Published in final edited form as:
J Cell Physiol. 2014 November ; 229(11): 1607–1617. doi:10.1002/jcp.24602.

Loss of Jab1 in Osteochondral Progenitor Cells Severely Impairs Embryonic Limb Development in Mice

Lindsay A. Bashur[1], **Dongxing Chen**[1], **Zhijun Chen**[1], **Bojian Liang**[1,5], **Ruggero Pardi**[4], **Shunichi Murakami**[1,2], and **Guang Zhou**[1,2,3,*]

[1]Department of Orthopaedics, Case Western Reserve University, Cleveland, OH, USA

[2]Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, OH, USA

[3]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

[4]San Raffaele University, School of Medicine and Scientific Institute San Raffaele, Milan, Italy

Abstract

The transcriptional cofactor Jab1 controls cell proliferation, apoptosis, and differentiation in diverse developmental processes by regulating the activity of various transcription factors. To determine the role of Jab1 during early limb development, we developed a novel *Jab1*$^{flox/flox}$*; Prx1-Cre* conditional knockout (*cKO*) mutant mouse model in which *Jab1* was deleted in the osteochondral progenitor cells of the limb buds. *Jab1 cKO* mutant mice displayed drastically shortened limbs at birth. The short limb defect became apparent in *Jab1 cKO* mutants at E15.5 and increasingly worsened thereafter. By E18.5, *Jab1 cKO* mutant mice exhibited significantly shorter limbs with: very few hypertrophic chondrocytes, disorganized chondrocyte columns, much smaller primary ossification centers, and significantly increased apoptosis. Real-time RT-PCR analysis showed decreased expression of *Sox9*, *Col2a1*, *Ihh*, and *Col10a1* in *Jab1 cKO* mutant long bones, indicating impaired chondrogenesis. Furthermore, in a micromass culture model of early limb mesenchyme cells, alcian blue staining showed a significant decrease in chondrogenesis in *Jab1 cKO* limb bud cells. The expression of *Sox9* and its downstream targets *Col2a1* and *Aggrecan*, as well as BMP signaling downstream targets, *Noggin*, *Id1*, and *Ihh*, were significantly decreased in *Jab1 cKO* micromass cultures. Moreover, over-expression of *SOX9* in *Jab1 cKO* micromass cultures partially restored *Col2a1* and *Aggrecan* expression. *Jab1*-deficient micromass cultures also exhibited decreased BMP signaling response and reduced BMP-specific reporter activity *ex vivo*. In summary, our study demonstrates that Jab1 is an essential regulator of early embryonic limb development *in vivo*, likely in part by co-activating Sox9 and BMP signaling.

Keywords

Jab1; Sox9; COP9 signalosome; limb development; BMP

[*]Corresponding author: Guang Zhou, PhD, Department of Orthopaedics, BRB, Room 328, Case Western Reserve University, 2109 Adelbert Road, Cleveland, OH 44106, Tel: (216) 368-2260, Fax: (216) 368-1332, guang.zhou@case.edu.
[5]Present address: Department of Orthopaedics, China-Japan Union Hospital, Jilin University, Changchun, Jilin Province, P.R. China

Disclosure
All authors state that they have no conflicts of interest.

Introduction

Limb development is a complex process where a balance of cell proliferation, cell cycle progression, cell fate determination, and cell differentiation have to be maintained under well-controlled, multilayered regulation (Kronenberg, 2003; Long and Ornitz, 2013). Limb abnormality is the second most common congenital disorder and accounts for around 2% of human live-birth defects (Dolk et al., 2010; Warman et al., 2010). The size of the limbs is determined by intrinsic factors including the number of osteochondral progenitor cells (OPCs), and extrinsic signals such as BMP, hedgehog, Wnt, and Notch signaling that can control the fate, survival, and proliferation of OPCs (Akiyama et al., 2005; Dong et al., 2010; Kobayashi et al., 2005; Mak et al., 2006; Shu et al., 2011). OPCs are skeletal progenitor cells located in the condensed mesenchyme during early limb development (Akiyama et al., 2005). They proliferate and further differentiate into chondrocytes to form cartilage or into osteoblasts to form bone. OPCs express master transcription factor Sox9, a potent transcriptional activator of chondrocyte-specific genes such as *Col2a1* and *Aggrecan*. *SOX9* heterozygous mutations in humans cause campomelic dysplasia, a severe skeletal disorder characterized by generalized hypoplasia of endochondral bones (Zhou et al., 2006). Sustained high-level expression of *Sox9* in the center of limb bud condensation is essential for chondrocyte differentiation and the successive steps of cartilage formation. Indeed, the inactivation of Sox9 in limb bud OPCs results in the complete absence of mesenchymal condensations and the subsequent lack of cartilage and bone formation in the limbs (Akiyama et al., 2002). Interestingly, a recent study in a chicken model indicates that defects in OPC differentiation, accompanied by decreased expression of *Sox9*, have been implicated in the etiology of phocomelia, a devastating human birth defect in which long bones fail to develop properly (Galloway et al., 2009). Thus, Sox9-mediated OPC differentiation, proliferation, and survival are critical components of embryonic limb development.

The transcriptional co-regulator Jab1 was originally cloned as a Jun activation domain-binding protein 1 (Claret et al., 1996). Jab1, also named Csn5 or Cops5, is the 5th subunit of the evolutionarily conserved proteolysis regulator COP9 signalosome (Chamovitz and Segal, 2001; Kato and Yoneda-Kato, 2009). Jab1 is highly conserved with over 60% identity between animal and plant counterparts (Wei and Deng, 2003). Jab1 plays critical roles in a variety of developmental processes by regulating the stability and activity of numerous transcription factors to control cell cycle progression, apoptosis, and proliferation (Chamovitz, 2009; Shackleford and Claret, 2010; Wei et al., 2008). The constitutive deletion of *Jab1* in mice results in early embryonic lethality by E8.5, with impaired proliferation and accelerated apoptosis, thus underscoring the essential role of Jab1 in overall early embryogenesis (Tian et al., 2010; Tomoda et al., 2004). JAB1 is also over-expressed in various cancers and has emerged as a novel and critical player in tumorigenesis in recent years (Shackleford and Claret, 2010) (Yang et al., 2011).

We recently reported that Jab1 is critical for chondrocyte differentiation *in vivo*. The chondrocyte-specific *Jab1* knockout (*Jab1*$^{flox/flox}$*; Col2a1-Cre*) mutants exhibited neonatal lethal chondrodysplasia with severe dwarfism (Chen et al., 2013). Furthermore, Jab1 represses chondrocyte hypertrophy *in vivo*, likely in part by down-regulating BMP signaling

(Chen et al., 2013). However, the specific role of Jab1 in early skeletal development is still unknown due to early embryonic death in constitutive *Jab1* knockout mice. Therefore, in this study, we bred *Jab1*$^{flox/flox}$ mice with the *Prx1-Cre* transgene to delete *Jab1* in the OPCs of the limb buds in order to determine the specific role of Jab1 during early limb development.

Materials and Methods

Mouse breeding

All animal protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University. All mice were maintained and housed at the Case Western Reserve University animal facility under standard conditions. *Jab1*$^{flox/flox}$ mice (Panattoni et al., 2008) were crossed with the *Prx1-Cre* transgene (Logan et al., 2002) to generate *Jab1*$^{flox/flox}$*; Prx1-Cre* conditional knockout (*cKO*) mutant mice. All mice were maintained on C57/BL6J background. All controls were *Cre* negative wild-type littermates of *Jab1* cKO mutants. Mouse genotyping was performed as described using GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) (Chen et al., 2013). For all matings, embryonic day (E) 0.5 was designated as noon of the day that a plug appeared, and embryos were collected at indicated times.

Skeletal Staining, Histology, and Immunohistochemistry

Mouse skeletal preparations were stained with alcian blue for cartilage and alizarin red for bone as described (Zhou et al., 2006). For histology, mouse skeletons were fixed in 10% formalin overnight, embedded in paraffin, and sectioned. Tissue sections were stained with hematoxylin and eosin for general morphological analysis. Von Kossa staining was performed as described previously (Kyono et al., 2012). Briefly, slides were stained with a 5% silver nitrate solution until mineralized matrix turned black, followed by a 5% sodium thiosulfate solution staining until mineralized matrix turned brown, and then counterstained with nuclear fast red (Vector, Burlingame, CA, USA).

Immunohistochemistry was performed as previously described (Chen et al., 2013). Briefly, slides were heated in a steamer in 1× antigen unmasking solution (Vector), blocked with normal serum, incubated overnight with primary antibodies against type × collagen (dilution 1:200, Quartett, Berlin, Germany), Sox9 (dilution 1:100, Millipore, Billerica, MA, USA), cleaved caspase-3 (dilution 1:100, Cell Signaling, Danvers, MA, USA), and phospho-Smad1/5 (dilution 1:100, Epitomics, Burlingame, CA, USA), incubated with a biotinylated secondary antibody (dilution 1:100, Vector),and treated with ABC reagent (Vector), followed by developing with DAB substrate (Vector).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed with ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Billerica, MA, USA) according to the manufacturer's instructions. For bromodeoxyuridine (BrdU) labeling, pregnant mice were intraperitoneally injected with BrdU labeling reagent (Invitrogen, Carlsbad, CA, USA). After 4 hours, embryos were harvested, processed for histology, and then stained using a BrdU staining kit (Invitrogen) according to the

manufacturer's instructions. Cell proliferation was quantified by the ratio of the number of BrdU-positive chondrocytes to total number of chondrocytes in the growth plates. All images were obtained with a Leica DC500 digital camera with either Leica DM 6000B, DM IRB, or MZ16 microscopes using Leica Application Suite 1.3 software.

