scap deficient and control mice (Fig 5A). We then examined whether exogenous cholesterol could reverse the
changes in primary chondrocyte cultures from mice lacking Scap in collagen two expressing cells. Treatment of control chondrocytes with exogenous cholesterol increased intracellular levels, but reached a threshold at 2 ug/ml. Treatment with 3ug/ml was thus used for the remainder of the studies. The level of intracellular cholesterol in Scap deficient chondrocytes did not reach that of controls (Fig. 5B). Filipin is a naturally fluorescent polyene antibiotic that binds to unesterified cholesterol (Bornig and Geyer, 1974). Staining for filipin was substantially decreased in cells lacking Scap expression. Treating cells with cholesterol increased staining, but not to the level observed in cells expressing Scap. While the amount of staining increased, the intracellular localization of cholesterol did not differ between exogenous and endogenous sources, and staining was located primarily in the cytoplasm. (Fig. 5C). Interestingly, Ldlr expression is low in chondrocytes lacking Scap (Fig. 5D), and the product of this gene can regulate the ability of cells to uptake extracellular cholesterol(Brown et al., 1983; Zhang et al., 2016). Thus, we examined if overexpression of Ldlr could increase intracellular cholesterol level. By overexpression Ldlr, the level of intracellular cholesterol approached control cell levels with exogenous cholesterol supplementation (Fig. 5E). While exogenous cholesterol increased the expression of Acan, Col2 and Sox9 expression in control mice, in mutant chondrocytes, exogenous cholesterol did not significant alter expression of these genes. However, in Scap deficient cells in which Ldlr was also overexpressed, the level of expression of these genes increased, but not nearly to the level observed in cells expressing Scap (Fig. 5F). These results suggest that exogenous cholesterol is insufficient to maintain chondrocyte homeostasis in the absence of either intracellular biosynthesis, or overexpression of Ldlr. Our data is also consistent with the notion that overexpression of Ldlr is not as effective as intracellular biosynthesis in the maintenance of chondrocyte physiologic function.
Deletion of *Insig* genes cause dwarfism.

Since a lack of intracellular cholesterol production caused such a dramatic phenotype, we examined if overproduction of intracellular cholesterol affected skeletal development. *Insig1* and *Insig2* (Engelking et al., 2005) encode proteins with redundant functions. Reduction of both Insig proteins, leads to an increase in nuclear SREBPs and production of cholesterol. While the articular chondrocyte phenotype of mice lacking both *Insig* genes has been previously reported (Ali et al., 2016), the developmental phenotype has not been analyzed. *Insig1*^{fl/fl}; *Insig2*^{−/−}; *Prx1cre* mice were generated and compared with control mice. Mutant mice were viable and showed dwarfism and a cleft palate (Fig. 6 A, to D), and exhibited postnatal lethality. Next, we compared the phenotype of *Insig1*^{β/}; *Insig2*^{−/−}; *Col2cre* with that of *Insig1*^{β/}; *Insig2*^{−/−} control mice. The length of Type X collagen staining was decreased in mice lacking both *Insig* genes in chondrocytes (Fig 6 E to I). The mutant mice had shorter limbs than that of controls. While a lack of intracellular cholesterol and lipid biosynthesis had a more robust phenotype that increasing cholesterol levels, we found a subtle phenotype, illustrating that intracellular cholesterol biosynthesis needs to be maintained at an optimal level for normal chondrocyte function.

