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Neural EGFL-Like 1 Regulates Cartilage Maturation through Runt-Related Transcription Factor 3–Mediated Indian Hedgehog Signaling

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At the time of publication, author Chenshuang Li was affiliated with the School of Dentistry, University of California and the Peking University, School and Hospital of Stomatology. Currently, (s)he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

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Abstract

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Keywords

Animals, Calcium-Binding Proteins, Cartilage, Cell Differentiation, Cells, Cultured, Chondrocytes, Chondrogenesis, Core Binding Factor Alpha 1 Subunit, Core Binding Factor Alpha 3 Subunit, Glycoproteins, Hedgehog Proteins, Mice, Knockout, Signal Transduction, cyclopamine, neural EGFL like 1 protein, regulator protein, sonic hedgehog protein, transcription factor RUNX3, unclassified drug, calcium binding protein, glycoprotein, ihh protein, mouse, Nell1 protein, mouse, Runx2 protein, mouse, Runx3 protein, mouse, sonic hedgehog protein, transcription factor RUNX2, transcription factor RUNX3, adenovirus infection, animal cell, animal experiment, Article, cartilage, cell differentiation, cell isolation, chondrocyte, chondrogenesis, controlled study, in vitro study, in vivo study, maturation, mouse, nonhuman, priority journal, protein expression, real time polymerase chain reaction, RNA interference, signal transduction, Western blotting, animal, cartilage, cell culture, chondrogenesis, cytology, deficiency, knockout mouse, physiology, signal transduction

Disciplines

Dentistry | Orthodontics and Orthodontology | Other Dentistry

Comments

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GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Neural EGFL-Like 1 Regulates Cartilage Maturation through Runt-Related Transcription Factor 3—Mediated Indian Hedgehog Signaling

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The pro-chondrogenic function of runt-related transcription factor 2 (Runx2) was previously considered to be dependent on direct binding with the promoter of Indian hedgehog (Ihh)—the major regulator of chondrocyte differentiation, proliferation, and maturation. The authors' previous studies identified neural EGFL like 1 (Nell-1) as a Runx2-responsive growth factor for chondrogenic differentiation and maturation. In this study, it was further revealed that the pro-chondrogenic activities of Nell-1 also rely on Ihh signaling, by showing: i) Nell-1 significantly elevated Ihh signal transduction; ii) *Nell-1* deficiency markedly reduced Ihh activation in chondrocytes; and iii) Nell-1—stimulated chondrogenesis was significantly reduced by the specific hedgehog inhibitor cyclopamine. Importantly, the authors demonstrated that Nell-1—responsive Ihh signaling and chondrogenic differentiation extended to *Runx2*$^{-/-}$ models *in vitro* and *in vivo*. In *Runx2*$^{-/-}$ chondrocytes, Nell-1 stimulated the expression and signal transduction of Runx3, another transcription factor required for complete chondrogenic differentiation and maturation. Furthermore, knocking down *Runx3* in *Runx2*$^{-/-}$ chondrocytes abolished Nell-1's stimulation of Ihh-associated molecule expression, which validates Runx3 as a major mediator of Nell-1—stimulated Ihh activation. For the first time, the Runx2 → Nell-1 → Runx3 → Ihh signaling cascade during chondrogenic differentiation and maturation has been identified as an alternative, but critical, pathway for Runx2 to function as a pro-chondrogenic molecule via Nell-1. *(Am J Pathol 2018, 188: 392—403; https://doi.org/10.1016/j.ajpath.2017.09.020)*

Originally identified as a craniosynostosis-associated molecule from resected human fusing and fused coronal sutures,[1] neural EGFL like 1 (Nell-1) is recognized as a critical regulator of osteogenesis.[2,3] The human *NELL1* gene, located on chromosome 11 at 11p15.1-15.2, encodes an 810—amino-acid open reading frame with a 90-kD core protein, before N-glycosylation and oligomerization, that constitutes part of the secreted extracellular matrix.[4] As a highly conserved

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C.L. and Z.Z. contributed equally to this work.

Disclosures: X.Z., K.T., and C.S. are holders of Nell-1—related patents (Patents US9598480, US9511115, US9447155, US9301976, US8207120, US8053412, US8048646, US8044026, US7884066, US7833968, US7807787, US7776361, US769,607, US7687462, US7544486, and US7052856). C.T.C. is a holder of Nell-1—related patents filed from Oak Ridge National Laboratory (ORNL; Patent US7910542). X.Z., K.T., and C.S. are founders and/or past board members of Bone Biologics Inc./Bone Biologics Corp., which sublicenses Nell-1 patents from the University of California Regents, and also holds equity in the company. C.T.C. is a founder of NellOne Therapeutics, Inc., which licensed Nell-1—related patent applications from ORNL.