Micromass Culture

Limb buds from E11.5 embryos were dissected, and cells were dissociated by trypsin digestion in a 1:5 mixture of 0.05% trypsin to PBS. Digested limb bud solutions were diluted 1:1 with growth media (DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamax), passed through a 70μm cell strainer, and centrifuged. Cells were then resuspended in growth media at 2×10^7 cells/mL and plated in 20μL droplets on 24-well tissue culture plates (ThermoScientific, Waltham, MA, USA). Cells were incubated for 1.5 hours at 37°C in a humidified CO_2 incubator and then fed with 1 mL of growth media. After 6 days, cultures were fixed in 10% formalin, then stained with 1% alcian blue (Sigma, St. Louis, MO, USA), or collected for RNA or protein analysis.

Jab1 cKO mutant and wild-type micromass cultures were infected with adenoviruses expressing human *SOX9* transgene (Ad-SOX9) or red fluorescent protein control (Ad-RFP) (SignaGen Laboratories, Rockville, MD, USA) at multiplicity of infection (MOI) 100. Adenoviruses were added at the time of initial plating and the media was changed on the following day. Cultures were collected for RNA on day 7. *Jab1*$^{flox/flox}$ micromass cultures were infected with adenoviruses expressing Cre recombinase (Ad-Cre) or green fluorescent protein control (Ad-GFP) (Gene Transfer Vector Core, University of Iowa) at MOI 50. Adenoviruses were added at the time of initial plating and the media was changed on the following day to media containing BMP-7 (100ng/mL, Peprotech, Rocky Hill, NJ, USA) or BMP-2 (400ng/mL, Invitrogen). The micromass cultures were collected for RNA on day 5.

For transfection of micromass cultures, *Jab1 cKO* mutant and wild-type littermate E11.5 limb bud cells were first plated in micromass cultures as described above. On day 4, cells were lifted with 0.05% trypsin, and cell suspensions of the same genotype were combined and transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were transfected with 150 ng *pcDNA*, 150 ng *ID1-luc*, or 150 ng *4x48-p89Col2a1-luc* plasmid along with 25ng *pSV2βgal* control plasmid per micromass, then plated in 20 μL droplets containing 200,000 cells for micromass culture on 24-well plates. The micromass cultures were incubated for one hour at 37°C in a humidified CO_2 incubator, and then fed with DMEM containing 5% FBS to cover the well. After an additional 3 hr incubation, the media was replaced with growth media (DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamax). The cultures were incubated for another 48 hrs, and then collected for luciferase and β-galactosidase activity measurements using the Dual-Light Reporter Gene Assay System (Applied Biosystems, Carlsbad, CA, USA). The relative reporter activity was determined as described previously (Zhou et al., 1999).

Primary Osteoblast and C3H10T1/2 Cell Culture

Calvarias were dissected from 5-day-old *Jab1*$^{flox/flox}$ mice and digested with αMEM containing 1% penicillin/streptomycin, 0.04% trypsin, and 0.1mg/mL collagenase P as described (Zhou et al., 2006). Cells were plated at 50,000 cells/cm^2 in 12-well tissue culture plates in osteoblast growth media (αMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamax) containing adenoviruses Ad-Cre or Ad-GFP (Gene Transfer Vector Core) at MOI 500. The media was changed 48 hrs later to mineralization media (osteoblast growth media containing 5mM β-glycerophosphate and 100μg/mL ascorbic acid). After 3 weeks, osteoblast cultures were stained with alizarin red or collected for RNA analysis. C3H10T1/2 cell culture and transient transfection with *Jab*1 and *Sox9* expression plasmids and a *4x48-p89Col2a1-luc* reporter plasmid were performed as described (Zhou et al., 1999).

Real-time Quantitative RT-PCR

Total RNA was extracted from E11.5 limb buds, E18.5 long bones (including epiphysis, metaphysis, and diaphysis with connective tissue and muscle carefully removed), or micromass cultures using TRIzol reagent (Invitrogen) and PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. 500ng of RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Real time RT-PCR was performed in an ABI 7500 real time PCR system (Applied Biosystems) using Power SYBR Green Master Mix (Applied Biosystems) and gene-specific primer pairs (Supplemental Table 1), or an Osteogenesis PCR array (#PAMM-026 SABiosciences, Valencia, CA, USA). Quantification of gene expression was performed using the comparative threshold cycle ($\Delta\Delta C_t$) method with *Gapdh* as the internal reference as described (Liang et al., 2012). Relative gene expression was reported as $2^{-\Delta\Delta Ct}$. Each reaction was performed in triplicate and repeated on at least three independent samples per genotype.

Western Blot

Total protein was extracted from micromass cultures using 50mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM PMSF, and 1% Halt protease and phosphatase inhibitor cocktail (ThermoScientific). Total protein was extracted from E18.5 long bones (epiphysis, metaphysis, and diaphysis with connective tissue and muscle carefully removed) using 62.5 mM Tris (pH 7.0), 5% SDS, and 1% Halt protease and phosphatase inhibitor cocktail (ThermoScientific). Western blotting was performed as described (Chen et al., 2013). Briefly, proteins were separated on a 4–15% Tris-HCl SDS-PAGE gel (Bio-Rad) and transferred onto PVDF nitrocellulose membranes. Membranes were probed with primary antibody against tubulin (dilution 1:300, Sigma), Jab1 (dilution 1:150, Santa Cruz), Sox9 (dilution 1:300, Millipore), or Csn8 (dilution 1:300, Epitomics) overnight at 4°C followed by horseradish peroxidase-conjugated secondary antibody (dilution 1:300, ThermoScientific Pierce) for 1 hr. Protein bands were visualized by ECL western blotting substrate (ThermoScientific Pierce). Membranes were then stripped and reprobed with another primary antibody.

Statistical analysis

Embryos were collected individually from 2–3 separate litters, analyzed separately, and then combined for n=3–6 per genotype. Statistical analysis was performed using the student's t-test to compare the differences between *Jab1 cKO* mutant mice and wild-type controls. $p \leq 0.05$ is considered to be statistically significant. Results were presented as mean ± standard error.

Results

Jab1$^{flox/flox}$;Prx1-Cre mutant mice display drastically shortened limbs

To determine the specific role of Jab1 during early limb development, we developed a novel *Jab1$^{flox/flox}$;Prx1-Cre* conditional knockout (*cKO*) mutant mouse model in which *Jab1* was deleted specifically in the mesenchymal cells, including OPCs of the limb buds, starting at E9.5 (Logan et al., 2002). The *Jab1$^{flox/+}$; Prx1-Cre* heterozygous mice appeared grossly normal, fertile, and had a normal life span. However, the *Jab1 cKO* mutant mice displayed drastically shortened limbs at birth (Fig. 1A, B, C). The majority of *Jab1 cKO* mutant mice died within 5 days of birth, likely due to malnutrition. There was a noticeable lack of milk in the newborn *Jab1 cKO* mutant mice compared with wild-type littermates (Fig. 1A), indicating the mutants' failure to obtain nutrition for survival. E18.5 *Jab1 cKO* mutant mice skeletal preparations exhibited abnormalities in *Prx1-Cre* targeted areas, with the most dramatic effect in the limbs (Fig. 1B). This limb defect becomes even more severe in the postnatal stage, as shown in the very few surviving mutants (Fig. 1C). Thus, Jab1 is required for both embryonic and postnatal limb growth. Immunohistochemistry and western blot analysis of newborn limbs confirmed the loss of Jab1 protein in *Jab1 cKO* mutant limbs (Fig. 1D, E). In contrast, the expression of Csn8, another key component of the COP9 signalosome, appeared to be relatively unchanged (Fig. 1E).

Histological analysis revealed an increasingly worsened short-limb defect during embryonic development in *Jab1 cKO* mutant mice. At E12.5, *Jab1 cKO* mutant mice and wild-type littermates exhibited no gross morphological differences (Fig. 2). However, starting at E13.5, *Jab1 cKO* mutant limb development became progressively impaired. At E15.5, *Jab1 cKO* mutant mice exhibited a much smaller cartilage element and a reduced primary ossification center (Fig. 2). At E18.5 and postnatal day 5, *Jab1 cKO* mutant mice exhibited significantly shorter limbs with: very few hypertrophic chondrocytes, disorganized chondrocyte columns, and much smaller primary ossification centers (Fig. 2). At postnatal day 21, *Jab1 cKO* mutant mouse limbs showed an even more drastic size difference with: the lack of a clear hypertrophic zone, disorganized proliferating chondrocytes, and missing secondary ossification centers (Fig. 2). Immunohistochemical staining of type × collagen, a specific marker for hypertrophic chondrocytes, confirmed the severely reduced zone of hypertrophic chondrocytes in newborn *Jab1 cKO* mutant mouse limbs (Fig. 3A). In addition, von Kossa staining showed reduced mineralization in the primary ossification center of newborn *Jab1 cKO* mutant mouse limbs compared with the wild-type littermate controls (Fig. 3B). Thus, there is a severe ossification defect in *Jab1 cKO* mutant mouse limbs. To determine the direct effect of the loss of *Jab1* on osteoblast differentiation, we isolated *Jab1$^{flox/flox}$* calvarial osteoblasts and infected them with adenoviruses expressing Cre

recombinase (Ad-Cre) to delete *Jab1*, or a control adenovirus expressing GFP (Ad-GFP). Alizarin red staining and gene expression analysis of *osteocalcin*, *bone sialoprotein*, *osterix*, and *Runx2* revealed a significant decrease in osteoblast differentiation in Ad-Cre infected cells compared with Ad-GFP infected control cells (Fig. 3C, D). Therefore, Jab1 is required for both chondrogenic and osteogenic differentiation.

