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The Transcriptional Cofactor Jab1/Cops5 is Crucial for BMP-Mediated Mouse Chondrocyte Differentiation by Repressing p53 Activity

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Abstract

We previously reported that the evolutionary conserved transcriptional cofactor Jab1/Cops5 is critical for mouse chondrocyte differentiation by selectively repressing BMP signaling. In this study, we first uncovered that the endogenous Jab1 interacts with endogenous Smad1/5/8. Furthermore, although Jab1 did not directly interact with Acvr1 (Alk2), a key type I BMP receptor, the interaction between endogenous Smad1/5/8 and Acvr1 was increased in *Jab1*-null chondrocytes. Thus, Jab1 might negatively regulate BMP signaling during chondrocyte differentiation in part by sequestering Smad1/5/8 away from Acvr1. Next, to identity Jab1 downstream targets in chondrocytes, we performed RNA-Sequencing analysis of *Jab1*-null chondrocytes and discovered a total of 1993 differentially expressed genes. Gene set enrichment analysis revealed that key targets inhibited by *Jab1* includes p53, BMP/TGF-β, and apoptosis pathways. We confirmed that endogenous Jab1 interacts with endogenous p53. There was significantly elevated p53 reporter activity, an enhanced expression of phospho-p53, and an increased expression of a key p53 downstream target, Puma, in *Jab1*-null chondrocytes. Moreover, treatments with a p53-specific inhibitor and/or a BMP type I receptor-specific inhibitor reversed the elevated p53 and BMP signaling activities in *Jab1*-null chondrocytes and partially restored columnar growth plate structure in E17.5 *Jab1*-null mouse tibia explant cultures. Finally, we

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The authors declare no potential conflicts of interest.

demonstrated that the chondrocyte-specific *Jab1* overexpression in mice resulted in smaller-sized embryos with disorganized growth plates. In conclusion, our data showed that the delicate Jab1-mediated crosstalk between BMP and p53 pathways is crucial to maintain proper chondrocyte survival and differentiation. Moreover, the appropriate Jab1 expression level is essential for proper skeletal development.

Keywords

Jab1/Csn5; COP9 Signalosome; p53; BMP; Chondrocytes

Introduction

Jun activation domain-binding protein 1 (JAB1), also known as COP9 signalosome subunit 5 (COPS5/CSN5), is the only catalytic subunit of the highly conserved macromolecular complex the COP9 Signalosome (CSN) (Kato & Yoneda-Kato, 2009). The importance of the COP9 Signalosome is underscored by the fact that the knockout of any individual subunits, so far, in mice, results in early embryonic lethality (Kato & Yoneda-Kato, 2009; G. Liu, Claret, Zhou, & Pan, 2018). Indeed, the constitutive deletion of *Jab1* in mice results in early embryonic lethality by E8.5 with impaired proliferation and increased apoptosis (Tian et al., 2010; Tomoda, Yoneda-Kato, Fukumoto, Yamanaka, & Kato, 2004). Furthermore, previous studies from our lab demonstrated that *Jab1* is required for the successive stages of skeletogenesis (Bashur et al., 2014; Chen et al., 2013).

As a versatile transcriptional cofactor, Jab1 plays an important role in cellular differentiation, cell cycle regulation, apoptosis, and DNA damage repair (Bashur et al., 2014; Chen et al., 2013; Claret, Hibi, Dhut, Toda, & Karin, 1996; G. Liu et al., 2018; Panattoni et al., 2008; Sitte et al., 2012). Previously we showed that Jab1 negatively regulates BMP signaling in chondrocytes (Chen et al., 2013). Canonical BMP signaling is primarily mediated by the downstream effectors Smad1/5/8. The activation of Smad1/5/8 is mediated by the BMP receptors including Alk1, 2, 3, and 6 (Song, Estrada, & Lyons, 2009). The activated R-Smads (Smad1/5/8) then form a complex with Smad4 (co-Smad) and translocate into the nucleus to initiate the transcription of BMP target genes. The chondrocyte-specific conditional knockout (cKO) of Smad1/5/8 led to lethal chondrodysplasia (Retting, Song, Yoon, & Lyons, 2009), confirming the essential role of BMP signaling in chondrocyte differentiation. BMP type 1 receptor Acvr1 (Alk2) is one of the seven known BMP/TGF-β type 1 receptors that transduce the extracellular signal to nucleus (Akhurst & Hata, 2012). Notably, the gain of function mutations in ACVR1 result in increased pSmad1/5/8, leading to Fibrodysplasia Ossificans Progressiva (FOP), a severe skeletal dysplasia in humans (Meyers et al., 2019; Shore et al., 2006). Moreover, the inhibitory Smad6 inhibits BMP signaling whereas Smad7 inhibits both BMP and TGFβ signaling. Thus, BMP signaling is extremely complex for chondrocyte differentiation and skeletal development. Previously we showed that Jab1 selectively modulates BMP signaling in chondrocytes and osteochondro-progenitor cells *in vivo* (Bashur et al., 2014; Chen et al., 2013). However, it remains to be determined how Jab1 modulates BMP signaling in chondrocytes.

The tumor suppressor p53 tightly regulates cell survival in response to genotoxic damage and other types of cellular stress (Kruiswijk, Labuschagne, & Vousden, 2015). Upon stress, p53 stabilizes in the cell and activates key apoptotic pathways (Lee, Kim, Choi, Ha, & Kim, 2006). The constitutive p53 activation results in segmental progeria in mice (D. Liu et al., 2010). The stabilization of p53 is largely mediated through posttranslational modifications, such as phosphorylation and acetylation, as well as by protein-protein interactions (Appella & Anderson, 2001; Lee et al., 2006). In recent years, it has been revealed that increased p53 activity underlies the developmental defects in a wide range of genetic syndromes (Bowen & Attardi, 2019). Interestingly, Jab1 was also reported to regulate p53, either by directly promoting p53 cytoplasmic translocation and degradation, or by promoting the stability of the Mdm2 complex, which is a major E3 ubiquitin ligase for p53 (Bech-Otschir et al., 2001; Oh et al., 2006; Zhang, Chen, Su, Yang, & Lee, 2008). But whether Jab1 regulates p53 in chondrocytes was unknown.

Our previously published study showed that in mice, the chondrocyte-specific conditional knockout of *Jab1* (*Jab1 cKO*) resulted in a lethal chondrodysplasia phenotype at birth (Supplemental Fig. S1A–1C). *Jab1 cKO* chondrocytes displayed increased apoptosis, accelerated chondrocyte hypertrophy, G2 phase cell cycle arrest, and increased BMP response (Chen et al., 2013). In the present study, we performed Jab1 immunoprecipitations to determine whether Jab1 interacts with the key components of the BMP and p53 signaling pathways in chondrocytes. Then, the unbiased RNA sequencing analysis was conducted to identify key Jab1 downstream targets in *Jab1 cKO* chondrocytes. A reporter screening assay was also performed to identify the drastically altered downstream signal transduction pathways in *Jab1 cKO* chondrocytes. Both p53 and BMP inhibitors were also tested *ex vivo* and in whole explant organ cultures to rescue the *Jab1 cKO* phenotype. Finally, we generated a novel, chondrocyte-specific Jab1 overexpression mouse model, which resulted in smaller-sized embryos with disorganized growth plate. In summary, this study significantly advances our understanding of the complex Jab1 regulatory network in chondrocyte differentiation.

Materials and Methods

Animals and Transgenic Construct

All mice were housed and maintained at Case Western Reserve University's animal facility under standard conditions. Animal protocols were approved by the IACUC of Case Western Reserve University. The full-length FLAG-tagged Jab1 cDNA was cloned into a *Col2a1-WPRE* transgenic expression vector as described previously (Weinstein, Tompson, Chen, Lee, & Cohn, 2014; Zhou et al., 1995). Mice genotyping was performed as previously described (Chen et al., 2013).