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protein, Nell-1 contains a laminin G domain, which overlaps with a NH_2-terminal thrombospondin-1 domain, at the N-terminus, followed by five von Willebrand factor C domains and six epidermal growth factor-like domains.[5,6] To date, Nell-1's pro-osteogenic function has been studied extensively by various independent research groups and demonstrated in both small and large calvarial defects, osteoporosis, spinal fusion, and ectopic bone formation animal models.[5,7–15] Moreover, previous studies have shown that Nell-1 can induce the expression and activation of the critical osteogenic inducer runt-related transcription factor 2 (Runx2, also known as Cbfa1),[16] and vice versa, in osteoblast lineage cells, such as neonatal mice calvarial cells and osteoblasts[2,3,17]. Moreover, the Runx2 → Nell-1 → mitogen-activated protein kinase (MAPK)/Wnt osteogenic differentiation pathway has previously been identified.[2,3,10,18–20]

In addition to bone tissues, Nell-1 is also expressed in uncalcified human articular cartilage.[6] Further investigations showed that Nell-1 enhances the chondrogenic marker expression and cartilage nodule formation in rabbit chondrocytes.[21] It was also found that Nell-1 induces hyaline cartilage regeneration in subchondral defects of rabbit knees,[22] and implanting Nell-1–overexpressed bone marrow mesenchymal stem cells in critical-sized goat mandibular condyle osteochondral defects results in articular cartilage re-establishment.[23] Moreover, neonatal *N*-ethyl-*N*-nitrosourea (ENU)-induced Nell-1–deficient mice, in comparison with *Nell-1*$^{+/+}$ littermates, have markedly deformed and shorter rib cages, and vertebral bodies with compressed intervertebral spaces and reduced cartilage matrices.[24] Lastly, Nell-1 deficiency leads to reduced expression of multiple cartilage-related genes.[24] All of these findings reveal the essential role of Nell-1 during chondrogenic differentiation, maturation, and regeneration. However, unlike the function of Nell-1 in osteogenesis, the chondrogenic bioactivity of Nell-1 has only recently garnered interest. Thus, Nell-1's mechanism of action used for stimulating chondrogenic differentiation has remained largely elusive.

Recently, it was demonstrated that the expression of Nell-1 during chondrogenic differentiation and maturation is tightly regulated by Runx2; however, Nell-1's pro-chondrogenic activity is not dependent on the presence of Runx2.[25] To better understand the potential regulatory roles of Nell-1 in chondrogenesis, this study focused on using *Runx2*-deficient models to eliminate the pivotal contribution of Runx2. Since Runx2 regulates chondrogenic differentiation and maturation primarily through the Indian hedgehog (Ihh) signaling pathway,[26–28] examining whether Nell-1, a functional downstream mediator of Runx2,[25] also acts through the Ihh signaling pathway to exhibit its prochondrogenic properties is critical in discerning the interplay of these two regulators.

Previous studies have shown that *Runx2*$^{-/-}$ mice have a complete blockade of chondrocyte hypertrophy in the proximal limbs (femur and humerus), but only a mild disturbance of chondrocyte differentiation in the distal limbs (radius, fibula, and tibia) during the late development stages.[27] However, a double-knockout of *Runx2* and *Runx3* resulted in a complete absence of hypertrophic chondrocytes in the entire skeleton.[27] In addition, *Runx3*$^{-/-}$ mice have slightly delayed chondrocyte maturation during development, but have normal gross skeletal development at the neonatal stage.[27] These phenomena suggest that Runx3 is required for complete chondrogenic differentiation and maturation.[29,30] Moreover, the expression of Ihh in chondrocytes is only partially reduced in *Runx2*$^{-/-}$ neonatal mice, but undetectable in age-matched *Runx2*$^{-/-}$/*Runx3*$^{-/-}$ animals.[27] This finding indicates that, in addition to Runx2, Runx3 can also potently regulate Ihh expression in chondrocytes.[27]

In this study, by using 2-dimensional and 3-dimensional cultured mouse chondrocytes, as well as by overexpressing *Nell1* in *Runx2*$^{-/-}$ mice *in vivo*, Nell-1's ability to activate Ihh signaling transduction, even in Runx2 deficient conditions, was examined. Lastly, in *Runx2*$^{-/-}$ chondrocytes, the role of runt-related transcription factor 3 (Runx3) in Nell-1–stimulated Ihh signaling transduction during chondrogenesis was assessed. In summary, the aim was to further reveal the mechanism by which Nell-1 compensates for a Runx2 deficiency during chondrogenesis.