Increased apoptosis and impaired differentiation in *Jab1$^{flox/flox}$; Prx1-Cre* mutant mice

In order to determine the cause of severe short-limb defect in *Jab1 cKO* mutant mice, we first evaluated their cell proliferation status. BrdU labeling revealed no significant difference in cell proliferation between *Jab1 cKO* mutant mice and wild-type controls at both E15.5 and E18.5 (Fig. 4A, B). This is consistent with our previous work showing that *Jab1* deletion also did not affect overall proliferation in differentiating chondrocytes (Chen et al., 2013). Next, we performed TUNEL staining to evaluate cell survival in *Jab1 cKO* mutant mice. We identified a significant increase in TUNEL positive apoptotic cells in *Jab1 cKO* mutant mice compared with wild-type controls at E18.5 (Fig. 4C). Moreover, the expression of cleaved caspase-3, a critical apoptosis mediator, was also increased in *Jab1 cKO* mutant mice compared with wild-type controls at E18.5 (Fig. 4D). Thus, there is increased apoptosis in *Jab1 cKO* mutant mice, which likely contributes in part to their severe limb shortening phenotype.

To determine the effect of Jab1 deletion on the expression of skeletal differentiation markers, we extracted RNA from E11.5 limb buds and E18.5 long bones of *Jab1 cKO* mutant mice and wild-type controls. Real-time RT-PCR analysis revealed no significant changes in the expression of chondrocyte differentiation markers *Sox9* and *Col2a1*, and proliferation marker *cyclinD1*, in E11.5 *Jab1 cKO* mutant limb buds compared with wild-type controls (Fig. 5A, B). These results are consistent with our histological findings at E12.5 that showed no gross morphology change in *Jab1 cKO* mutant mice (Fig. 2). However, at E18.5 the expression of various chondrocyte differentiation markers, including *Col2a1*, *Ihh*, and *Col10a1*, were all down-regulated in *Jab1 cKO* mutant long bones, indicating impaired chondrogenesis (Fig. 5C, D). At E18.5, the expression of osteoblast differentiation markers *Runx2* and *Col1a1* was also significantly decreased in *Jab1 cKO* mutant long bones, indicating impaired ossification (Fig. 5C, D) and consistent with the critical role of Jab1 in osteoblast differentiation as described above (Fig. 3C, D). Furthermore, *Vegf-A* expression was decreased in E18.5 *Jab1 cKO* mutant long bones compared with wild-type controls (Fig. 5C, D), suggesting an angiogenesis defect and also consistent with impaired ossification center formation in *Jab1 cKO* mutants (Fig. 2, 3B). The altered expressions of these critical skeletal differentiation markers correlate with severely impaired endochondral ossification in E18.5 *Jab1 cKO* mice (Fig. 2).

Decreased chondrogenesis in *Jab1$^{flox/flox}$; Prx1-Cre* mutant micromass culture

To delineate the underlying mechanism for the severe short-limb defect in *Jab1 cKO* mutants, we utilized a widely applied micromass culture model of E11.5 limb mesenchyme cells to study chondrogenesis *ex vivo* (Bruce et al., 2010; Dong et al., 2010; Dranse et al., 2011). In both *Jab1 cKO* mutant and wild-type micromass cultures, nodules appeared by day 3 and persisted thereafter, indicating grossly normal mesenchymal condensations in *Jab1*

cKO mutant micromass cultures (Fig. 6A). Peanut agglutinin (PNA) staining of micromass cultures showed comparable formation of mesenchymal condensations in *Jab1 cKO* mutants and controls (Supp. Fig. 1). Real-time RT-PCR analysis showed no significant change in proliferation marker *cyclin D1* between *Jab1 cKO* mutant and wild-type cultures (Fig. 6C). Thus, Jab1 does not appear to have a significant effect on overall limb bud cell condensation and proliferation *ex vivo*. However, alcian blue staining showed a significant decrease in chondrogenesis in *Jab1 cKO* mutant micromass cultures compared with wild-type controls in both forelimb and hindlimb cultures on day 6 (Fig. 6B). The expression of *Sox9* and its downstream targets *Col2a1*, *Comp*, and *Aggrecan* were all significantly decreased in *Jab1 cKO* mutant micromass cultures compared with wild-type controls (Fig. 6C). The BMP downstream targets *Ihh*, *Id1*, and *noggin* were also significantly decreased in *Jab1 cKO* mutant micromass cultures (Fig. 6C). In addition, early markers of osteoblast differentiation *Osterix* and *Dlx5* were also significantly decreased in *Jab1 cKO* mutant micromass cultures (Fig. 6C). The expression of *Bmp7* and *Bmpr1b* was only modestly decreased (Fig. 6C). Moreover, western blotting analysis of the micromass culture extracts revealed the loss of Jab1 expression and down-regulation of Sox9 expression, but the relatively unchanged expression of Csn8, another critical subunit of the COP9 signalosome, in *Jab1 cKO* mutant cultures (Fig. 6D). In agreement with our *in vivo* data, above, showing impaired chondrocyte differentiation in E18.5 *Jab1 cKO* mutant limbs (Fig. 5C, D), the *ex vivo* micromass culture results validate that Jab1 is essential for chondrogenesis.

Jab1 likely promotes Sox9 and BMP signaling activity during early limb development

To determine whether Sox9 is a downstream target of Jab1, we infected *Jab1 cKO* mutant and wild-type micromass cultures with adenoviruses Ad-SOX9, to over-express the human *SOX9* transgene, or Ad-RFP as a control. We first confirmed that the *SOX9* transgene was over-expressed in both wild-type and mutant cultures infected with Ad-SOX9 (Fig. 7A). *Jab1* expression remained decreased in *Jab1 cKO* micromass cultures after infection with Ad-SOX9 or Ad-RFP (Fig. 7A). As expected, chondrocyte differentiation markers *Col2a1* and *Aggrecan* were both significantly decreased in *Jab1 cKO* +Ad-RFP group compared with WT+Ad-RFP group (Fig. 7A). Both *Col2a1* and *Aggrecan* expressions were increased in wild-type and mutant *Jab1 cKO* micromass cultures when infected with Ad-SOX9, compared with those infected with control Ad-RFP (Fig. 7A). More importantly, *Col2a1* and *Aggrecan* expressions in *Jab1 cKO* +Ad-SOX9 group were increased from virtually non-existent to the levels comparable to or higher than those in WT+Ad-RFP group (Fig. 7A). Thus, over-expression of *SOX9*, the master regulator of chondrogenesis, was able to compensate in part for the loss of *Jab1* in terms of the key chondrogenic marker expression in micromass cultures. Next, to determine whether Jab1 has any direct effect on Sox9 transcriptional activity, *Jab1* and *Sox9* expression plasmids were co-transfected with a *4x48-p89Col2a1-luc* reporter into mesenchymal-like C3H10T1/2 cells. *4x48-p89Col2a1-luc* is a well-characterized and widely used Sox9 reporter construct and contains four tandem repeats of a 48-bp Sox9-binding enhancer element (Lefebvre et al., 1997) (Zhou et al., 1998). While Sox9 alone activated the reporter significantly and Jab1 had no effect by itself, the co-transfection of Jab1 and Sox9 increased the reporter activity by two-fold (Fig. 7B). In addition, *Jab1 cKO* mutant and wild-type E11.5 micromass cultures were transfected for 48 hrs with the *4x48-p89Col2a1-luc* reporter plasmid (Zhou et al., 1998). *Jab1 cKO* limb bud

cells had significantly decreased Sox9 reporter activity compared with the wild-type littermate controls (Supp. Fig. 2). Moreover, immunohistochemical staining revealed decreased Sox9 expression in *Jab1 cKO* mutants compared with wild-type controls (Fig. 7C). Taken together, our results suggest that Jab1 might sustain Sox9 expression and facilitate Sox9 activity during early chondrogenesis. Interestingly, *Col2a1* and *Aggrecan* expressions in the *Jab1 cKO* +Ad-SOX9 group were still significantly lower than those in the WT+Ad-SOX9 group (Fig. 7A), indicating that besides Sox9, there are likely other critical factors also mediating Jab1 function in OPCs.