**Hedgehog signaling and intracellular cholesterol production regulate each other during chondrocyte differentiation.**

Our data shows that intracellular cholesterol needs to be maintained at an optimal level for normal growth plate chondrocyte differentiation and the same holds true for hedgehog signaling. Since hedgehog signaling and intracellular cholesterol biosynthesis cholesterol can interact on several levels (Luchetti et al., 2016; Porter et al., 1996; Xiao et al., 2017), this raises the possibility that they could act in part through a feedback loop regulating chondrocyte differentiation. Ihh and its target genes were examined in primary chondrocytes from *Scap* or *Insig* deficient mice. There was a significant decrease in the expression of *Ihh* and its target genes, *Gli1* and *Ptch1*, in *Scap* deficient limbs, while expression
of the Hh regulated genes were upregulated in \textit{Insig} deficient limbs (Fig. 7A). Since cholesterol can modify Hh ligands or regulate its intracellular signaling, we examined primary chondrocyte cultures from Scap deficient or control littermate mice. Treatment with 3ug/ml exogenous cholesterol slightly increased Hh target genes in \textit{Scap} deficient cells, but had no effect on cells from littermate controls (Fig. 7B). This suggests that the differences in of Hh signaling levels between \textit{Scap} deficient, \textit{Insig} deficient, and control littermate cells are more likely due to intracellular signaling changes than to extracellular changes in how cholesterol modifies Hh activity. Treatment with hedgehog ligand increased the cholesterol level, while treatment with cylopamine decreased the cholesterol level in organ cultures from E16.5 metatarsal bones, but there was little change in cholesterol level in limbs lacking \textit{Scap} (Fig. 7C). Next, we examined how Hh activation and cholesterol biosynthesis might interact in the regulation of chondrocyte differentiation. In control explant cultures, treatment with Hh ligand results in a smaller zone of type X collagen expression, a marker of hypertrophic chondrocytes. In contrast, this regulation is lost with treatment with Hh ligand in explants from limbs lacking \textit{Scap} in chondrocytes (Fig. 7D and E). To determine the role of Hh signaling in the phenotype of \textit{Scap} deletion, \textit{Scap}^{floxflox}; \textit{Col2a1-Cre} mice were crossed with \textit{Tg(Gli2; Col2a1)} mice, in which the Gli2 mediated Hh transcriptional activator is overexpression in \textit{Col2}-expressing cells. Since Gli2 is not efficiently processed to a repressor form, this constitutively activates Hh signaling downstream of Hh ligand activation (Hopyan et al., 2002). The overexpression of Gli2 partially rescued the short limb phenotype associated with \textit{Scap} deficiency in chondrocytes, with a 27% increase in upper limb length and a 23% increase in lower limb length at E16.5 (Fig. 7F). Type X collagen expression in the E16.5 metatarsals was rescued by overexpression of Gli2 (Fig. 7G and H). Filipin staining and cholesterol levels showed that Gli2 overexpression increased cholesterol levels (Fig 7I and J). Interestingly, there is an increase in cholesterol levels in cells lacking \textit{Scap} with \textit{Gli2} overexpression. One possibility is that Gli2 expression would increase cholesterol uptake, and indeed, \textit{Ldrl} expression is increased in these cells (Fig. 7K).
Discussion:

Here we demonstrate the importance of genes regulating intracellular cholesterol and lipid biosynthesis in mesenchymal and chondrocyte differentiation. Mice lacking Scap in mesenchymal precursors showed reduced mesenchymal condensation and differentiation to chondrocytes, associated with reduced proliferation and increased apoptosis. Moreover, a balance in intracellular cholesterol and lipid synthesis is critical to maintain normal chondrocyte differentiation, as illustrated by the phenotype observed in both Scap and Insig deficient animals. Interestingly, the mice have a normal serum cholesterol level and in-vitro studies showed that there was only a mild rescue of the Scap deficient phenotype with exogenous cholesterol treatment, even when intracellular levels reached that of controls. Thus, our data show that cell autonomous intracellular cholesterol and lipid biosynthesis is critical for normal enchondral ossification and maintenance of chondrocyte homeostasis.