Limb explants and primary cell culture

E18.5 wild-type (WT) and *Jab1 cKO* primary rib chondrocytes were isolated as previously described (Chen et al., 2013). When indicated, primary chondrocytes were also treated with doxorubicin (Sigma) to induce p53 activation. For limb explant cultures, mouse E17.5 tibiae were micro-dissected, and placed in non-treated polystyrene 96-well plates, and cultured in

α-minimal essential medium (Gibco), supplemented with 0.2% bovine serum albumin, 1mM β -glycerophosphate, and 50 g/ml ascorbic acid. WT and *Jab1 cKO* mutant tibiae were treated with the p53-specific inhibitor, Pifithrin-α hydrobromide (PFTα) (Santa Cruz Biotech), and/or the BMP type I receptor-specific inhibitor, LDN-193189 (Selleckchem), for two weeks. The tibiae were subsequently analyzed for total length and growth plate histology.

RNA isolation, cDNA synthesis and real-time RT-PCR

RNA was extracted from primary chondrocytes using the TRIzol reagent (Invitrogen) and PureLink RNA Mini kit (Invitrogen) as previously described (Chen et al., 2013). 1μg of total RNA was reverse transcribed to cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The ABI 7500 real-time PCR System (Applied Biosystems) was used to perform real-time qPCR analysis with the Power SYBR Green Master Mix (Applied Biosystems) and gene-specific primers. Gene expression was quantified using the comparative threshold cycle ($\Delta\Delta C_t$) method as described in (Liang, Cotter, Chen, Hernandez, & Zhou, 2012). *Gapdh* was used as an internal control.

RNA-sequencing analysis

RNA-sequencing was performed at the Genomics Core at Case Western Reserve University, using three WT and three *Jab1 cKO* mutant littermates. The bioinformatics analysis was performed at the Case Computational Biology Core Facility. RNA sequencing libraries were prepared using the TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, San Diego, CA, USA). This kit allows the generation of strand-specific data while simultaneously removing both cytoplasmic and mitochondrial rRNA. Libraries were sequenced on the HiSeq 2500 instruments and 50 million 50-base pair single end reads were produced per sample. Data was extracted, de-convoluted based on dual index reads, and analyzed using CLC Genomics Workbench software (Qiagen, Redwood City, CA, USA). Sequencing reads generated from the Illumina platform were assessed for quality using FastQC (Babraham Institute, Babraham, UK). The reads were then trimmed for adapter sequences using TrimGalore (Babraham Institute). Reads that passed quality control were then aligned to the human reference genome (hg38) using STAR aligner. The alignment for the sequences was guided using the Gencode gene annotation for human version 25. The STAR aligned reads were then analyzed for differential gene expression using cufflinks, a RNASeq analysis package which reports the fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. Differential genes were identified using a significance cutoff of FDR < 0.05. These genes were then subjected to gene set enrichment analysis using the MSigDB from the Broad Institute to determine any relevant processes that may be differentially over-represented for the conditions tested. For GSEA, FDR q-value < 0.25 is significant.

Co-immunoprecipitation

Primary chondrocytes and rat chondrosarcoma (RCS) cell pellets were lysed, and immunoprecipitation and subsequent western blotting were performed as described (Chipuk et al., 2002) with Protein G Dyanabeads (Life Technologies). Antibodies and their dilutions used in this study can be found in Supplementary Table 1.

Cignal Reporter Array

The Cignal Reporter Array (Qiagen # CCA-901L-12) was performed according to the manufacturer's protocol. Briefly, primary chondrocytes were reverse transfected in 96-well plates. The reporter activity was measured 48 hours after transfection, using the Dual Luciferase Assay (Promega) according to the manufacturer's protocol. A detailed list of the pathways and the targeted transcription factors in the Cignal Reporter Array is provided in Supplementary Table 2.

Statistical Analysis

All experiments were performed in at least triplicates. The statistical analysis was performed using a two-tailed Student's t-test. P values < 0.05 were considered statistically significant.

Results

Endogenous Jab1 selectively interacts with endogenous BMP downstream effectors in mouse primary rib chondrocytes.

To understand how Jab1 regulates BMP/TGFβ signaling pathways, we first performed Jab1 immunoprecipitation (IP) in primary chondrocytes, and found that endogenous Jab1 interacted with endogenous Smad1/5/8 but not with endogenous Smad2/3, Smad4 and Smad7 in chondrocytes (Fig. 1A). This suggests that Jab1 acts through BMP signaling by specifically interacting with endogenous Smad1/5/8 in mouse primary chondrocytes. Furthermore, we observed the co-localization of Jab1 and Smad1/5/8 (Fig. 1B), which confirms endogenous Jab1 interaction with Smad1/5/8 in mouse chondrocytes. These results are consistent with our previously published results that Jab1 likely acts mainly through BMP signaling but not via TGF-β signaling in mouse chondrocytes (Chen et al., 2013).

The enhanced pSmad1/5/8 levels in *Jab1 cKO* chondrocytes are likely partly due to increased binding of Smad1/5/8 to Acvr1

In our previous study, we found that chondrocyte-specific *Jab1* deletion resulted in increased levels of phosphorylated Smad1/5/8 and it is partly BMP type 1 receptor-dependent (Chen et al., 2013). To further understand the underlying mechanism for increased pSmad1/5/8 levels in *Jab1 cKO* chondrocytes, we collected primary rib chondrocyte from E18.5 WT and *Jab1 cKO* mutant littermates, performed Smad1/5/8 IP experiments, and subsequently immunoblotted the membranes with anti-Smad1/5/8, Jab1, Acvr1 and Pp2a antibodies. As expected, we confirmed that the endogenous Smad1/5/8 interacts with endogenous Jab1 in the WT chondrocytes (Fig. 1C). Most importantly, we also noticed there was a drastically enhanced binding of Acvr1, a key BMP type I receptor, to Smad1/5/8 in *Jab1 cKO* chondrocytes compared with WT chondrocytes (Fig. 1C). In contrast, between WT and *Jab1 cKO* chondrocytes, we observed no significant differences of Smad1/5/8 interaction with Pp2a, a protein phosphatase known to dephosphorylate both Smads1/5/8 and the BMP type I receptors to downregulate BMP signaling pathway (Fig. 1C). Furthermore, we observed similar expression levels of total Acvr1 and Pp2a in WT and *Jab1 cKO* chondrocytes (Fig. 1D). Thus, the stronger interaction of Acvr1 with Smad1/5/8 in *Jab1 cKO* chondrocytes might at least partially account for the enhanced pSmad1/5/8 expression. Interestingly, the

expression of Bmpr1b, another major BMP type I receptor, was decreased in *Jab1 cKO* chondrocytes (Supplemental Figure S1D), suggesting a differential role of Jab1 on different BMP signaling components.

RNA-sequencing revealed that the p53, apoptosis, and BMP signaling pathways are major Jab1 downstream targets in mouse primary chondrocytes

To identify the critical Jab1 downstream targets in primary chondrocytes, we conducted an unbiased RNA-sequencing analysis. We identified a large Jab1-mediated transcriptome in *Jab1 cKO* chondrocytes, with 1060 and 933 genes that were up- and downregulated, respectively (Fig. 2A). We then used the standard bioinformatics tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang da, Sherman, & Lempicki, 2009), to identify the most significantly altered Gene Ontology (GO) terms regarding molecular functions, cellular components, and biological processes in *Jab1 cKO* chondrocytes. For the significantly upregulated genes, a large number of genes were involved in protein binding and cell adhesion, and mainly localized in the extracellular matrix (Fig. 2B). On the other hand, for the significantly downregulated genes, a large number of genes were involved in protein binding and mainly localized in the plasma membrane and cytoplasm (Fig. 2C). We also performed the standard Gene Set Enrichment Analysis (GSEA) to identify the key biological pathways that are altered in *Jab1-cKO* chondrocytes (Fig. 3A). GSEA analysis demonstrated that the p53, apoptosis, and BMP/TGFβ signaling pathways are the top three most significantly enhanced key developmental pathways (Fig. 3A, Supplemental Fig. S2 and S3). GSEA analysis also demonstrated that the Heme Metabolism and Complement pathways are significantly downregulated in *Jab1 cKO* chondrocytes (Supplemental Fig. S4). However, their significance in Jab1-mediated chondrocyte differentiation remains to be further analysed.