BMP signaling affects all aspects of skeletal development and regeneration (Chen et al., 2004) (Song et al., 2009) (Chen et al., 2012). To determine whether *Jab1* deletion in OPCs might affect BMP signaling during chondrogenesis, we analyzed BMP signaling response upon the immediate loss of *Jab1* in limb bud cells using micromass cultures. We infected $Jab1^{flox/flox}$ E11.5 limb mesenchyme micromass cultures with adenoviruses Ad-Cre, to delete *Jab1*, and Ad-GFP, as a control. BMP-7 was then added to the media after 24 hrs of culture (day 1). The addition of BMP-7 significantly increased the expression of BMP downstream targets *noggin*, *Col2a1*, and *Ihh*, in Ad-GFP micromass cultures (Fig. 8A). The addition of BMP-7 in Ad-Cre micromass cultures increased the expression of *noggin*, *Col2a1*, and *Ihh*, but at levels significantly lower than those of the Ad-GFP+BMP-7 group. The addition of BMP-2 was also tested under the same micromass culture conditions and resulted in the same trends as did the addition of BMP-7 (Supp. Fig. 3). Thus, the loss of *Jab1* in limb bud cells impaired their response to various BMP ligands. In order to determine the direct and broad effect of Jab1 on BMP signaling at the transcriptional level, *Jab1 cKO* mutant and wild-type E11.5 micromass cultures were transfected for 48 hrs with a BMP-specific reporter *ID1-luc* (Shola et al., 2012). *Jab1 cKO* limb bud cells had significantly decreased BMP reporter activity compared with wild-type littermate controls (Fig. 8B). Moreover, immunohistochemical staining showed decreased phospho-Smad1/5 expression in E14.5 *Jab1 cKO* limbs as compared with wild-type controls (Supp. Fig. 4). Therefore, Jab1 regulates early limb development likely in part through promoting BMP signaling as well as Sox9 activity (Fig. 8C). Interestingly, the difference in phospho-Smad1/5 expression became less pronounced as development progressed in E15.5 and newborn limbs (Supp. Fig. 4). Thus, the effect of Jab1 on BMP during embryonic development is likely to be dynamic and stage-specific, and the underlying mechanism remains to be further studied in the future.

Discussion

Our study demonstrates for the first time that Jab1 is an essential regulator of early embryonic limb development *in vivo*. To overcome early embryonic death in constitutive *Jab1* knockout mice, we developed a novel mouse model in which *Jab1* was specifically deleted in the mesenchymal cells, including osteochondral progenitor cells (OPCs), of limb buds. The deletion of *Jab1* in limb bud OPCs impairs cell survival and inhibits chondrogenesis, consequently leading to a severe limb shortening phenotype in *Jab1 cKO* mice. In addition, Jab1 regulates early limb development likely in part through co-activating Sox9 and BMP signaling in OPCs (Fig. 8C).

Our data indicates that *Jab1 cKO* OPCs were able to condense similarly to the wild-type controls without a gross patterning defect (Fig. 2 and Supp. Fig.1). However, as *Jab1 cKO* limbs further developed, they became increasingly shorter than the wild-type littermates over time. By E18.5, *Jab1 cKO* mutant limbs had increased apoptosis, very few hypertrophic chondrocytes, disorganized chondrocyte columns, and much smaller primary ossification centers (Fig. 2). At postnatal day 21, *Jab1 cKO* mutant mice limbs showed an even more drastic size difference with the lack of a well-defined hypertrophic zone and no secondary ossification center (Fig. 2). It appears that *Jab1 cKO* OPCs are unable to expand sufficiently, partially due to increased apoptosis and potentially other defects such as cell cycle dysregulation as reported in several types of *Jab1*-deficient cells, including chondrocytes (Chen et al., 2013), T cells (Panattoni et al., 2008), and MEFs (Yoshida et al., 2010). Jab1 is also critical for DNA damage repair (Huang et al., 2007; Tian et al., 2010). Thus, the loss of Jab1 in limb bud OPCs might cause an OPC self-renewal deficiency; consequently only a significantly reduced number of OPCs would be available to differentiate into hypertrophic chondrocytes and subsequently induce ossification, resulting in severe limb shortening at birth. Cell fate mapping and lineage tracing of *Jab1 cKO* OPCs *in vivo* might help us to better understand the role of Jab1 in OPC survival, renewal, and differentiation. Furthermore, the striking short-limb phenotype in newborn *Jab1 cKO* mice is likely due to the accumulated effect of the loss of Jab1 in limb buds starting at E9.5 (Logan et al., 2002). The precise function of Jab1 in OPCs *in vivo* is very likely to be complex and stage-specific. Future work is needed to address the specific role of Jab1 at different stages of limb development, early versus late, using spatiotemporal-specific inducible genetic tools such as a recently developed *Prx1-CreER-GFP* mouse model (Kawanami et al., 2009).

Jab1 can directly interact with various transcription factors to regulate cell differentiation. In micromass cultures, *Jab1 cKO* mutant OPCs failed to undergo overt chondrogenesis with greatly reduced Sox9 expression (Fig. 6C). The master transcription factor Sox9, expressed in skeletal precursor cells during early embryonic development, is a crucial regulator of OPC differentiation and survival and is therefore essential for proper skeletal development (Akiyama, 2008). The inactivation of *Sox9* in limb bud OPCs results in the complete absence of mesenchymal condensations and subsequent loss of skeletal formation in the limbs (Akiyama et al., 2002). In addition, heterozygous mutations in the human *SOX9* gene cause the human disease campomelic dysplasia (Zhou et al., 2006). However, the mechanisms controlling the expression and activity of Sox9 itself are still poorly understood (Akiyama and Lefebvre, 2011). A recent study has provided some insight into the role of Sox9 in BMP-2-induced chondrogenesis *in vitro* (Pan et al., 2008). Their findings show that the BMP-2-mediated p38 pathway regulates both the expression and activity of Sox9, while the BMP-2-dependent Smad pathway regulates only Sox9 transcriptional activity. Furthermore, mouse mutants deficient in both *Bmpr1a* and *Bmpr1b* in cartilage develop severe chondrodysplasia with drastically reduced Sox9 expression (Yoon et al., 2005). Interestingly, in our study, the over-expression of human *SOX9* transgene in *Jab1 cKO* micromass cultures partially restored *Col2a1* and *Aggrecan* expression, suggesting that Sox9 is a downstream target of Jab1 in OPCs (Fig. 7A). Conversely, *Jab1 cKO* limb bud cells had significantly decreased Sox9 reporter activity compared with wild-type littermate controls

(Supp. Fig. 2). Additionally, Jab1 can increase Sox9 transcriptional activity in 10T1/2 cells (Fig. 7B). Notably, Jab1 by itself did not activate Sox9 reporter in 10T1/2 cells (Fig. 7B). Over-expression of Jab1 via adenovirus in 10T1/2 cells and E11.5 mouse limb bud cells did not enhance Sox9 expression (data not shown). This is likely due to the very low endogenous Sox9 expression in 10T1/2 cells and the lack of an identifiable DNA binding domain in Jab1 itself (Claret et al., 1996) (Lefebvre et al., 1998). Therefore, Jab1 is likely to be required for the sustenance, but not the initiation, of Sox9 expression and activity in OPCs. Jab1's effect on Sox9 is likely to be multi-layered and complex, and remains to be further elucidated.

BMP is a key signaling pathway regulating all aspects of cartilage and bone development. Interestingly, the expression of BMP downstream targets, *Noggin*, *Id1*, and *Ihh*, were all significantly decreased in *Jab1 cKO* micromass cultures (Fig. 6C). *Jab1*-deficient micromass cultures also exhibited a decreased BMP signaling response compared with the controls (Fig. 8A, Supp. Fig. 3). These observations suggest that Jab1 may promote BMP signaling in OPCs during early limb development. In contrast, previously we have shown that Jab1 represses BMP signaling in differentiating chondrocytes, using a *Col2a1-Cre* transgene to achieve chondrocyte-specific Jab1 knockout (Chen et al., 2013). Jab1 is a very versatile molecule that can interact with numerous factors in various cell types (Shackleford and Claret, 2010). Thus, the contrasting effect of Jab1 on BMP signaling in *Col2a1-Cre*- vs *Prx1-Cre*- targeted cells is likely due to the different factors that Jab1 interacts with at different stages of limb development. Taken together, these two studies indicate that Jab1 may play different roles at different stages of skeletal development. Indeed, Jab1 can interact with Smad4, a co-activator of TGF-β/BMP signaling (Wan et al., 2002), and also Smad7, an inhibitor of TGF-β/BMP signaling (Kim et al., 2004). By degrading Smad4 or Smad7, Jab1 can repress or increase TGF-β/BMP signaling, respectively, *in vitro*. These conflicting results underscore the complex role of Jab1 in TGF-β/BMP signaling. Given the crucial roles of both TGF-β/BMP signaling and Jab1 in various developmental processes, it will be important to investigate the interaction between Jab1 and various TGF-β/BMP signaling components in the differentiation of diverse cell types.

In conclusion, our study provides novel and important insights into the role of Jab1 during early limb development. We demonstrate for the first time that Jab1 is an essential regulator of early embryonic limb development *in vivo*, likely in part by co-activating Sox9 and BMP signaling in OPCs. Since *Jab1 cKO* mice display a significant short-limb phenotype accompanied by severe OPC defects, this novel mouse model can serve as an excellent tool to study the critical role of OPCs in the pathogenesis of limb abnormalities. Further elucidation of Jab1 function in OPCs can potentially lead to the development of Jab1-based novel therapies for the treatment of severe limb-shortening birth defects.