Intracellular cholesterol and lipid synthesis regulates the differentiation, proliferation, and apoptosis of undifferentiated mesenchymal cells in the developing limb. In the growth plate, Scap expression and intracellular cholesterol levels decrease with differentiation. Insig1 is also differentially regulated during enchondral ossification (Li et al., 2016). As such, cholesterol and lipid biosynthesis regulate limb development and enchondral bone growth at multiple stages during limb development. LDL receptors provide a mechanism for delivery of cholesterol to cells (Brown et al., 1983). Ldlr expression is low in hypertrophic chondrocytes, and in Scap deficient chondrocytes. When Ldlr is over expressed, intracellular cholesterol levels can reach that observed in chondrocytes expressing in Scap. Intriguingly, however, this is insufficient to maintain expression of genes important in chondrocyte homeostasis. Thus, our data is also consistent with the notion that intracellular cholesterol in chondrocytes can derive from exogenous and endogenous sources, but that exogenous sources alone cannot overcome the phenotypic effect of Scap deficiency.
Previous work shows that Hh signaling regulates genes important for intracellular cholesterol and lipid synthesis in chondrocytes (Ali et al., 2016; Wu and De Luca, 2004). Furthermore, cholesterol can regulate Hh signaling at multiple levels in its signaling cascade, from ligand processing to modification of receptors and intracellular transducers of transcriptional activity (Huang et al., 2016; Luchetti et al., 2016; Porter et al., 1996). We found that Scap expression also regulates Hh transcriptional activity in chondrocytes. With differentiation, cholesterol and lipid biosynthesis decreases, associated with Hh signaling inhibition. This finding and our explant data is consistent with the notion that Hh and cholesterol biosynthesis regulate each other during chondrocyte differentiation. The addition of Hh did not completely rescue the Scap-deficient phenotype, while activation of Gli did rescue the Scap-deficient phenotype. In addition, exogenous cholesterol is not as effective in regulating Hh signaling as modulating intracellular biosynthesis. While our findings are consistent with cholesterol modulating Hh signaling at multiple levels in the Hh signaling cascade, it suggests that Scap acts primarily intracellularly to transmit the Hh signaling to the Gli transcription factors. Cholesterol also regulates other signaling pathways, such as Wnt signaling (Sheng et al., 2014) that can regulate chondrocyte differentiation (Hulce et al., 2013). Thus, there are also multiple mechanisms by which cholesterol and Hh might interact in regulating differentiation during enchondral ossification.

The major site of cholesterol production is the liver (Turley, 2004). Yet mice lacking Scap in hepatocytes are relatively healthy (McFarlane et al., 2015). We found that mice lacking Scap in mesenchymal cells do not survive after birth. Our results in mesenchymal cells and chondrocytes are reminiscent to that found in the intestine, where depletion of Scap causes severe enteropathy and death (McFarlane et al., 2015). The proliferation and differentiation of chondrocytes requires multiple signaling pathways, and cholesterol and lipid biosynthesis can regulate many of these
pathways (Incardona and Eaton, 2000; Mann and Beachy, 2000; Mann and Beachy, 2004). While exogenous cholesterol can cause changes in chondrocytes expressing Scap, this capacity is lost in chondrocytes lacking Scap. This suggests that the proliferation and differentiation of chondrocytes requires high levels of intracellular cholesterol and lipids that cannot be completely compensated for exogenously. The decreased levels of Scap in cells near terminal differentiation of the growth plate is consistent with the notion that these cells are slowing their metabolism and do not require such high levels of cholesterol and lipids. Furthermore, the Hh regulation of Scap expression raises the possibility that this signaling pathway maintains a balance of high cholesterol synthesis in the less differentiated cells, and as Hh activity declines, so does intracellular cholesterol biosynthesis.

Cholesterol biosynthesis can be pharmacologically targeted, and as such represents a therapeutic opportunity. Our data showing dwarfing with either activation or inactivation of this biosynthetic pathway is consistent with the notion that its level needs to be precisely regulated for normal bone growth. Such a notion is consistent with being part of a feedback loop regulating the pace of cell differentiation. Interestingly, such modulation was shown to play a role modulating endochondral growth in achondroplasia (Yamashita et al., 2014). While we do not have data on achondroplasia, it is intriguing to speculate that cholesterol level is deregulated in achondroplasia. This could explains how pharmacologic manipulation of cholesterol synthesis improves bone growth in this condition. It is also possible that other conditions associated with abnormal bone growth might be treated in a similar manner. There are neoplastic processes, such as enchondromas and chondrosarcomas that can arise from growth plate chondrocytes. The inhibition of cell proliferation by modulation of this biosynthetic pathway raises the possibility that pharmacologically targeting cholesterol synthesis could be developed into an effective therapeutic approach.
Methods:

Experimental animals

Scap^{flox/flox} (B6;129-Scap^{tm1Mbjg}J) (Matsuda et al., 2001) and Insig1^{flox/flox}; Insig2^{-/-} (B6;129S6-Insig1^{tm1Mbjg} Insig2^{tm1Mbjg}J) mice (Engelking et al., 2005) were obtained from Jackson laboratory. Scap^{flox/flox} mice were crossed with Col2a1-Cre mice producing mice conditionally lacking one or both alleles of Scap. Similarly, Scap^{flox/flox} mice were crossed with Prx1-Cre mice. Likewise, we generated Insig1^{flox/flox}; Insig2^{-/-}; Col2-cre and Insig1^{flox/flox}; Insig2^{-/-}; Prx1-cre. We also used Tg(Gli2;Col2a1) mice, which are characterized by the expression of the Hh-activated growth factor, Gli2 in growth plate chondrocytes driven by the regulatory elements of Col2(Hopyan et al., 2002). These mice were crossed with Scap^{flox/flox};Col2a1 mice to generate Scap^{flox/flox};Col2a1-Cre;Tg(Gli2;Col2a1) mice. All of the mice were on a B6 background and equal numbers of male and female embryos examined. Mice were analyzed with whole mount staining using Alcian blue and Alizarin red, histology using Hematoxylin and Eosin (H&E) staining, Safranin O staining and immunohistochemistry. All animals were performed according to the approved protocol by Institutional Animal Care and Use committee of Duke University.

Micromass cultures of Limb mesenchyme

Mouse embryos E12.5 are harvested and fore and hind limb buds collected. Limb buds were incubated in 0.25% Trypsin at 4°C for 1 hour. Cells were placed (1 x 10^5 cells) in the center of culture dish and allowed cells to attach for 3 hours. DMEM F/12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA) and 1% penicillin and streptomycin were gently added to the plate. Cells were incubated for various time periods, after which expression analysis or Alcian Blue staining was performed.
Analysis of gene expression

Total RNA was extracted from cells or tissues using RNA easy mini kits (Qiagen) according to the manufacturer’s instructions. Total RNA was reverse transcribed in PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio USA, Mountain View, CA) to make single-stranded cDNA. Quantitative real-time RT-PCR (Biorad, Hercules, CA) was performed using SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Bio). Analysis of gene expression was performed using the $\Delta\Delta^\text{Ct}$ method. Data were normalized to expression of the HPRT mRNA levels. Each experiment was performed in triplicate.

Western Blotting

Proteins were extracted from cultured cells using RIPA Lysis and Extraction Buffer (Pierce, Rockford, IL) with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). The samples were centrifuged at 14,000 rpm during 15 min at 4°C. BCA colorimetric method was performed to check the concentration of the proteins. Twenty-Microgram protein samples were electrophoresed in NuPAGE 4–12% Bis-Tris gel electrophoresis (Invitrogen). The gels were transferred to Immune-Blot PVDF Membrane (Biorad) in Tris/glycine buffer (pH 8.3) containing 20% methanol. After blocking nonspecific binding sites with 4% nonfat milk or 4% BSA in 0.1% Tween 20/phosphate buffered saline or Tris buffered saline (PBS-T or TBS-T) for 1 hour. The membranes were treated with primary antibody against polyclonal rabbit anti Scap antibody (Thermo Scientific, Waltham, MA) diluted 1:200, polyclonal rabbit anti SREBP2 antibody (abcam, Cambridge, England) diluted 1:400, rabbit polyclonal anti cyclin D antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1000, polyclonal rabbit anti cleaved caspase 3 antibody (CST) diluted 1:1000 and polyclonal rabbit anti Bax antibody (CST) diluted 1:1000 in blocking buffer
at 4°C overnight. After washing, horseradish peroxidase–conjugated secondary antibody (BioSource International, Chicago, IL) was added for 1 hour at room temperature. The immune reactive blots were detected using ECL Plus (Amersham Pharmacia Biotech).