Our RNA-sequencing analysis revealed that the expression of p53 downstream targets such as *Cdkn1a* and *Gadd45a* were also increased in *Jab1 cKO* chondrocytes (Fig 3B). Indeed, in rat chondrosarcoma cells (Mukhopadhyay et al., 1995), p53-IP confirmed a direct interaction between Jab1 and p53 (Fig. 3C). We next performed an unbiased reporter screening assay to identify the significantly altered pathways in *Jab1 cKO* chondrocytes, which also confirmed p53 reporter activity was increased 1.6 fold (Fig. 3D). Apart from p53, the activities of four other reporters, including endoplasmic reticulum stress, interferon regulation, xenobiotic stress response, and the androgen pathway, were also clearly upregulated in *Jab1 cKO* chondrocytes. However, the significance of those pathways in Jab1-mediated chondrocyte differentiation remains to be investigated.

Inhibition of p53 and BMP signaling partially reversed the *Jab1 cKO* chondrocyte phenotype

Next, we used a p53-specific inhibitor, Pifithrin-α (PFT-α), and a BMP type I receptor-specific inhibitor, LDN-193189, to confirm that p53 and BMP signaling pathways are indeed major Jab1 downstream targets in *Jab1 cKO* chondrocytes. As expected, the hypertrophic chondrocyte-related genes, *Col10a1, Ihh*, and *Id1*, all of which are also BMP downstream targets, were elevated in *Jab1 cKO* chondrocytes (Fig. 3E) (Chen et al., 2013). Upon treatment with PFT-α and/or LDN-193189, the expression levels of all these genes were

restored to close to the WT levels in *Jab1 cKO* chondrocytes (Fig. 3E) (Chen et al., 2013). Western blot analysis revealed that the increased phospho-p53 and phospho-Smad1/5/8 expressions in *Jab1 cKO* chondrocytes were also restored to similar levels as in WT chondrocytes upon treatment with PFT-α and/or LDN-193189 (Fig. 4A). The increased expressions of Puma, a key p53 target gene, and Bax, a key pro-apoptotic signal, in *Jab1 cKO* chondrocytes were also reduced upon treatment with PFT-α and/or LDN-193189 (Fig. 4A), as confirmed by the quantification of the western blot results (Fig. 4B). Additionally, *Jab1 cKO* chondrocyte cultures were more prone to lose the typical chondrocyte morphology (Fig. 4C). However, after treatment with PFT-α and/or LDN-193189, *Jab1 cKO* chondrocytes appeared to regain typical chondrocyte morphology (Fig. 4C). Immunofluorescence analysis revealed that the expression of the most abundant chondrocyte-specific cartilage matrix protein, Col2a1, was significantly reduced in *Jab1 cKO* chondrocytes (Fig. 4C). However, Col2a1 expression was greatly restored in *Jab1 cKO* chondrocytes upon treatment with PFT-α and/or LDN-193189 (Fig. 4C). Our previous study reported the increased apoptosis in *Jab1 cKO* chondrocytes (Chen et al., 2013). Indeed, the expression of a p53 downstream target and pro-apoptotic marker, cleaved caspase 3, was also increased in *Jab1 cKO* chondrocytes compared with WT chondrocytes. Again, the treatment with PFT-α and LDN-193189, either separately or together, diminished the cleaved caspase 3 expression in *Jab1 cKO* chondrocytes (Fig. 4C). Moreover, E17.5 *Jab1 cKO* tibia explants, upon treatment with PFT-α and/or LDN-193189, exhibited increases in whole tibia length compared with untreated controls (Fig. 5A). The immunofluorescence analysis of pSmad1/5/8 and Col10a1 expressions revealed elevated pSmad1/5/8 (BMP signaling) and Col10a1 (chondrocyte hypertrophy) expressions in *Jab1 cKO* limb (tibia) chondrocytes (Fig. 5B), which were also reversed upon treatment with PFT-α and/or LDN-193189 (Fig. 5B). Furthermore, histological examinations revealed that the completely disorganized growth plates in *Jab1 cKO* tibiae were partially restored to normal columnar structure after treatment with PFT-α and/or LDN-193189 (Fig. 6A). The decreased ratio of the length of proliferating zone chondrocytes to hypertrophic zone chondrocytes in *Jab1 cKO* tibiae growth plates was also partially restored to that of the wild-type tibiae upon treatment with PFT-α and/or LDN-193189 (Fig. 6B). Taken together, these results indicate that the inhibition of p53 and BMP signaling pathways can partially rescue the major defects in *Jab1 cKO* chondrocytes.

Jab1 overexpression in chondrocytes results in perturbed mouse growth plates

Finally, to study the effect of *Jab1* overexpression in chondrocytes *in vivo*, we generated *Col2a1-Jab1* transgenic mice using a well-characterized, chondrocyte-specific *Col2a1* promoter-enhancer element (Weinstein et al., 2014; Zhou et al., 1995) (Fig. 7A). The *Col2a1-Jab1* transgenic mice appeared smaller compared with wild-type littermate controls at E18.5 (Fig. 7B). Compared with wild-type littermates, the proximal tibiae of E18.5 *Col2a1-Jab1* transgenic mice appears to be much thinner and shorter with an increased ratio in the length of the proliferating zone to hypertrophic zone chondrocytes (Fig. 7C). Hematoxylin and eosin staining of the proximal tibiae of E18.5 *Col2a1-Jab1* transgenic mice showed much fewer cells compared with the wild-type littermates. Moreover, there was a mixture of proliferating and hypertrophic chondrocytes in *Col2a1-Jab1* transgenic mice (Fig. 7D; enlarged). Interestingly, all *Col2a1-Jab1* transgenic mice died at birth, suggesting that a

proper and finely tuned Jab1 expression level is critical for normal cartilage formation (Supplemental Figures S1E and S5).

To determine the effect of Jab1 overexpression *ex vivo,* mouse primary rib chondrocytes were infected with adenoviruses expressing Jab1 (Ad-Jab1) or red fluorescent protein as a control (Ad-RFP). Real-time RT-PCR analysis revealed a significant decrease in the hypertrophic chondrocyte markers *Col10a1* and *Ihh* in Ad-Jab1 infected chondrocytes (Fig. 7E). Finally, immunohistochemistry results confirmed the decreased pSmad1/5/8 and Col10a1 expressions in the growth plates of E18.5 *Col2a1-Jab1* transgenic mice compared with wild-type littermates (Fig. 7F). These results are consistent with an overall inhibitory role of Jab1 on BMP-mediated chondrocyte hypertrophy and indicate that Jab1 overexpression in mouse chondrocytes can lead to dysregulated chondrocyte differentiation and abnormal cartilage formation.

Discussion

Our present study provides significant and novel insights into how Jab1 regulates chondrocyte differentiation by repressing p53 and BMP signaling. Our results demonstrate that endogenous Jab1 interacts with endogenous Smad1/5/8 and p53 in chondrocytes. Mechanistically, increased pSmad1/5/8 levels in *Jab1 cKO* chondrocytes might be, at least in part, due to an increased interaction between Smad1/5/8 and a key BMP type I Receptor, Acvr1. Our RNA-sequencing analysis uncovered key roles of p53/BMP/apoptotic pathways in *Jab1 cKO* chondrocytes. Indeed, we showed that the inhibition of both the BMP and p53 pathways could partially rescue the *Jab1 cKO* phenotype *ex vivo* and in whole tibia organ cultures, confirming that both the BMP and p53 pathways are indeed major downstream targets repressed by Jab1 in chondrocytes (Fig. 8). Thus, we hypothesize that the increased BMP signaling activity in *Jab1 cKO* chondrocytes might also enhance p53 transcriptional activity, which leads to the activation of p53 downstream targets Puma, Bax, and cleaved caspase 3 (Figure 8).

BMP signaling is required not only for the early stages of chondrocyte condensation, but is also essential for chondrocyte proliferation and maturation at later stages (Wang, Rigueur, & Lyons, 2020). For BMP signaling, there is a considerable degree of combinatorial mixing and matching at the levels of the ligands, receptors, antagonists, effectors, and a wide variety of Smad-interacting proteins. This regulatory wiring produces a large diversity in transcriptional outputs, to exert the precise cell context-dependent control during skeletal development.