Finally, JAB1 expression is significantly increased in a wide variety of cancers (Richardson and Zundel, 2005) (Shackleford and Claret, 2010) (Yang et al., 2011). In breast cancer, hepatocellular carcinoma, and pancreatic adenocarcinoma, JAB1 is a negative regulator of p27, and high JAB1 expression is associated with poor prognosis and decreased p27 expression (Shackleford and Claret, 2010). However, the underlying mechanisms of JAB1 involvement in tumor development remains poorly understood. Cancer stem cells and

embryonic stem/progenitor cells share some key characteristics. Thus, further elucidation of the crucial roles of JAB1 in OPCs might also provide deeper insights into the effect of JAB1 on key cancer stem cell properties, such as stem cell maintenance and renewal, and eventually lead to the development of JAB1-targeted cancer therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health grant R03-DE019190-1A1S1 and Case Department of Orthopaedics research fund to GZ, and T32 AR007505 to LB. We thank Dr. James F. Martin for the *Prx1-Cre* transgenic mice, Teresa Pizzuto for expert histology work, Dr. David Danielpour for the BMP reporter plasmid *ID1-luc*, and Valerie Schmedlen for editorial assistance.

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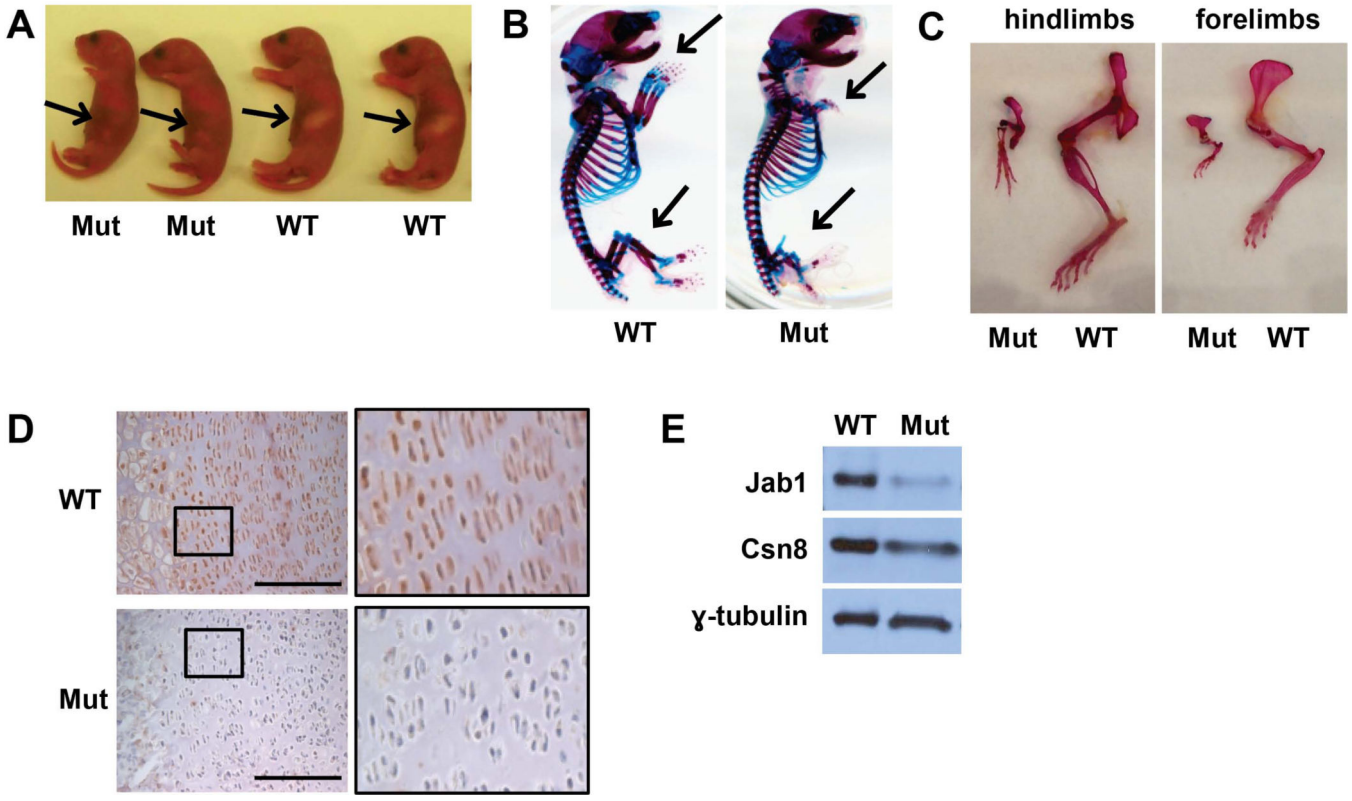

Figure 1. Deletion of Jab1 in the osteochondral progenitor cells (OPCs) of limb buds results in drastically shortened limbs

(A) Newborn *Jab1*$^{flox/flox}$*;Prx1-Cre* conditional knockout (*cKO*) mutants and wild-type littermates. Arrows indicate the lack of milk in the mutants. (B) Skeletal preparations of E18.5 *Jab1 cKO* mutant and wild-type mice. Arrows indicate a severe short-limb defect in *Jab1 cKO* mutants compared with wild-type controls. (C) *Jab1 cKO* mutant and wild-type forelimbs and hindlimbs at 6 weeks. (D) Jab1 immunohistochemical staining in newborn tibia sections, showing the loss of Jab1 expression in *Jab1 cKO* mutants. Right-column images are enlargements of the highlighted sections in the left-column images. Scale bars, 50 μm. (E) Western blot analysis of Jab1 and Csn8 in E18.5 hindlimbs. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

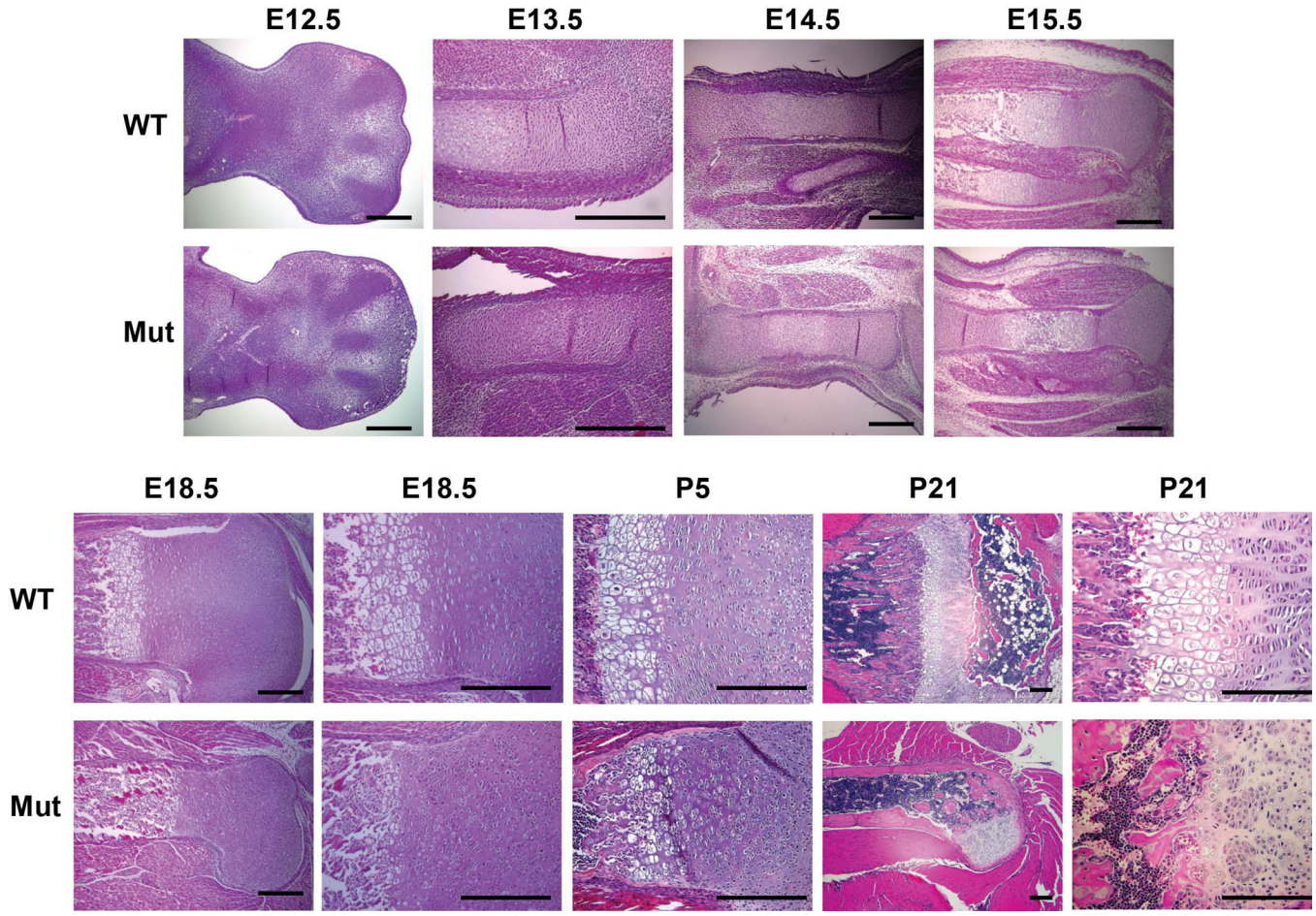

Figure 2. Progressively impaired endochondral ossification in *Jab1 cKO* mutant mice

Hematoxylin and eosin staining of *Jab1 cKO* mutant and wild-type tibias at E12.5, E13.5, E14.5, E15.5, E18.5, postnatal day 5, and postnatal day 21. Scale bars, 100 μm. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