**Immunohistochemistry**

Samples were fixed in 10% neutral buffered formalin or 4% paraformaldehyde and performed decalcification in formic acid or 14% EDTA. Antigen retrieval was performed by Proteinase K for 15min. Endogenous peroxidase activity was blocked by DAKO (Agilent Technologies, Santa clara, CA) kit for 30 minutes. The specimen was placed on 2% goat serum in PBS-T for blocking for 30 minutes and then incubated overnight at 4°C with primary anti-SCAP antibody (Thermo Scientific) diluted 1:200, anti-sox9 antibody (Merck Millipore, Billerica, MA) diluted 1:1000,  anti-Col10 (LSL Cosmo Bio, Tokyo, Japan) diluted 1:800, or anti-MMP13 (Thermo Scientific) diluted 1:1000, respectively. Finally, the samples were counterstained with hematoxylin.

**Proliferation and apoptosis analysis**

DNA synthesis of cells was assayed by bromodeoxyuridine (BrdU). Pregnant females were intraperitoneally injected with BrdU 100mg /Kg. A BrdU Staining Kit (Invitrogen, Camarillo, CA) was used for analysis. Detection of apoptosis was performed by TUNEL staining using the In situ Apoptosis Detection Kit (Takara Bio USA) according the manufacture’s protocol.

**Primary culture of costal chondrocytes from mice**

E18.5 and P3–P5 neonatal pups were used for primary chondrocytes as described previously (Gosset et al., 2008). The sternum and ribs were washed with PBS and digested in 15 ml of 2 mg/ml pronase (Roche, Basel, Switzerland) at 37 °C for 1 h with constant agitation. Ribs were washed three times with
PBS and digested in 3 mg/ml of collagenaseD (Plaisant et al.) in a 37 °C humidified cell culture chamber for 1 hour. Fresh collagenase D was transferred the ribs to a petri dish and Incubate at 37 °C in a humidified cell culture chamber for 4–6 total hours, and filtered using a 45 μM cell strainer.

**Ihh treatment and overexpression of Ldlr.**

To overexpress Ldlr, cells were infected with Lenti-CMV and Lenti-CMV-Ldlr virus (LDLR Lentivirus, Mouse, from Applied Biological Materials Inc., LVP536261). After 24 hours, the cells were treated with puromycin and then studied with and without cholesterol (water soluble, Sigma C4951) added to the media for 24 hours. IHH recombinant protein (1705-HH-025 R&D systems) was used at a concentration of 250 ng/ml, as in prior studies (Veistinen et al., 2017).

**Metatarsal Organ cultures**

E16.5 mouse embryos were prepared. Three central metatarsal bones (2nd, 3rd and 4th digit) were dissected from the hind limbs of the embryos and were placed in 24-well plates in 300 μl of α-MEM (Invitrogen) supplemented with 50 μg/ml ascorbic acid, 1 mM beta-glycerophosphate, and 0.2% bovine serum albumin. Explants were grown at 37°C in a humidified 5% CO2 incubator. The medium was changed every 2 days. The cultured rudiments were harvested on day 5 and then fixed in fresh 10% neutral buffer formalin (NBF) overnight at room temperature. Each metatarsal bone obtained from identical mouse embryos were transfected with adenovirus vectors expressing SCAP or GFP control and cultured at 37 °C in a humidified 5% CO2 incubator for 4 days. Safranin O and immunohistochemical staining was performed.
Cholesterol supplementation and intracellular cholesterol level analysis.

To supplement media, between 1 an 4 ug/mg of cholesterol (Sigma-Aldrich) was added to the media as previously reported (Corwin et al., 1977). Cholesterol levels were analyzed using Ultraperformance Liquid Chromatography /Electrospray Ionization/Tandem Mass Spectrometry (UPLC/ESI/MS/MS) as previously reported (Ayciriex et al., 2012). 110 uL of PBS were added to sonicated samples. Extracts were spiked with an internal standard in MeOH and saponified with 0.9 M NaOH. Saponified material was extracted with hexanes, derivatized with DMAPi in DMF, quenched, and re-extracted with hexanes. Cholesterol level was measured by a targeted UPLC-MS/MS method. (Ayciriex et al., 2012). Semi-quantitative values are reported for cholesterol in micromole per gram concentrations (umol/g). Filipin staining detects intracellular cholesterol and lipids and was undertaken as previously reported (Bornig and Geyer, 1974).