Materials and Methods

Animal Maintenance and Skeletal Analysis

Mice were bred and maintained as previously described,[2] and all of the experiments on live mice were performed under the institutionally approved protocol provided by the Chancellor's Animal Research Committee at UCLA (protocol number: 2012-041). Heterozygous *Runx2*-deficient mice (*Runx2*$^{+/-}$; generated by embryonic stem cells derived from the 129 mouse strain backcrossing with the C57BL/6 strain[31]) were mated with *Nell1*-overexpressing mice (*CMV-Nell1*; derivative of the B6C3 strain[32]) to generate *Runx2*$^{-/-}$/*CMV-Nell1* mice.[2] Nell-1 deficiency was obtained by ENU induction, resulting in a *Nell1*^{6R} point mutation truncating the 810–amino-acid Nell-1 protein at residue number 502.[24,33] Because the homozygous *Nell1*$^{6R/6R}$ mice have severely reduced Nell-1 expressions leading to neonatal death,[24] *Nell1*^{6R} heterozygous mice (*Nell1*$^{+/6R}$) were used to produce *Nell1*$^{6R/6R}$ fetuses. Mouse genotypes were determined by PCR, and expression levels of *Nell1* and *Runx2* were monitored using real-time quantitative PCR.

Mice used in this study were euthanized with an overdose of phenobarbital (Piramal Healthcare, Maharashtra, India). The harvested neonatal mouse hind limbs and ribs were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at 4°C overnight before paraffin embedding and sectioning at a thickness of 5 μm. Primary antibodies against type II collagen (II-II6B3, no antigen retrieval, dilution 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA), glioma-associated oncogene homolog 1 (Gli1, sc-20687, 0.125% trypsin digestion 20 minutes, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Ihh (ab39634, 0.125% trypsin

Table 1 Primer Sequences Used for Real-Time PCR

Gene	Primer sequence
Acan[25]	F: 5′-CCAGGCTCCACCAGATACTC-3′
	R: 5′-TGCTCATAGCCTGCCTCATA-3′
Col2α1[25]	F: 5′-GTCCTGAAGGTGCTCAAGGT-3′
	R: 5′-TTTGGCTCCAGGAATACCAT-3′
Gapdh[39]	F: 5′-ATTCAACGGCACAGTCAAGG-3′
	R: 5′-GATGTTAGTGGGGTCTCGCTC-3′
Gli1[40]	F: 5′-CCAAGCCAACTTTATGTCAGGG-3′
	R: 5′-AGCCCGCTTCTTTGTTAATTTGA-3′
Ihh[41]	F: 5′-CTCAGACCGTGACCGAAATAAG-3′
	R: 5′-CCTTGGACTCGTAATACACCCAG-3′
Nell-1[25]	F: 5′-TCCTGGGTAGATGGTGACAA-3′
	R: 5′-CATTGGCCAGAAATATGCAC-3′
Ptch1 (encoding Patched 1)[42]	F: 5′-TGCCACAGCCCCTAACAAAAA-3′
	R: 5′-ACCCACAATCAACTCCTCCTG-3′
Runx2[43]	F: 5′-AACGATCTGAGATTTGTGGGC-3′
	R: 5′-CCTGCGTGGGATTTCTTGGTT-3′
Runx3[44]	F: 5′-CAGGTTCAACGACCTTCGATT-3′
	R: 5′-GTGGTAGGTAGCCACTTGGG-3′
Sox9[45]	F: 5′-ACGGCTCCAGCAAGAACAAG-3′
	R: 5′-TTGTGCAGATGCGGGTACTG-3′

F, forward; R, reverse.

digestion 20 minutes, dilution 1:100; Abcam, Cambridge, MA), Runx3 (ab49117, no antigen retrieval, dilution 1:150; Abcam), and patched1 (sc-6149, no antigen retrieval, dilution 1:75; Santa Cruz Biotechnology) were used for immunohistochemical and/or immunofluorescent staining, respectively. DAPI (Sigma-Aldrich) was used for nuclear counterstaining.