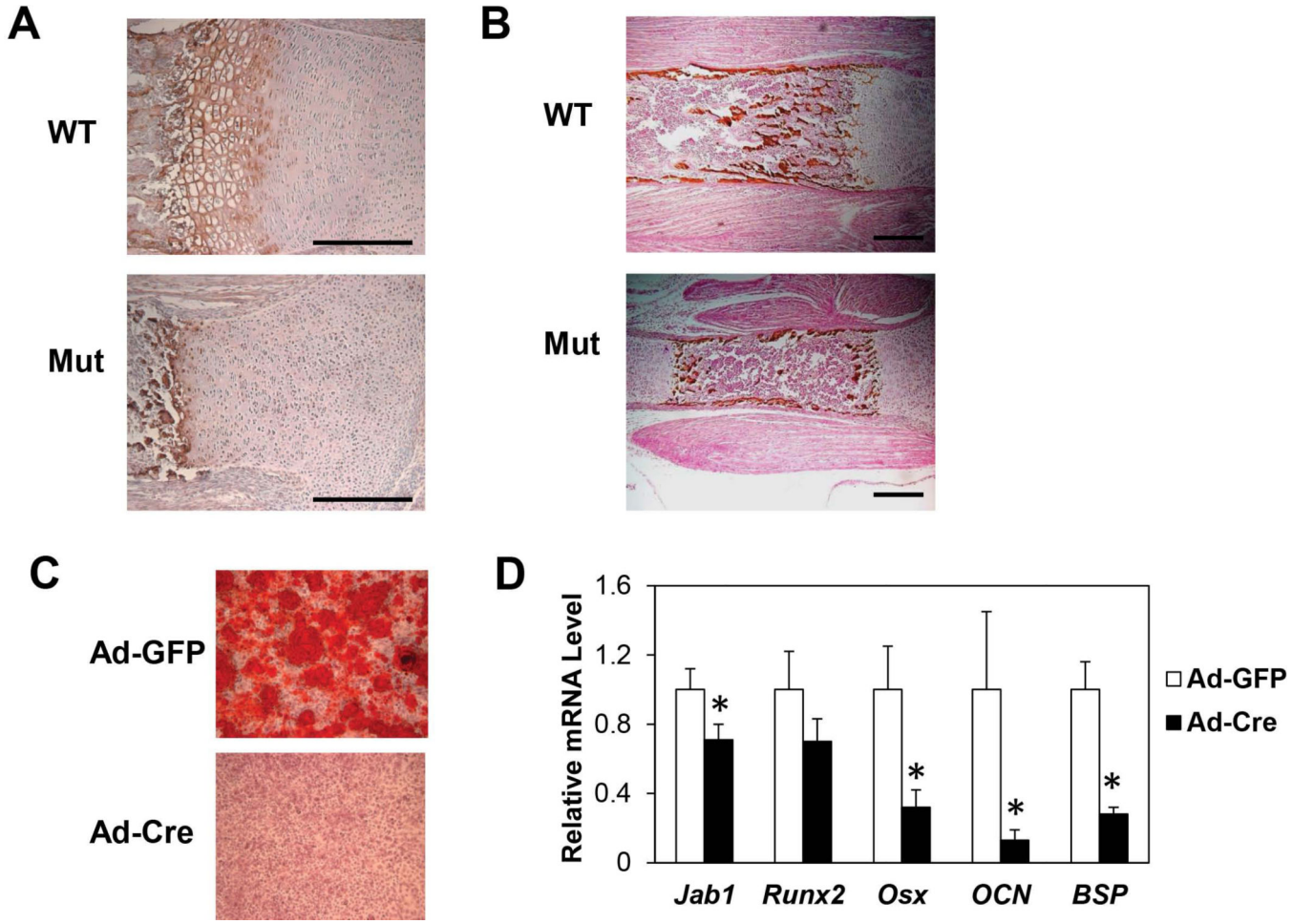

Figure 3. Impaired mineralization and osteoblast differentiation in *Jab1 cKO* mutant mice

(A) Reduced immunohistochemical staining of type × collagen in newborn *Jab1 cKO* mutant and wild-type tibias. Scale bars, 100 μm. (B) Reduced von Kossa staining in newborn *Jab1 cKO* mutant and wild-type tibias. Scale bars, 100 μm. (C) Alizarin red staining in $Jab1^{flox/flox}$ calvarial osteoblasts infected with adenoviruses Ad-Cre and Ad-GFP after 3 weeks of culture. (D) Real-time RT-PCR analysis of osteoblast differentiation markers in $Jab1^{flox/flox}$ calvarial osteoblasts infected with adenoviruses Ad-Cre or Ad-GFP after 3 weeks of culture (*osteocalcin, OCN; bone sialoprotein, BSP; osterix, Osx*). Data represents mean ± standard error, n=3. An asterisk (*) denotes a statistically different level of expression between Ad-Cre and Ad-GFP, $p \leq 0.05$. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

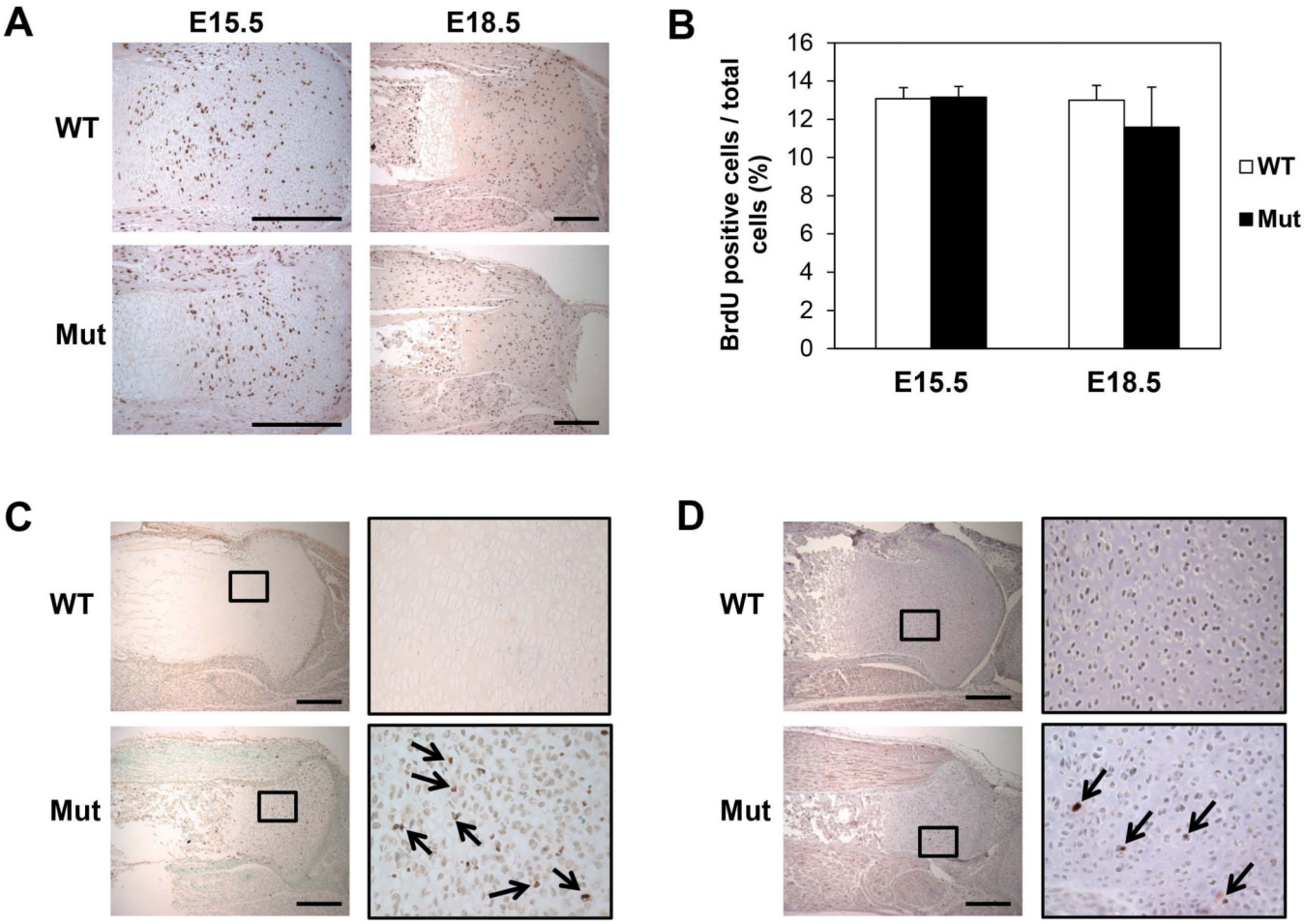

Figure 4. Increased apoptosis in *Jab1 cKO* mutant mice

(A) BrdU staining of *Jab1 cKO* mutant and wild-type tibias at E15.5 and E18.5. (B) Quantification of BrdU incorporation of E15.5 and E18.5. Data represents mean ± standard error, n=3 individual mice per group. (C) TUNEL-positive staining for apoptosis in E18.5 *Jab1 cKO* mutant and wild-type tibias. Right-column images are enlargements of the highlighted sections in the left-column images. Arrows point to positive staining for apoptosis. (D) Immunohistochemical staining of cleaved caspase-3 in newborn *Jab1 cKO* mutant and wild-type tibias. Right-column images are enlargements of the highlighted sections in the left-column images. Arrows point to positive staining of cleaved caspase-3. Scale bars, 100 μm (A, C, D). Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