Statistical analyses

For in vitro investigations, nonparametric comparisons were performed using the Mann-Whitney U-test. P-values less than 0.05 were considered significant.
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Competing Interests:

No competing interests declared.
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Figure 1: Phenotype of mouse embryos lacking \textit{Scap} in mesenchymal cells

A) RT-PCR data for \textit{Scap} expression in microdisected limb buds from embryos at different stages showing that \textit{Scap} is expressed during multiple stages of limb development (n=5 for each time point, Means and 95% confidence intervals are shown). B to F) Representative phenotype of embryos and mice in which \textit{Scap} is inactivated in \textit{Prx1} expressing cells. B) E11.5 embryos. b, d, g, and h are embryos lacking \textit{Scap} in \textit{Prx1} expressing cells, while the remainder of the images are controls. a, b, e,
and g are images of the embryos c, d, f, and h are backlight photographs of the embryos. e to h are magnified views of the forelimb bud. The limb bud from embryos lacking Scap is rounder in shape and contains a small hematoma compared to controls (n= 8 for mutants, 7 for controls). C) E13.5 embryos, showing a hematoma in the limb bud in embryos lacking Scap in Prx1 expressing cells. 3 of 11 embryos showed a phenotype as in image d with a large hematoma encompassing the entire limb bud (n= 6 for mutants, 8 for controls). D) E16.5 embryos. a and b are side views, c and d front views, and e and f skeletal preps. There is a short limb with malformed skeletal elements, more severe in the forelimbs (n= 7 for mutants, 6 for controls). E) E 18.5 embryos. c, d, g, and h show magnified views of the upper (c, g) and lower (g, h) extremities (n=6 for mutants, 7 for controls). F) Mice at P0. A magnified view of the upper extremity is shown in e and the lower in f (n=4 for mutants, 6 for controls). G) Histologic images from E11.75 to E16.5 limbs. 11.75 embryos are shown in a, b, and c. Low (b) and high (c) magnified views of the forelimb bud from an embryo lacking Scap in Prx1 expressing cells showing disorganized mesodermal differentiation. E12.5 embryos are shown in d and e, showing the formation of a vascular cyst in the limb bud from an embryo lacking Scap in Prx1 expressing cells in e. E13.5 embryos are shown in f, g, and h. A large cyst was found in 4 out of the 6 as shown in g, while in 2 there was an organized hematoma as shown in h. The humerus at E16.5 (i and j) and the tibia (k and l) showing a substantial lack of cartilage in the limbs from embryos in which Scap is inactivated in Prx1 expressing cells, and a hematoma at the distal aspect of the forearm.
Figure 2: *Scap* regulates mesenchymal cell proliferation and differentiation.

A) Representative Alcinian Blue staining from micromass cultures showing decreased glycosaminoglycan production in limbs from mice showing inactivation of Scap in Prx1 expressing cells (n=5 mutant and 5 controls). B) Relative RNA expression comparing micromass cultures lacking *Scap* in *Prx1* expressing cells with controls, showing decreased expression of markers of chondrogenesis at 10 days (n=6 mutant and 6 controls). Means and 95% confidence intervals are shown. C) BrdU uptake in limbs. Graphical representation of means and 95% confidence intervals on the lower panel, n=6 for each time point and condition. D) *Scap* deficient mice contained TUNEL
stained cells positive cells and cleaved Caspase-3 in the central regions of the developing bone, while TUNEL stained cells were restricted to the margins of digital rays (n=6 for each genotype). Graphical representation of means and 95% confidence intervals on the lower panel, n=6 for each, p<0.05 for each time point and condition. E) Representative Western blot for Cyclin D1, Caspase-3 and Bax. An asterisk shows a significant (P<0.05) difference.
Figure 3: Scap expression and cholesterol level is decreased in hypertrophic chondrocytes.