Mouse Primary Chondrocyte Isolation and Cultivation

After removing soft tissues with 2 mg/mL protease (Roche, Nutley, NJ) and 3 mg/mL collagenase II (Roche), the rib cages of the neonatal mouse embryos were digested in 1 mg/mL collagenase II for 3 hours to achieve single-cell suspensions.[34,35] After rinsing with Dulbecco's modified Eagle's medium, chondrocytes were cultured in a basal culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). The medium was changed every 3 days, and the cells were passaged at 70% to 90% confluence. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Passage 2 chondrocytes were seeded in 6-well plates at 5×10^4 cells/well with basal culture medium for 6 hours. Before Nell-1 treatment, the cells were synchronized by being cultured in starvation medium [Dulbecco's modified Eagle's medium + 1% ITS Universal Cell Culture Supplement Premix (BD Biosciences, San Jose, CA)] for 18 hours. The treatment recombinant human Nell-1 protein was synthesized by Aragen Bioscience Inc. (Morgan Hill, CA) with a purity of 92%.

Mouse Primary Mesenchymal Progenitor Cell Isolation and Cultivation

Primary embryonic mesenchymal progenitor cells were isolated from limb buds of E11.5 embryonic mice as previously described.[36] Briefly, limbs were dissected from embryos under a dissecting microscope and digested with 1 mg/mL Dispase (Roche) for 1.5 hours at 37°C. Cells were then filtered through a prewashed 40-μm cell strainer to generate single-cell suspensions. After washing with Puck's Saline A, cells were suspended in culture medium [Dulbecco's modified Eagle's medium:F12 = 2:3 (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mmol/L glutamine, 50 μg/mL vitamin C (Sigma-Aldrich), and 10 nmol/L β-glycerophosphate (Sigma-Aldrich)] with or without 2 μg/mL recombinant human Nell-1 protein at a density of 4×10^5 cells/mL. Cell suspension (0.5 mL) was then transferred into a 15-mL centrifuge tube, centrifuged at $500 \times g$ for 5 minutes, and incubated at 37°C in a 5% CO_2 humidified incubator as previously described.[37] The media were changed every 48 hours. Pellets were cultured for 7 days for further analyses.

Adenoviral Infection

Passage 2 mouse chondrocytes at 80% confluence were infected with either Ad*LacZ* or Ad*Runx2* at a multiplicity of infection of 500 plaque-forming units per cell, as previously described.[38]

RNA Interference

A plasmid package that contains four different plasmids harboring different shRNA sequences against mouse *Runx3* was purchased from Origene (Rockville, MD). Passage 2 chondrocytes isolated from *Runx2*$^{-/-}$ mice were transfected with the shRNA plasmids by Lipofectamine 3000 reagent (Invitrogen). A control shRNA plasmid provided by Origene was also used to transfect *Runx2*$^{-/-}$ mouse chondrocytes. Transfection efficiency was determined by Western blotting.

Real-Time Quantitative PCR

Total RNA was isolated by TRIzol Reagent (Invitrogen) followed by DNase (Invitrogen) treatment. RNA (1 μg) was injected for reverse transcription with the SuperScript II Reverse Transcriptase Kit (Invitrogen), as per the manufacturer's instructions. Real-time PCR was performed on the 7300 Real-Time PCR system with SYBR Green Master Mix (Invitrogen). All of the primer sequences used in the current study are listed in Table 1.[25,39–45] Concomitant glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was also evaluated in separate tubes for each reverse transcription reaction as a housekeeping standard. Relative gene expression was analyzed by the $_{\Delta\Delta}C_T$ method.[46]

Western Blotting Analysis

A total of 2×10^6 passage 2 mouse chondrocytes cultured in a 10-cm cell plate were starved and treated with Nell-1 as described above. Total protein was extracted by