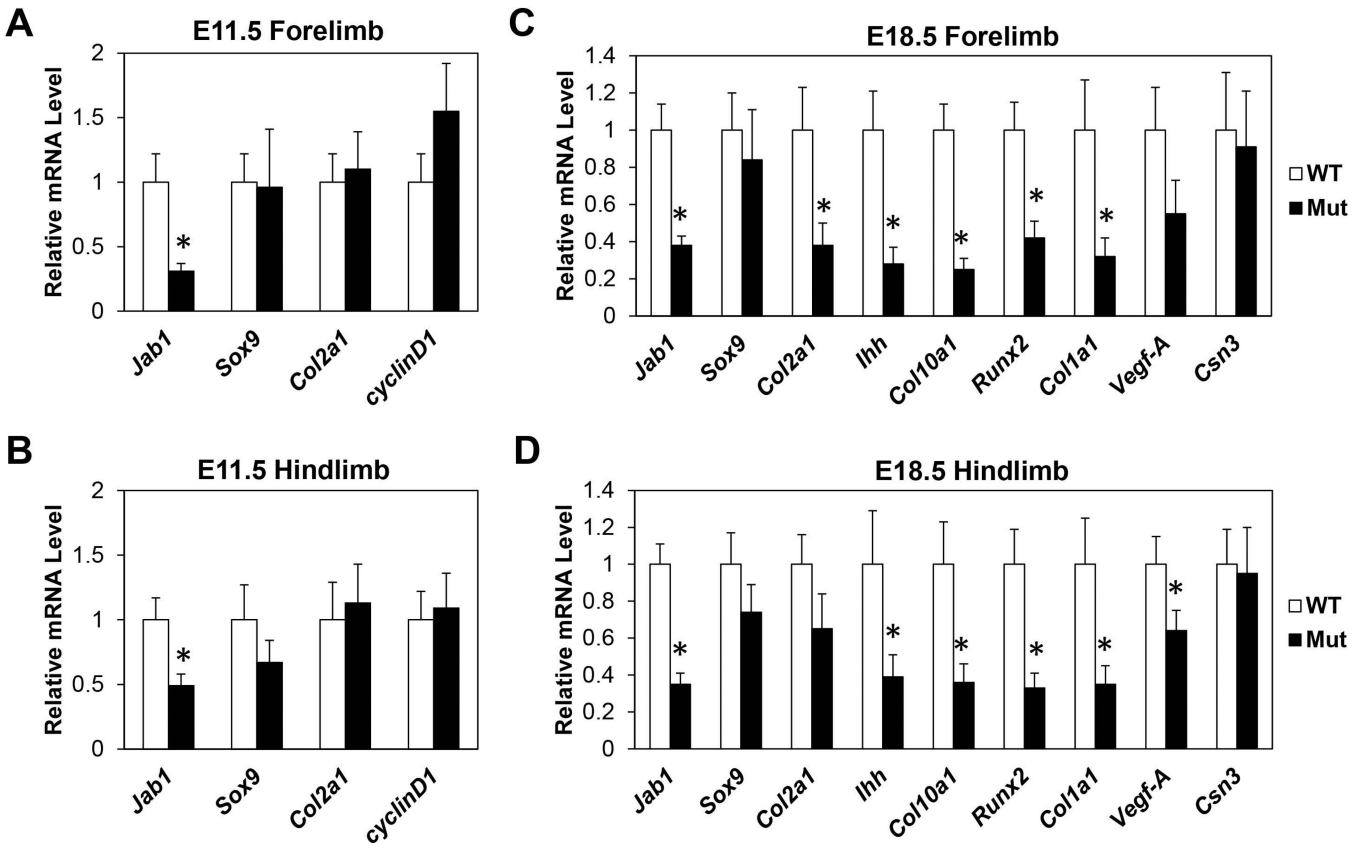

Figure 5. Decreased expression of skeletal differentiation markers in *Jab1 cKO* mutant mice

Real-time RT-PCR analysis of skeletal differentiation markers in *Jab1 cKO* mutant and wild-type E11.5 (A) forelimbs and (B) hindlimbs, and E18.5 (C) forelimbs and (D) hindlimbs. Data represents mean ± standard error, n≥4 individual mice per group. An asterisk (*) denotes a statistically different level of expression compared with wild-type, p≤0.05. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

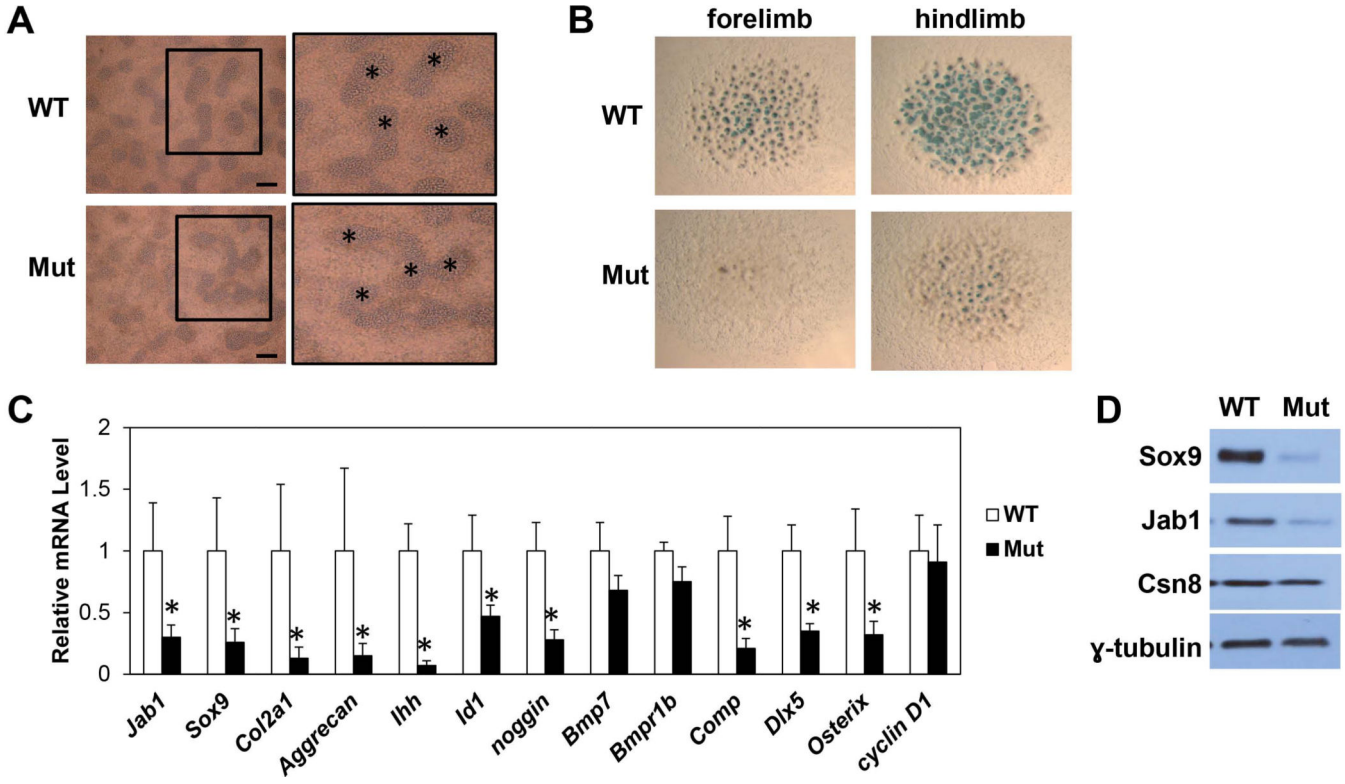

Figure 6. Decreased chondrogenesis in *Jab1 cKO* mutant micromass culture

(A) Mesenchymal condensation in both *Jab1 cKO* mutant and wild-type cultures on day 6. Right-column images are enlargements of the highlighted sections in the left-column images. An asterisk (*) represents a nodule. Scale bars, 100 μm. (B) Decreased alcian blue staining in *Jab1 cKO* mutant micromass cultures on day 6. (C) Real-time RT-PCR analysis of chondrocyte differentiation markers and BMP downstream targets on day 6. Data represents mean ± standard error, n≥4 individual mice per group. An asterisk (*) denotes a statistically different level of expression compared with wild-type, p≤0.05. (D) Western blot analysis of Sox9, Jab1, and Csn8 in *Jab1 cKO* mutant and wild-type hindlimb micromass cultures on day 6. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