Acvr1 is one of the seven known BMP type I receptors that transduce the extracellular signal to the nucleus (Rigueur et al., 2015; Akhurst et al., 2012). The gain of function mutation in ACVR1 results in increased pSmad1/5/8, leading to a severe skeletal dysplasia, fibrodysplasia ossificans progressiva (FOP), which is characterized by severe heterotopic ossification (Kaplan, Al Mukaddam, Stanley, Towler, & Shore, 2020; Meyers et al., 2019). Interestingly, the treatment with a BMP type I receptor-specific inhibitor LDN-193189 resulted in reduced heterotopic ossification in a mouse FOP model (Yu et al., 2008). Previously, we showed that the elevated pSmad1/5/8 expression levels in *Jab1 cKO*

chondrocytes were also partially rescued upon treatment with LDN-193189 (Chen et al., 2013). Therefore, in this study we focused on the effect of Jab1 loss on Acvr1-Smad1/5/8 signaling axis. Indeed, our overall results confirmed that the enhanced pSmad1/5/8 expression is likely to be partly due to the enhanced binding of Acvr1 with Smad1/5 in the *Jab1 cKO* chondrocytes. Since BMP signaling is highly complex and finely tuned at many different levels, it would be very interesting to determine the potential, specific effects of Jab1 on other key BMP signaling components in the future. Indeed, intriguingly, *Bmpr1b* expression was decreased in *Jab1 cKO* chondrocytes (Supplemental Figure 1D).

Although p53 knockout mice are grossly normal (Donehower et al., 1992), recent studies showed that p53, a well-known keeper of genome, is also activated in many developmental syndromes (referred to as p53-associated syndromes) (Bowen & Attardi, 2019; Bowen et al., 2019; Van Nostrand et al., 2014). Interestingly, our results showed that the inhibition of p53 in *Jab1 cKO* chondrocytes promotes their survival and partially rescues the differentiation defect. Therefore, it might be interesting to study the JAB1 status in clinical samples of p53-associated developmental syndromes to further elucidate the potential role of JAB1 in p53-associated developmental defects.

Interestingly, a previous study demonstrated that Smad1 interacts with p53 to modulate the DNA damage response in mouse embryonic fibroblasts (Chau et al., 2012). A recent study also showed that an increased BMP signaling in the neural crest impedes normal nasal cartilage morphogenesis by promoting p53-mediated apoptosis (Hayano, Komatsu, Pan, & Mishina, 2015). In this study, we found that endogenous Jab1 interacts with endogenous Smad1/5/8 and p53 to modulate chondrocyte differentiation and apoptosis (Fig. 1A, C and 3C). Thus, we hypothesize that increased pSmad1/5/8 levels in *Jab1 cKO* chondrocytes might also results in p53 stabilization. In summary, our present study revealed for the first time that Jab1-mediated BMP-p53 signaling is crucial for maintaining proper chondrocyte survival and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability Statement

The data that support the findings of this study are openly available in the Gene Expression Omnibus (GEO) at www.ncbi.nlm.nih.gov/geo/, reference number GSE143249.

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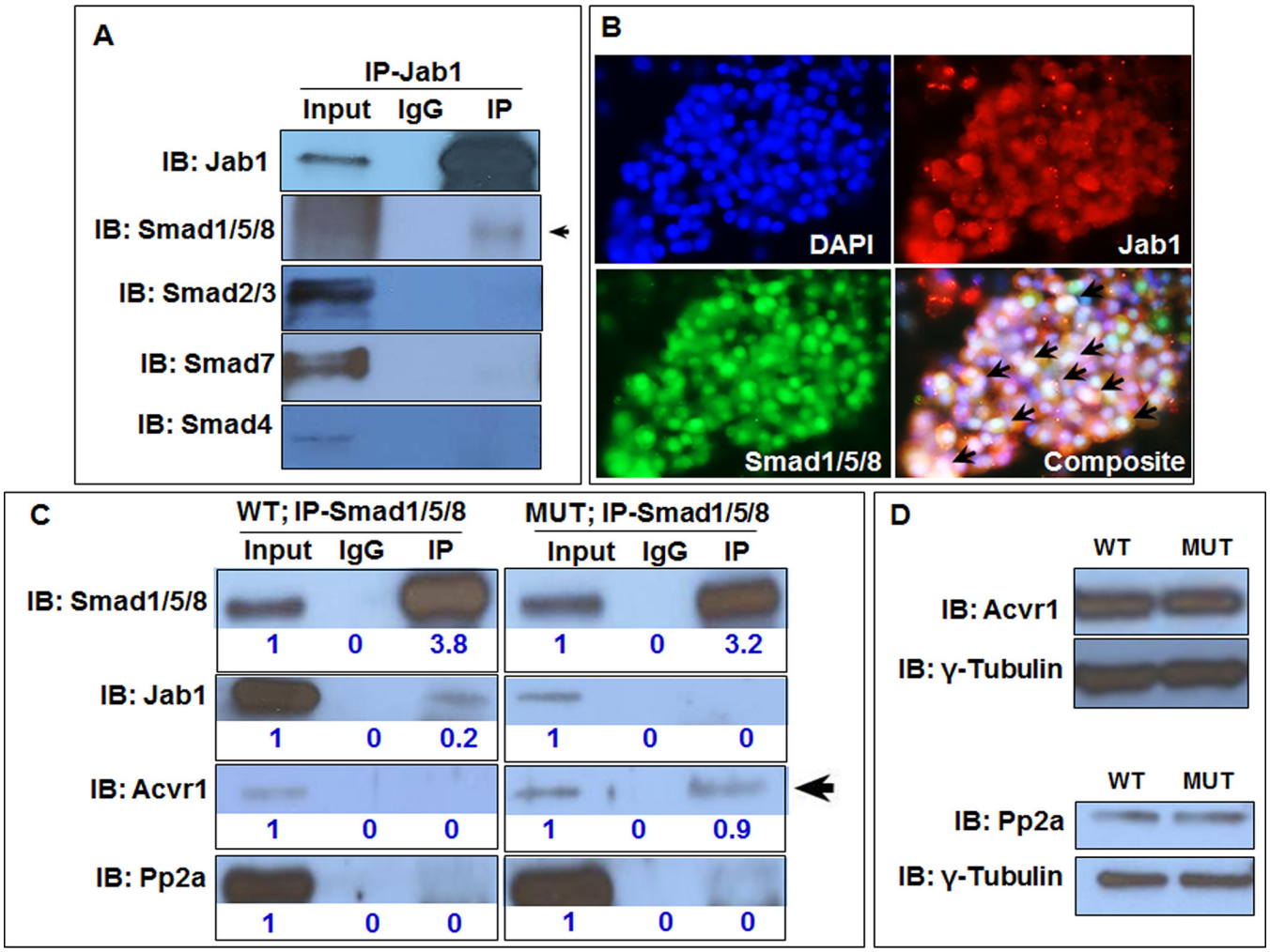

Figure 1. The endogenous Jab1 and Smad1/5/8 interaction in mouse primary chondrocytes.

(A) The immunoprecipitation (IP) was performed using a Jab1 antibody and the IP membrane was blotted with Smad1/5/8, Smad2/3, Smad7, and Smad4 antibodies, respectively. Input represents 5% protein lysates before IP. IgG was used as IP control. The pools of 3–5 embryos per genotype were used to perform the western blot analysis. (B) Co-localization of Jab1 and Smad1/5/8 in chondrocytes by immunofluorescence. Jab1, red; Smad1/5/8, green. Counter staining, DAPI, blue. (C) The Acvr1-Smad1/5/8 interaction. The pools of 3–5 embryos per genotype were used to perform the western blot analysis. Image J quantification values are shown in blue underneath the bands. The intensity of each input band is designated as 1. (D) Total Acvr1 and Pp2a protein levels in WT and *Jab1 cKO* chondrocytes.