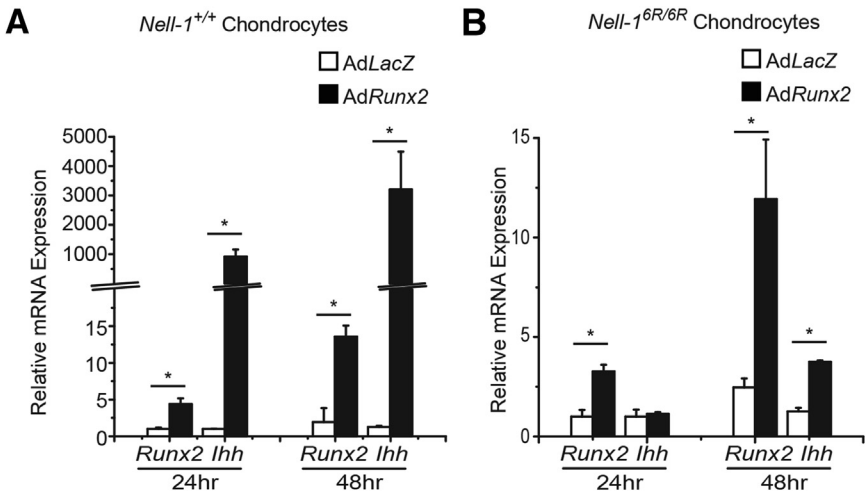

Figure 1 Nell-1 deficiency impairs Runx2-induced *Ihh* up-regulation in mouse chondrocytes. **A:** Twenty-four and 48 hours after transfection, Ad*Runx2* stimulates *Ihh* expression in *Nell1*$^{+/+}$ mouse chondrocytes. **B:** Under the same experimental circumstances, Runx2-responsive *Ihh* transcription is significantly delayed and weakened in *Nell1*$^{6R/6R}$ chondrocytes. Data are expressed as means ± SD. $n = 3$ independent experiments performed in triplicate (**A** and **B**). *$P < 0.05$.

radioimmunoprecipitation assay solution supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Invitrogen), whereas nuclear protein was isolated using the Nuclear Extraction Kit (Thermo Fisher Scientific Inc.). Protein (30 μg) was denatured by 5× loading buffer (Thermo Fisher Scientific), separated by SDS-PAGE, and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% nonfat milk in Tris-Buffered Saline with Tween 20 (TBST). Antibodies against Runx3 (ab49117, dilution 1:500 in 5% nonfat milk/TBST; Abcam), histone H3 (06-755, dilution 1:1000 in 5% nonfat milk/TBST; EMD Millipore, Billerica, MA), and Gapdh (sc-20357, dilution 1:1000 in 5% nonfat milk/TBST; Santa Cruz Biotechnology) were used as primary antibodies, respectively. All peroxidase-conjugated secondary antibodies were purchased from Abcam. Western blotting was visualized with the ECL kit (Thermo Fisher Scientific Inc.), and the blotting intensity was quantified by ImageJ software version 1.37v (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*).

Statistical Analysis

All statistical analyses were conducted in consultation with the UCLA Statistical Biomathematical Consulting Clinic. One-way analysis of variance and two-sample *t*-test analyses were performed by OriginPro 8 (OriginLab Corp., Northampton, MA) for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Nell-1 Is a Novel Mediator of the Runx2 → Ihh Signaling Pathway in Regulating Chondrocyte Differentiation

In accordance with previous findings,[26,27] overexpression of *Runx2* in *Nell-1*$^{+/+}$ mouse chondrocytes by Ad*Runx2* significantly up-regulated *Ihh* expression (Figure 1A). However, the increased expression of Runx2-responsive *Ihh* was significantly weakened and postponed in *Nell-1*$^{6R/6R}$ chondrocytes (Figure 1B).

We postulated that Nell-1, a functional downstream mediator of Runx2 during chondrogenesis,[25] exhibited its prochondrogenic properties through the Ihh signaling pathway. In a dose-dependent manner, Nell-1 protein stimulated the expression of Ihh-associated genes, such as *Ihh*, *Ptch1* (encoding patched homolog 1, Patched 1), and *Gli1*, in 2-dimensional monolayer-cultured *Runx2*$^{+/+}$ mouse chondrocytes (Figure 2A). Similar Nell-1–responsive stimulation of these genes was replicated in 3-dimensional *Runx2*$^{+/+}$ primary mesenchymal progenitor cell pellets (Figure 2B). These data indicate that Nell-1 participates in the Runx2 → Ihh signaling transduction in chondrocytes.