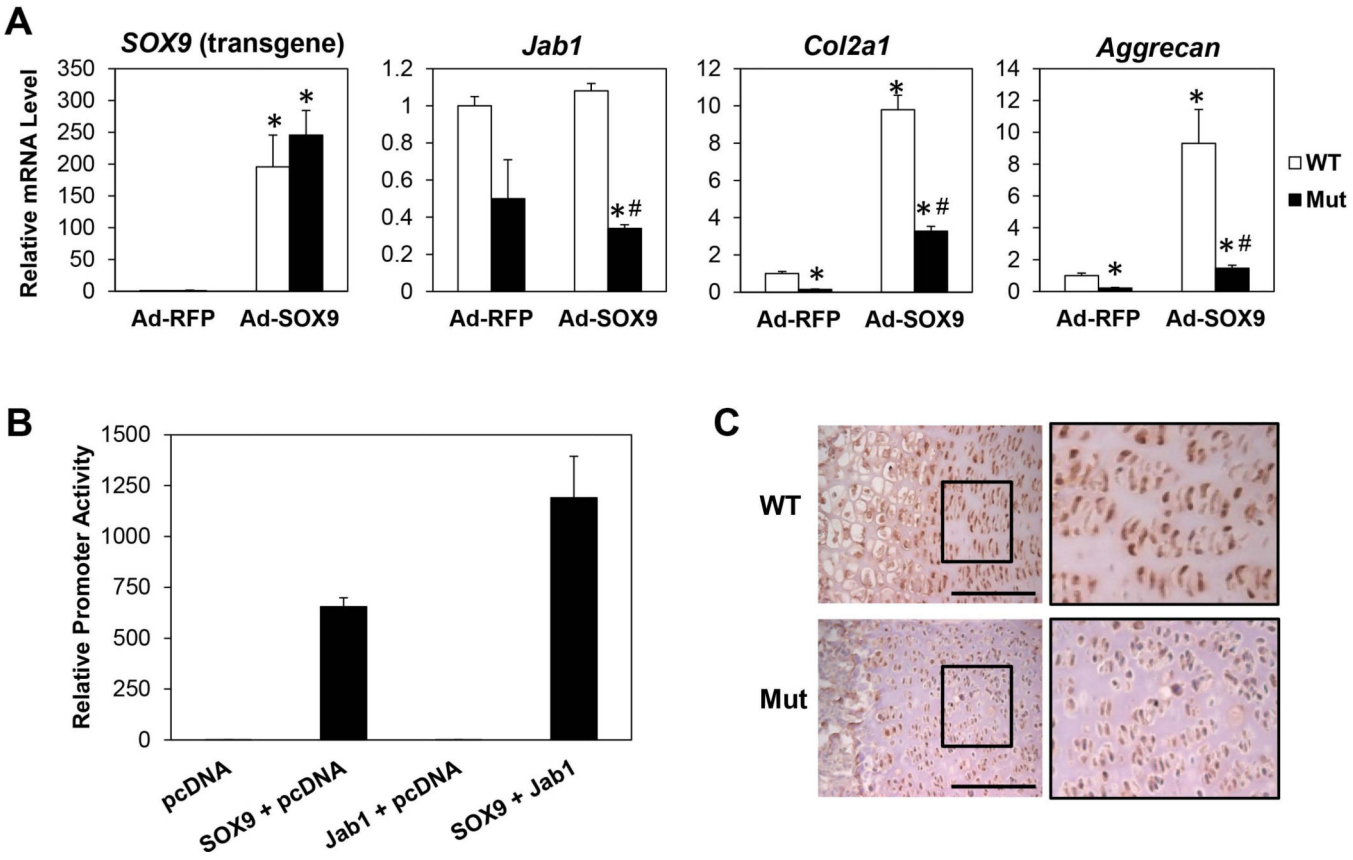

Figure 7. Jab1 likely promotes Sox9 activity during early limb development

(A) Real-time RT-PCR analysis of chondrocyte differentiation markers in *Jab1 cKO* mutant and wild-type hindlimb micromass cultures infected with adenoviruses Ad-RFP or Ad-SOX9 after 7 days of culture. Data represents mean ± standard error, n≥3 individual mice per group. An asterisk (*) denotes a statistically different level of expression compared with the WT + Ad-RFP group, p≤0.05. A pound sign (#) denotes a statistically different level of expression between the wild-type controls and mutant littermates with Ad-SOX9 infection, p≤0.05. (B) Jab1 co-activates Sox9 transcriptional activity *in vitro*. C3H/10T1/2 cells were co-transfected with a Sox9-responsive reporter *4x48-p89Col2a1-luc* and expression plasmids for Jab1 and SOX9. The promoter activity of C3H/10T1/2 cells transfected with pcDNA empty vectors was designated as 1. Data represents mean ± standard error, n=3 per group. (C) Decreased immunohistochemical staining of Sox9 in newborn *Jab1 cKO* mutant and wild-type tibias. Right-column images are enlargements of the highlighted sections in the left-column images. Scale bars, 50 μm. Mut, *Jab1 cKO* mutants; WT, wild-type littermates.

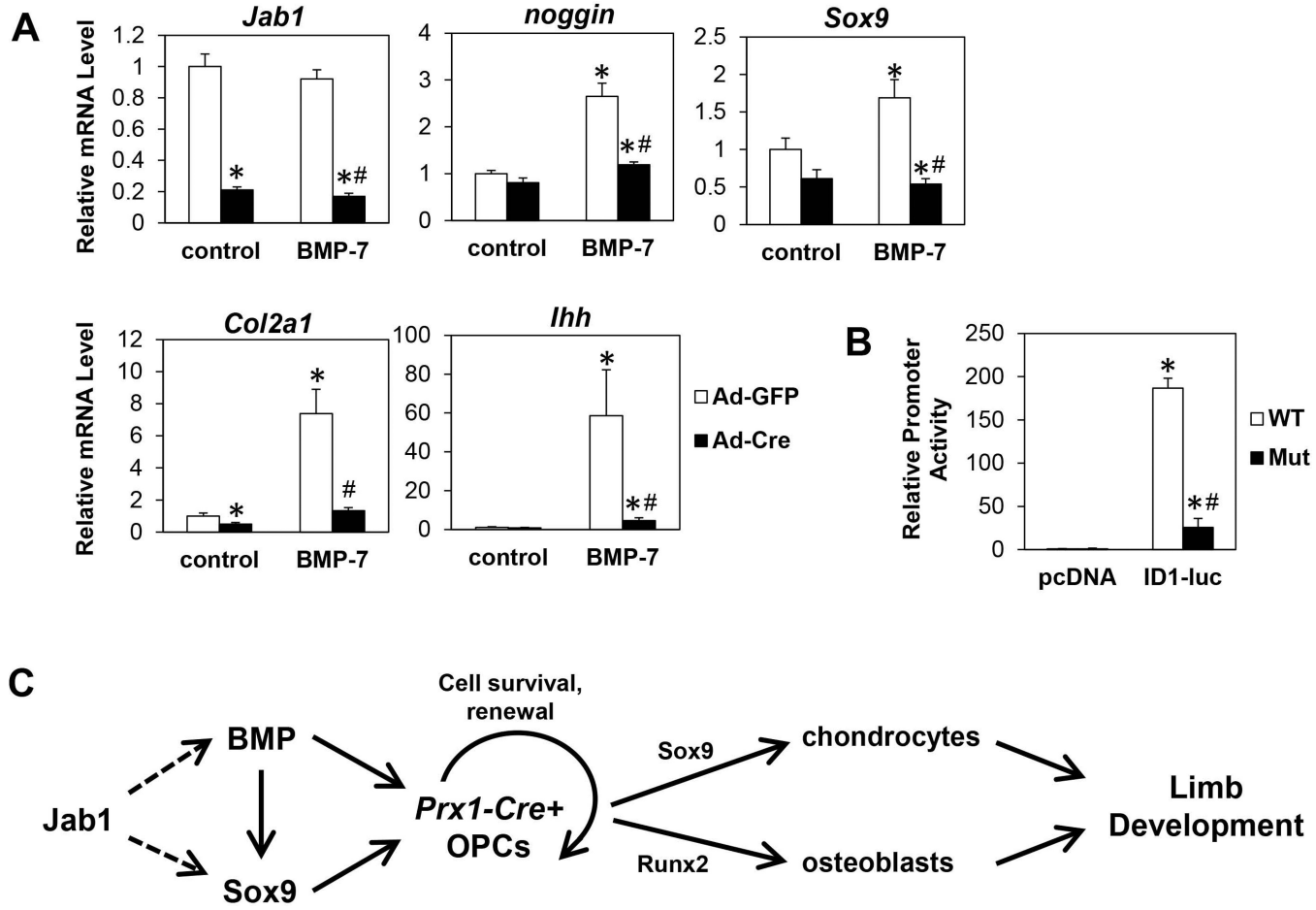

Figure 8. Jab1 likely promotes BMP signaling response in limb bud micromass culture

(A) Real-time RT-PCR analysis of chondrocyte differentiation markers and BMP downstream targets in *Jab1*$^{flox/flox}$ E11.5 limb bud cells infected with adenoviruses Ad-Cre or Ad-GFP. BMP-7 (100ng/mL) was added after 24 hrs of culture (day 1) and cells were collected on day 5. Data represents mean ± standard error, n=3 per group. An asterisk (*) denotes a statistically different level of expression with respect to the Ad-GFP control without treatment, p≤0.05. A pound sign (#) denotes a statistically different level of expression between the Ad-GFP control and Ad-Cre under BMP-7 treatment, p≤0.05. (B) *Jab1 cKO* mutant and wild-type E11.5 micromass cultures were transfected with a BMP-specific reporter *ID1-luc* or a *pcDNA* control plasmid and then cultured for 48 hrs. Data represents mean ± standard error, n=3 per group. An asterisk (*) denotes a statistically different level of expression compared with the WT + pcDNA group, p≤0.05. A pound sign (#) denotes a statistically different level of expression between the wild-type controls and mutant littermates with *ID1-luc* transfection, p≤0.05. (C) Proposed model for *the role of Jab1 in OPC-mediated embryonic limb development. Mut, Jab1 cKO* mutant; WT, wild-type littermates.