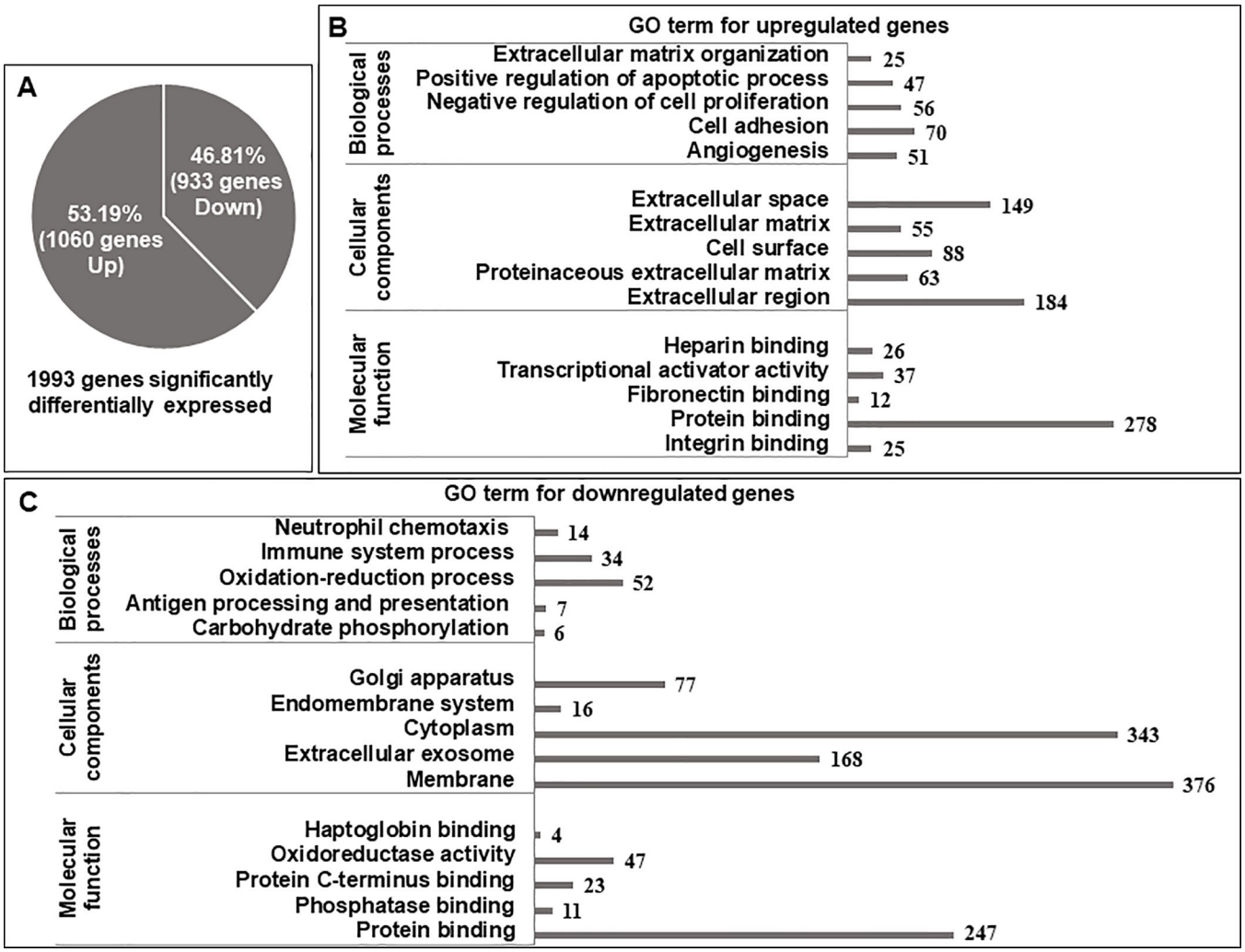

Figure 2. RNA-sequencing analysis in *Jab1 cKO* chondrocytes.

(A) A pie chart of significantly up- and downregulated genes in *Jab1 cKO* chondrocytes. (B) Significantly enriched Gene Ontology (GO) terms for upregulated genes in *Jab1 cKO* chondrocytes by DAVID analysis. (C) Significantly enriched GO terms for downregulated genes in *Jab1 cKO* chondrocytes by DAVID analysis. N = 3/genotype.

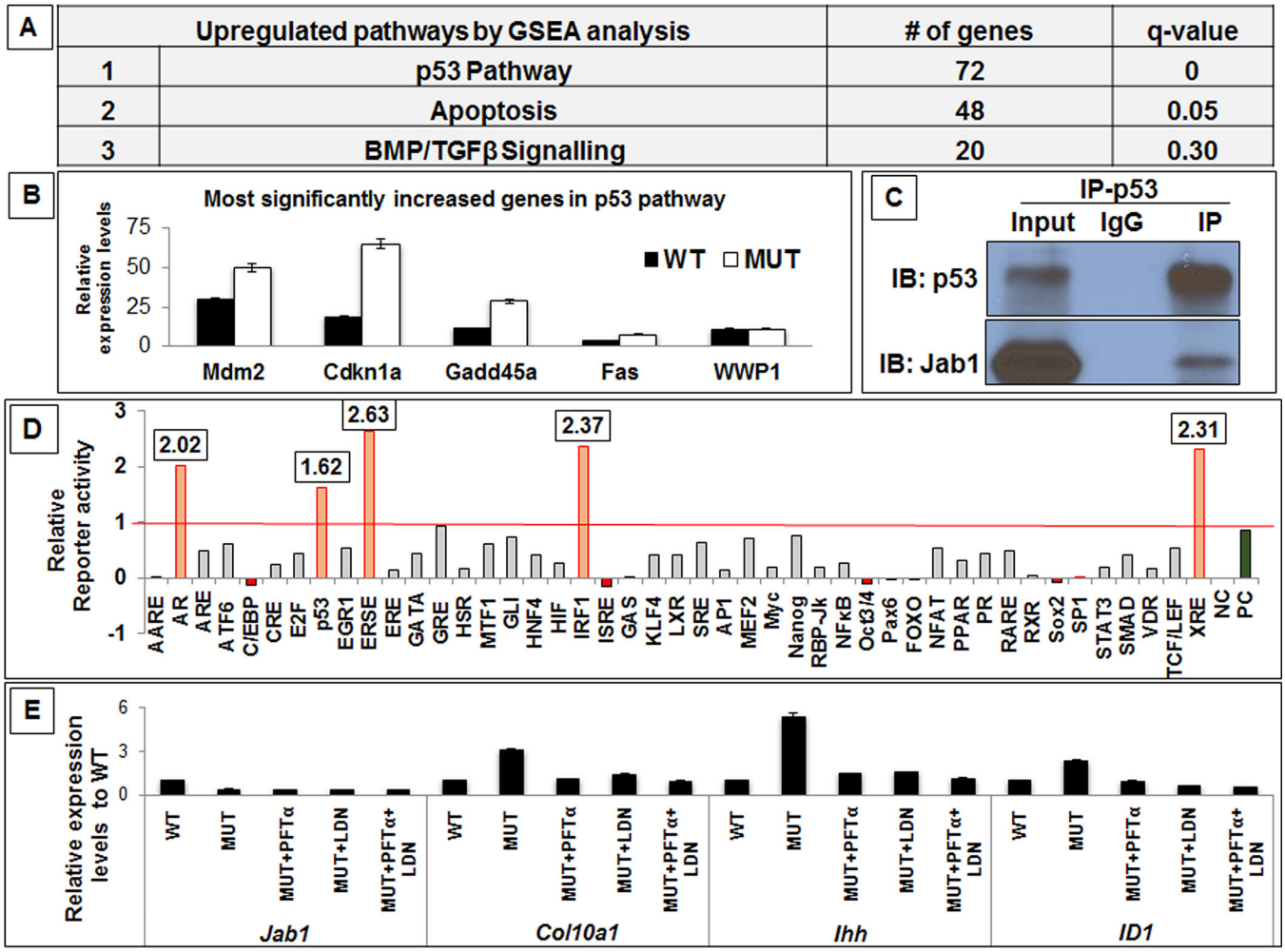

Figure 3. The p53 pathway is a key Jab1 downstream target in chondrocytes.