A) Immunofluorescent staining and in-situ hybridization from E16.5 embryos showed that Scap protein was expressed in round cell zone (resting) and proliferation zone, but decreased in hypertrophic zone. Left panel is immunoflourescent staining, middle panels are magnified views of the three zones, and the right panels shows an in-situ hybridization. B) RT-PCR data from microdisected regions of the proliferating zone (PZ) and hypertrophic zone (HZ) of growth plate showing differential regulation of Scap and other genes involved in intracellular biosynthesis of cholesterol and lipids (from E16.5 limbs, n=8 in each group). Black represents controls, and gray represents cells from limbs lacking Scap. Scap expression is also shown in panel B. C) Cholesterol levels in the proliferating zone (PZ) and
hypertrophic zone (HZ) of growth plate (n=4). D and E) Scap expression micromass cultures also showed that Scap expression was gradually decreased over time as cells progressed through chondrocytic differentiation, E shows RNA data (n=8), and E a representative Western blot. Means and 95% confidence intervals are shown. An asterisk shows a significant (P<0.05) difference.
Figure 4: Loss of Scap in chondrocytes results in a disordered growth plate and severe dwarfism.

A) Immunohistochemistry for SCAP (left) and filipin fluorescent staining (right) for control (top) and limbs from mice lacking Scap in Col2a1 expressing cells (bottom), confirming lack of SCAP expression and decreased intracellular cholesterol and lipids in mutant mice.  B and C) Whole mount and skeletal preps of representative E12.5 to P0 mice.  Top rows show mice expressing Scap in Col2a1 expressing cells, and the bottom rows show mice lacking Scap in these cells (n> 5 for each genotype at each age).  Scale line is 4mm long. P0 mice show images of the spine and rib showing a lack of spinal

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cartilage development and severely shortened long bones. D) Histologic sections of fetal limbs of E16.5 humerus sections with control limbs in right panel, limbs from a mouse lacking *Scap* in one allele in the middle, and limbs from a mouse lacking *Scap* in both alleles in *Col2a1* expressing cells on the left. E) Magnified views of 16.5 humeri showing views of the resting, proliferating, and hypertrophic zones. The mutant limb demonstrates ectopic hypertrophic cells. F) Alcian blue staining of the upper limb. G) Type X collagen staining of the upper limb. H) BrdU incorporation, with the limbs from mice lacking *Sacp*. I) Expression of various genes in the mutant and control limbs. Means and 95% confidence intervals are shown. An asterisk shows a significant (P<0.05) difference.
Figure 5: Exogenous cholesterol does not change the phenotype of chondrocytes lacking Scap in Col2 expressing cells.

A) Serum cholesterol levels in mice lacking Scap in chondrocytes at P0, showing no difference between serum cholesterol levels between Scap deficient and control mice (n=5 in each group). B) Intracellular cholesterol levels by mass spectroscopy in chondrocyte cultures from Scap deficient and control mice (n=8 at each time point for each genotype). Cholesterol levels increase with cholesterol supplementation, but in Scap deficient cells this did not reach the same level as observed in control cells. C) Filipin staining showing treatment with cholesterol increases staining in cultures, but cholesterol supplementation cannot increase the levels in Scap deficient cells to that observed in
controls. High power view shows intracellular localization. Cholesterol is primarily located in the cytoplasm in these cells, regardless of the genotype. D) Overexpression of \textit{Ldlr} in \textit{Scap} deficient chondrocytes increases intracellular cholesterol. E) RT-PCR for expression of \textit{Acan}, \textit{Col2a1} and \textit{Sox9} expression in cultures from \textit{Scap} deficient and control mice, with or without cholesterol supplementation or overexpression or overexpression of \textit{Ldlr} (n=5 in each group). Data given as means and 95\% confidence levels and an asterisk indicates that the difference observed is significant at p<0.05 compared to control (each genotype without cholesterol supplementation or \textit{Ldlr} overexpression).
Figure 6: Deletion of Insigs within mesenchymal cells or chondrocytes causes dwarfism.