Nell-1 Partially Re-Establishes the Diminished Ihh Signaling in Runx2 Deficiency Models

As expected, Runx2 deficiency resulted in a markedly reduced expression of Ihh-associated genes in chondrocytes (Figure 2C). As a chondrogenic regulator that exhibits prochondrogenic activities in *Runx2*$^{-/-}$ models,[25] Nell-1 elevated the transcription of Ihh-associated genes of *Runx2*$^{-/-}$ chondrocytes in a dose-dependent manner (Figure 2C). Nell-1 also induced the expression of Ihh-associated genes, as well as chondrogenic markers, in 3-dimensional–cultured primary *Runx2*$^{-/-}$ embryonic mesenchymal progenitor cell pellets (Figure 3, A–C). Particularly, the chondrogenic extracellular matrix type II collagen[47] staining was abundant in the cartilage nodule regions of the cell pellets, where apparent Ihh staining was also found (Figure 3D). In *Runx2*$^{-/-}$*/CMV-Nell-1* mouse femurs, cytomegalovirus (CMV)-driven Nell-1 overexpression partially rescued the impaired chondrogenic differentiation and maturation from Runx2 deficiency.[25] Furthermore, intense staining of Ihh-associated molecules was also observed in the pre-hypertrophic and hypertrophic zones of *Runx2*$^{-/-}$*/CMV-Nell1* mouse femurs, which closely

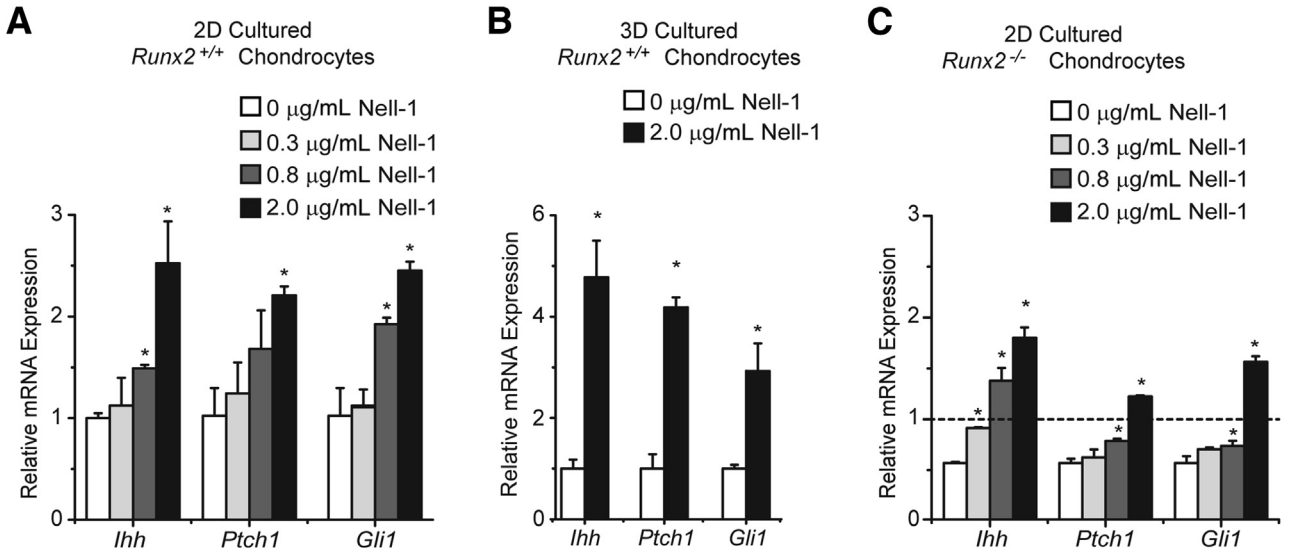

Figure 2 Nell-1 increases the expression of Ihh and its downstream molecules in chondrocytes. **A:** Twelve-hour Nell-1 protein treatment results in a dose-dependent increase of Ihh-associated gene expression in 2D monolayer–cultured *Runx2*$^{+/+}$ mouse chondrocytes. **B:** Nell-1 treatment enhances the expression of Ihh-associated molecules in 3D pellet–cultured primary *Runx2*$^{+/+}$ embryonic mesenchymal progenitor cells at day 7. **C:** In 2D monolayer–cultured *Runx2*$^{-/-}$ mouse chondrocytes, 12-hour Nell-1 stimulates the transcription of Ihh-associated genes in a dose-dependent manner. Data are normalized to 0 μg/mL Nell-1–treated chondrocytes (**A**), embryonic mesenchymal progenitor cell pellets (**B**), and Nell-1–treated *Runx2*$^{+/+}$ chondrocytes (**C**) (**dashed line**), respectively. Data are expressed as means ± SD. $n = 3$ independent experiments performed in triplicate (**A** and **C**); $n = 6$ pellets (**B**). *$P < 0.05$ versus 0 μg/mL Nell-1 control group. 2D, 2-dimensional; 3D, 3-dimensional.

resembled the expression pattern of Ihh signaling–related molecules in the wild-type littermates (Figure 4 and Supplemental Figure S1). Through these findings, Nell-1 appears to have rescued, at least partially, the diminished Ihh signaling resulting from Runx2 deficiency.