(A) Gene set enrichment analysis of RNA-sequencing data uncovered elevated p53 signaling activity in *Jab1 cKO* chondrocytes along with the apoptosis and BMP signaling pathways. (B) Enhanced expression of p53 target genes as identified by RNA-sequencing analysis. (C) The immunoprecipitation showed the interaction between endogenous Jab1 and p53 in rat chondrosarcoma cells. (D) A cignal reporter array confirmed that the *Jab1 cKO* chondrocytes display enhanced p53 signaling activity. Red line indicates the reporter activity of the positive control. (E) Real-time RT-qPCR analysis revealed that the increased expressions of the chondrocyte differentiation markers *Col10a1, Ihh,* and *Id1* in *Jab cKO* chondrocytes were reversed upon treatment with 1μM 53-specific inhibitor PFT-α and/or 100nM BMP type I receptor-specific inhibitor LDN-193189 (n = 3/genotype).

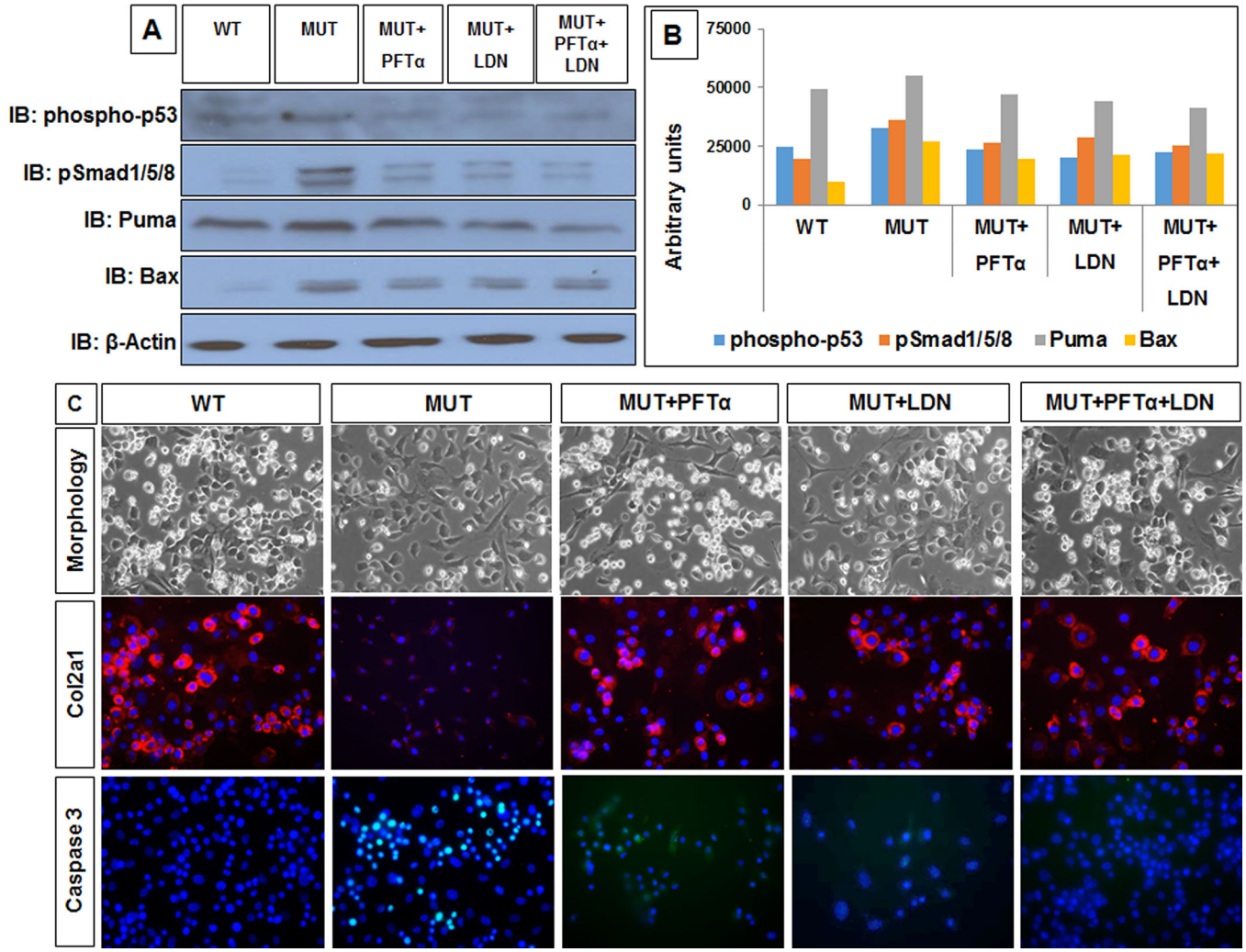

Figure 4. The inhibition of the p53 and BMP signaling pathways in *Jab1 cKO* chondrocytes partially restores typical chondrocyte morphology.

(A) Western blot analysis revealed that the elevated phospho-p53, pSmad1/5/8, Puma, and Bax expressions in *Jab1 cKO* rib primary chondrocytes were partially reversed upon treatment with PFT-α and/or LDN-193189. The pools of 3–5 embryos per genotypes were used to performed the western blot analysis. The primary chondrocytes were first treated with 2.0μM doxorubicin (Sigma) for four hours to induce p53 expression, then were incubated with 1μM PFT-α and/or 100nM LDN-193189 overnight. A representative blot from two independent experiments with similar results is shown. (B) The Image J quantification of western blot bands in Figure 4A. (C) Phase contrast images showing the morphology of WT and *Jab1 cKO* chondrocytes treated with PFT-α and LDN-193189 either alone or together. Counter staining, DAPI, blue. Immunofluorescence (IF) analysis confirmed the restored expression of Col2a1 (red) in *Jab1 cKO* rib primary chondrocytes after treatment with PFT-α and LDN-193189 either alone or together. The enhanced apoptosis was also confirmed in *Jab1 cKO* chondrocytes by IF staining of apoptotic marker cleaved caspase 3 (green). The treatment with PFT-α and/or LDN-193189 either alone or together resulted in the reduced or the absent expression of cleaved caspase 3.