A) Representative whole mounts, skeletons, and long bone histology of E18.5 embryos and mice, showing dwarfism but intact overall bone structure comparing mice lacking both Insig genes in Prx1 expressing cells with controls. B) Femur length of E18.5 Prx1-cre; Insig1lox/lox; Insig2/- and Insig1lox/lox; Insig2/- embryos. The length of Insig1lox/lox; Insig2/- is normalized to “1” (n=8 for Prx1-cre; Insig1lox/lox; Insig2/- and 9 for Insig1lox/lox; Insig2/-). Data shown as means and 95% confidence intervals. An asterisk indicates p<0.05 compared to data from Insig1lox/lox; Insig2/-.

C) The histology of the humeral growth plate at P0. R = resting zone, P = proliferative zone, and H = hypertrophic zone. Bottom panels show type X collagen staining, labeling the hypertrophic zone. D) Relative length of type X collagen expressing cells with the length of the growth plates in C. Insig1lox/lox; Insig2/- is
normalized to “1” (n=6 for Prx1-cre; Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}; and 5 for Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}). Data shown as means and 95% confidence intervals. An asterisk indicates p<0.05 compared to data from Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}. E) Representative skeletons of embryos and mice showing dwarfism in mice lacking both Insig genes in Col2a1 expressing cells, showing intact overall bone structure compared with controls. F) Femur length of P0 Col2-cre; Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-} and Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-} embryos. The length of Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-} is normalized to “1” (n=7 for Col2-cre; Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-} and 7 for Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}). Data shown as means and 95% confidence intervals. An asterisk indicates p<0.05 compared to data from Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}. G) Representative histology of an E16.5 humerus. Alcian blue and type X collagen staining of the same limbs are shown. R = resting zone, P = proliferative zone, and H = hypertrophic zone. H) Representative images of mice at P0. I) Relative length of type X collagen expressing cells with the length of the growth plates in P0 limbs. Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-} is normalized to “1” (n=5 for Col2-cre; Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}; and 6 for Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}). Data shown as means and 95% confidence intervals. An asterisk indicates p<0.05 compared to data from Insig1\textsuperscript{floxflox}; Insig2\textsuperscript{-/-}. 
Figure 7: Hedgehog and cholesterol regulating each other in the growth plate.

A) Expression of Ihh and Hedgehog target genes in cells from mice lacking Scap or the Insig genes in Col2 expressing cells and controls (n=6 for each genotype). Expression in littermate controls was arbitrarily normalized to “1”. B) Effect of treatment with 3ug/ml exogenous cholesterol on Hh target
gene expression on chondrocyte cultures from mice lacking Scap in chondrocytes or littermate controls. Data given as mean and 95% confidence intervals. An asterisk indicates significant difference, p<0.05). Data is normalized so that expression in littermate controls averages 1 (n=5 in each group).

C) Cholesterol levels in metatarsal explants treated with Ihh, or control. D) Treatment with Ihh-N ligand in mice lacking Scap in Col2 expressing cells resulted in a level of Type X collagen expression similar to that seen in control explants. E) Graph shows the length of collagen type X collagen expressing cells in the explants in mm (n=5 in each group). F) Crossing mice lacking Scap in Col2a1 expressing cells with mice overexpressing Gli2 in in Col2a1 expressing cells, results in a partial rescue of the phenotype shown in E16.5 mice in. G) Metatarsals from E16.5 mice showing rescue of type X collagen expressing cells in mice overexpressing Gli2 by depleting Scap. H) Length of type X collagen with data shown as means and 95% confidence intervals are shown from samples in G. An asterisk shows a significant (P<0.05) difference. n>8 for each genotype at each time point. I) Filipin staining from representative chondrocytes from mice in G. J) Cholesterol level from cells. Data given as mean and 95% confidence intervals. An asterisk indicates significant difference, p<0.05, and n=4 in each group. K) Ldlr expression, with level in littermate controls normalized to “1”. Data given as mean and 95% confidence intervals. An asterisk indicates significant difference, p<0.05, and n=6 in each group.