Nell-1's Prochondrogenic Activities Are Dependent on Ihh Signaling

To further confirm the importance of the Ihh signaling pathway in Nell-1–mediated chondrogenic differentiation,

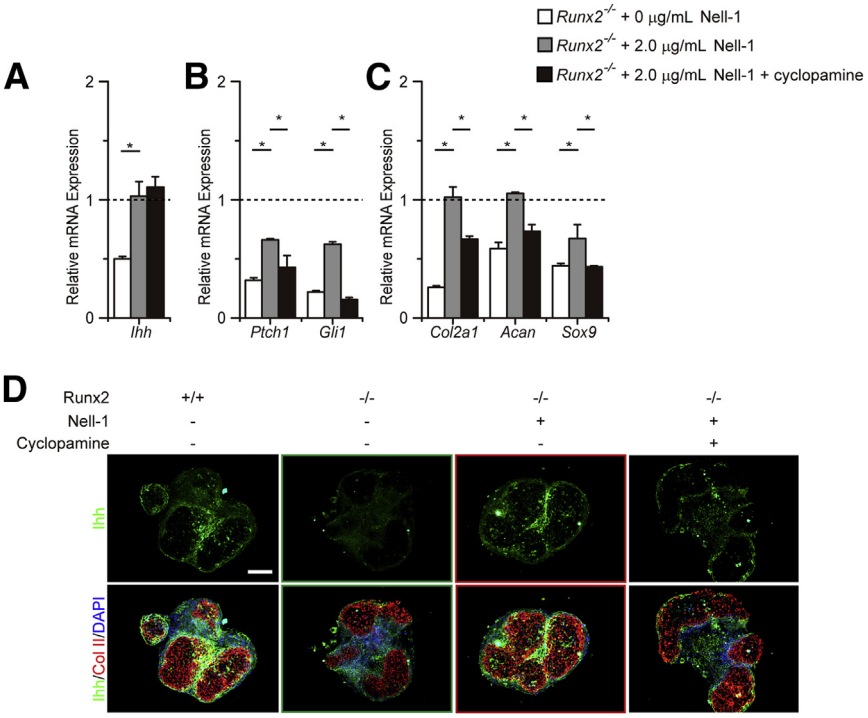

Figure 3 Nell-1 partially rescues the diminished expression of Ihh and its downstream molecules in 3-dimensional-cultured primary *Runx2*$^{-/-}$ embryonic mesenchymal progenitor cells. Nell-1 promotes the transcription of Ihh (**A**), Ihh-associated molecules (**B**), and chondrogenic markers (**C**) in *Runx2*$^{-/-}$ primary embryonic mesenchymal progenitor cell pellets, which is confirmed by immunofluorescent staining (**D**). Type II collagen staining is abundant in the cartilage nodule regions of the cell pellets. Administration of cyclopamine, a Hedgehog signaling inhibitor, eliminates Ihh signaling transduction stimulated by Nell-1 and Nell-1's induction of mesenchymal progenitor cell chondrogenic differentiation. Data are collected on day 7 of pellet cultivation, and normalized to 0 μg/mL Nell-1–treated primary *Runx2*$^{+/+}$ embryonic mesenchymal progenitor cell pellets (**dashed lines**). Data are expressed as means ± SD. $n = 6$ (**A**–**C**). *$P < 0.05$. Scale bar = 100 μm.