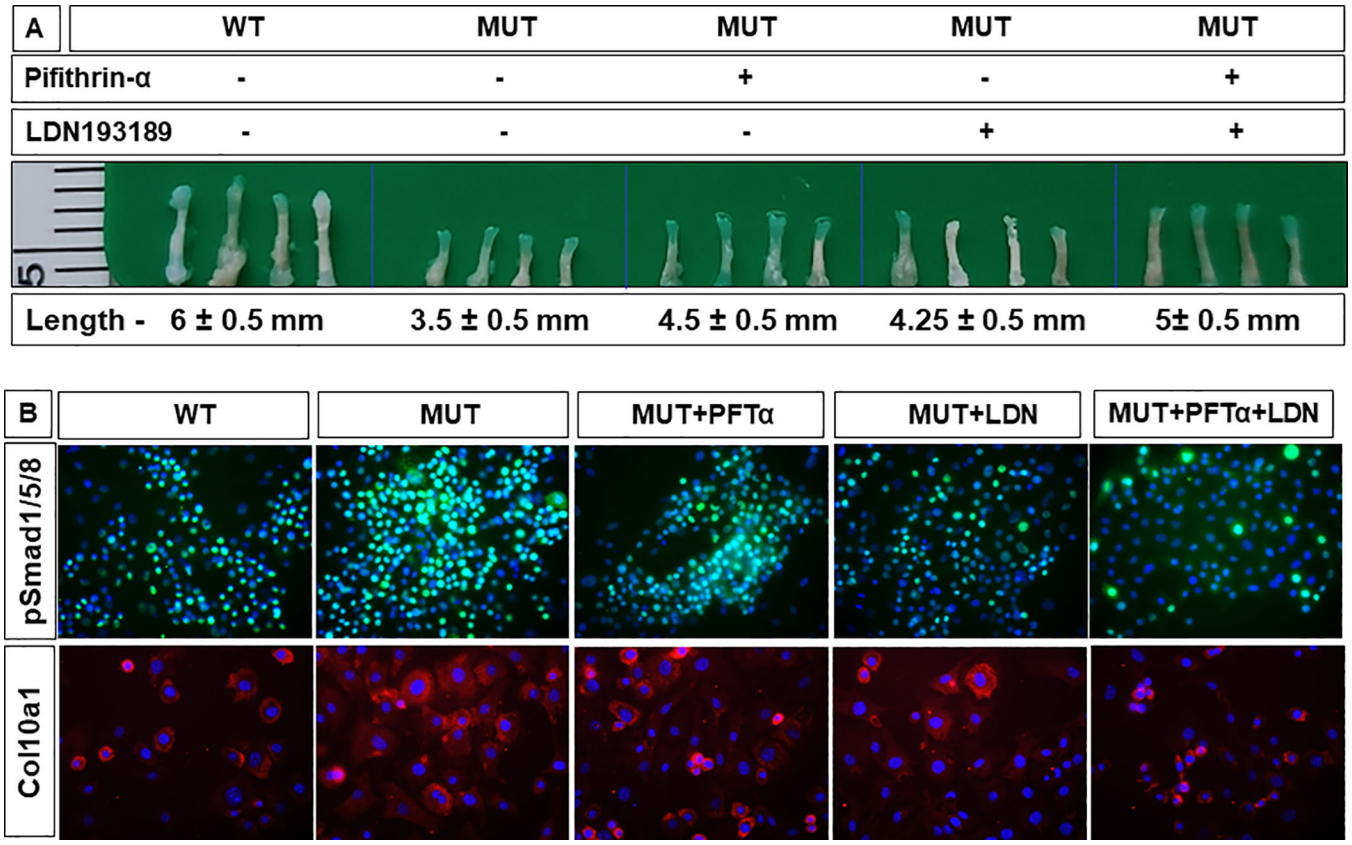

Figure 5. Whole tibia explant culture experiments confirmed that both the p53 and BMP signaling pathways are major Jab1 downstream targets in chondrocytes.

(A) The explant cultures of whole tibiae. WT, wild -type littermates; MUT, *Jab1 cKO* mutants. n =4/each. (B) Immunofluorescence (IF) analysis confirmed elevated pSmad1/5/8 (green) and Col10a1 (red) expressions in *Jab1 cKO* tibia primary chondrocytes with DAPI (blue) as counter staining. The treatment with 5μM PFT-α and 500nM LDN-193189 either alone or together reduced pSmad1/5/8 and Col10a1 expression.

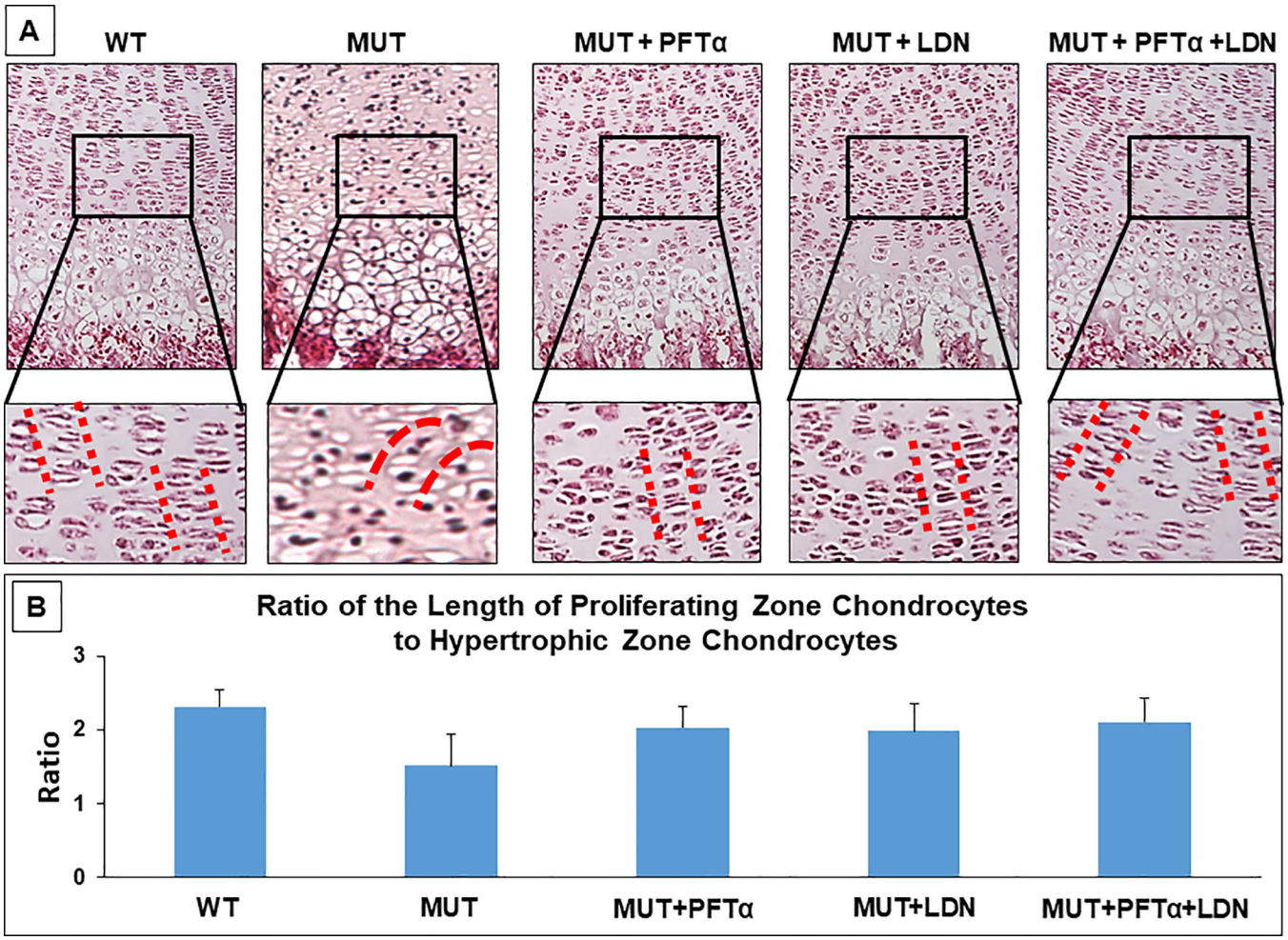

Figure 6. Partially restored normal growth plate columns in *Jab1 cKO* explant tibiae upon treatment with p53 and BMP inhibitors.

(A) Hematoxylin and eosin staining of E17.5 WT and *Jab1 cKO* explant tibiae sections. Normal growth plate column structures in *Jab1 cKO* tibiae were partially rescued upon treatment with 5μM PFT-α and 500nM LDN-193189 either alone or together as indicated by the red dotted lines in the enlarged boxes below). (B) The ratio of the length of proliferating zone to hypertrophic zone chondrocytes in *Jab1 cKO* explant tibiae was restored to be similar to that of WT littermates upon PFT-α and/or LDN-193189 treatment.

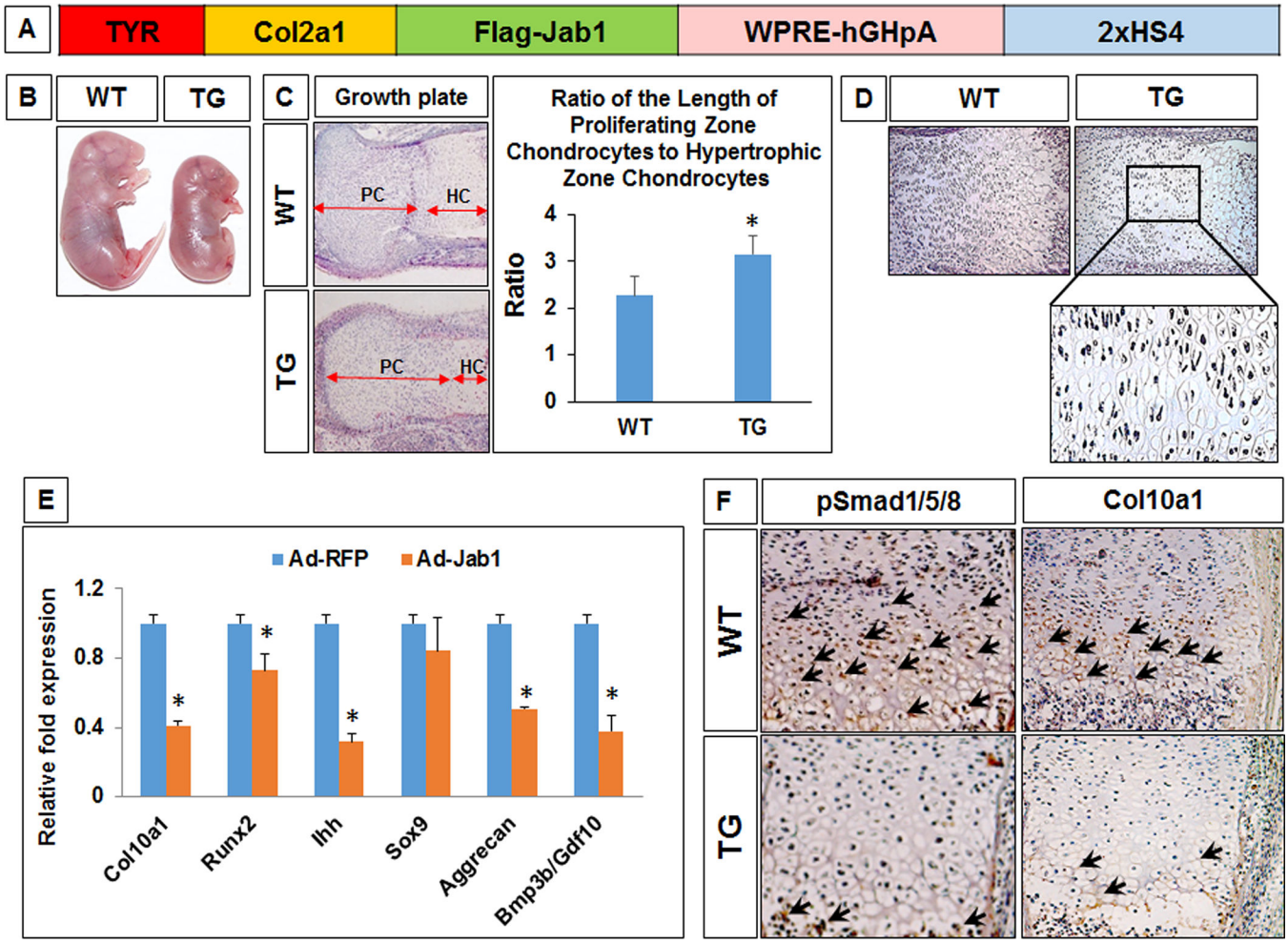

Figure 7. Chondrocyte-specific Jab1 overexpression led to disturbed chondrocyte growth plate morphology and abnormal chondrocyte differentiation in mice.

(A) The *Col2a1-Jab1* transgenic construct. (B) The smaller size of *Col2a1-Jab1* transgenic mice compared with wild-type littermate control at E18.5. (C) Hematoxylin and eosin staining of proximal tibiae showing a much smaller and thinner growth plate in E18.5 *Col2a1-Jab1* transgenic mice compared with wild-type littermate controls with an altered ratio of the lengthen of proliferation to hypertrophic zone. n=3, * indicates $p < 0.05$. (D) Hematoxylin and eosin staining showing the proliferating and hypertrophic zones in E18.5 *Col2a1-Jab1* transgenic and wild-type littermates. An enlarged image showing the mixture of proliferating and hypertrophic zones in the growth plates of E18.5 *Col2a1-Jab1* transgenic mice. (E) The real time RT-PCR analysis of hypertrophic chondrocyte markers *Col10a1, Runx2, Ihh,* BMP target genes *Sox9, Aggrecan,* and BMP ligand *Bmp3b/Gdf10* in Ad-Jab1 and Ad-RFP infected primary chondrocytes. n = 3, * indicates $p < 0.05$. (F) Immunostaining showing the decreased expression of pSmad1/5/8 and Col10a1 in E18.5 *Col2a1-Jab1* transgenic mice compared with wild-type littermate controls.

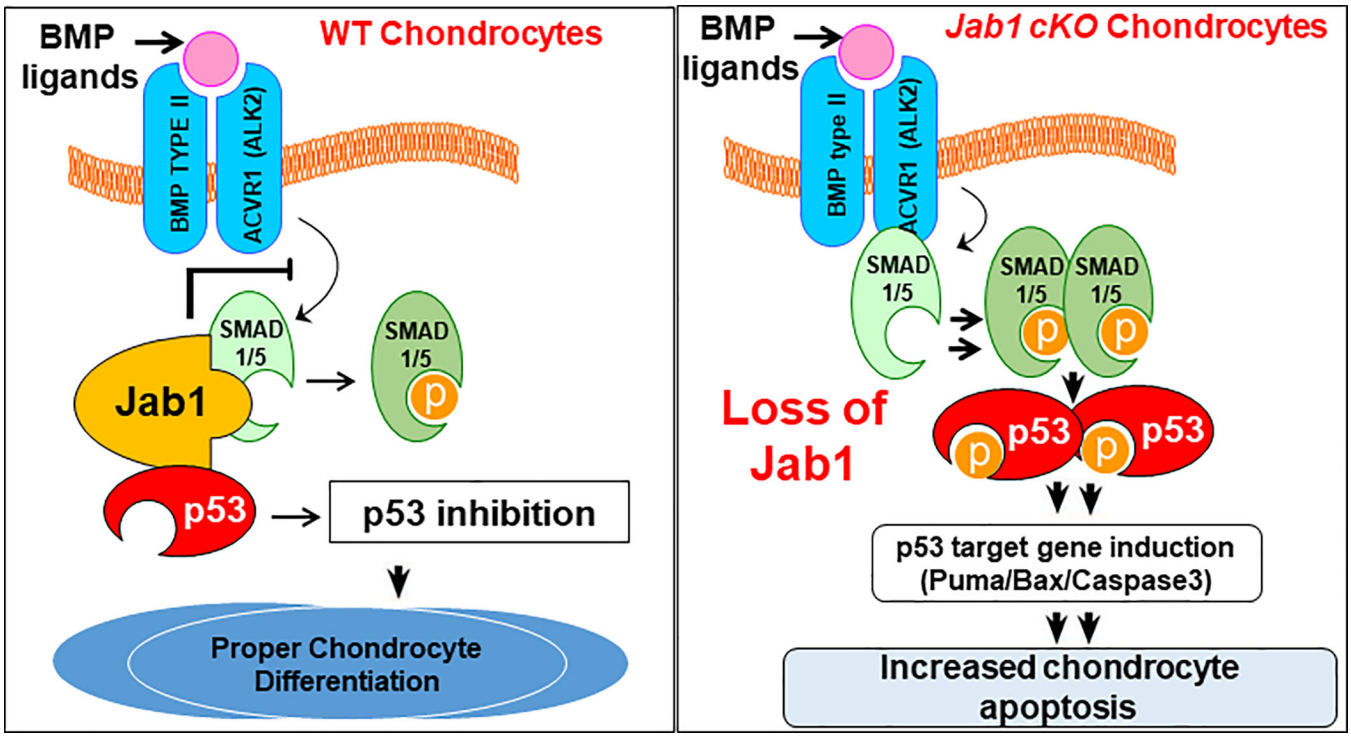

Figure 8. A working model of the Jab1-mediated cross-talk between p53 and BMP signaling in controlling chondrocyte survival and differentiation.

Jab1 negatively regulates BMP signaling in chondrocytes by competitively inhibiting the binding of Smad1/5/8 to Acvr1/Alk2 (WT, left panel). In the absence of Jab1, increased BMP signaling activity may result in increased p53 stabilization and subsequent target gene induction, which promotes chondrocyte apoptosis (*Jab1 cKO,* right panel).