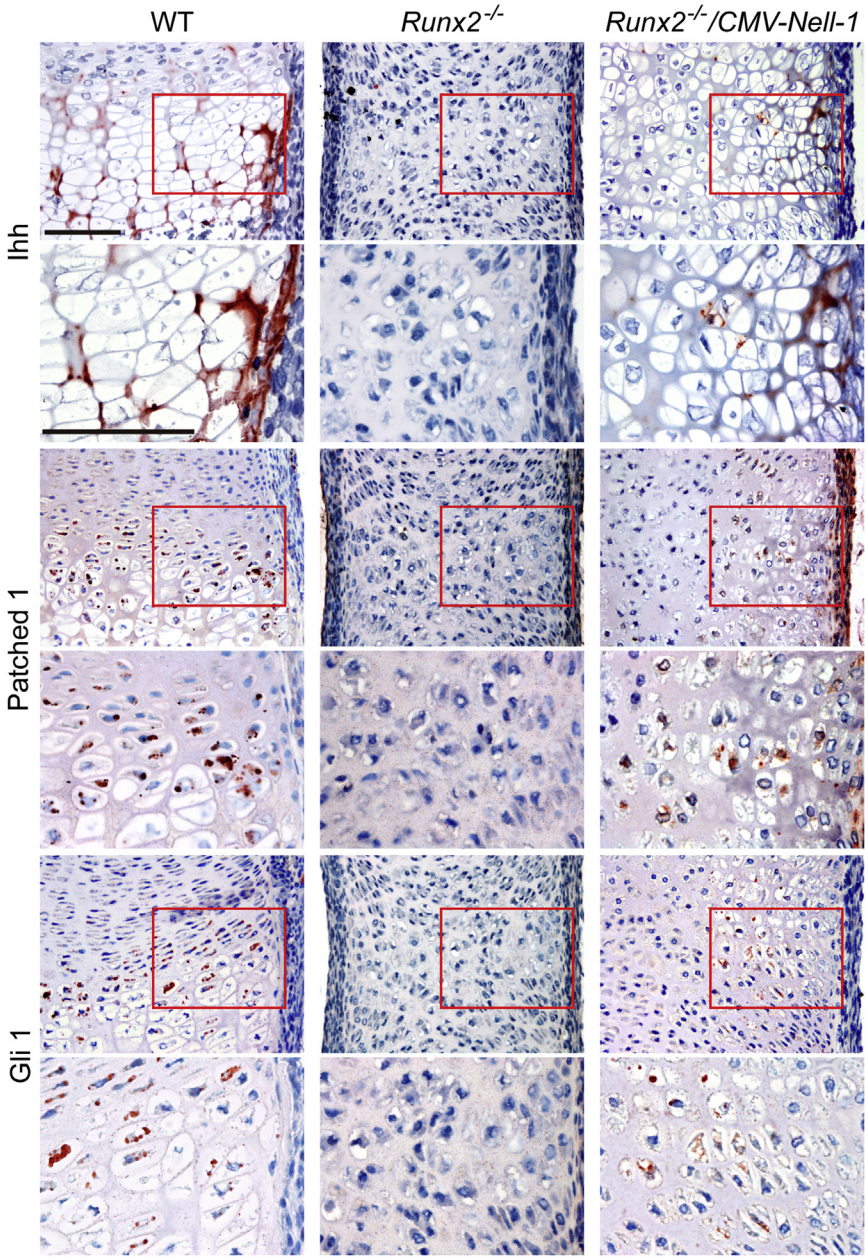

Figure 4 CMV-driven Nell-1 overexpression partially rescues the impaired expression of Ihh-associated molecules in Runx2-deficient neonatal mouse femurs (immunohistochemical staining). Intense staining of Ihh-associated molecules, including Ihh, patched 1, and Gli 1, is observed in the prehypertrophic and hypertrophic zones of *Runx2*$^{-/-}$*/CMV-Nell1* mouse femurs, which closely resemble the expression pattern of Ihh signaling–related molecules in the WT littermates. **Boxed areas** are shown at higher magnification below. Scale bars = 100 μm. WT, wild-type.

especially in Runx2 deficiency conditions, *Runx2*$^{-/-}$ pellets were treated with cyclopamine to block Ihh signaling transduction.[48] Analogous to previous reports,[49,50] cyclopamine effectively blocked Nell-1–responsive Ihh signaling pathway activation, but did not tamper with the Nell-1-responsive *Ihh* up-regulation in *Runx2*$^{-/-}$ pellets (Figure 3, A and B). Moreover, cyclopamine effectively inhibited Nell-1-stimulated expression of chondrogenic markers in *Runx2*$^{-/-}$ pellets, such as type II collagen (an abundant and specific protein in cartilage,[47] encoded by gene *Col2α1*), aggrecan (one of the major structural components in the cartilage matrix, also known as a cartilage-specific proteoglycan core protein or chondroitin sulfate proteoglycan 1,[47] encoded by the gene *Acan*), and SRY-Box 9 (the master transcription factor for chondrogenesis initiation,[47] encoded by the gene *Sox9*) (Figure 3, C and D). These findings indicate that the compensatory effects of Nell-1 on the chondrogenic commitment of *Runx2*$^{-/-}$ mesenchymal progenitor cells also rely on Ihh signaling transduction.

Although the direct binding of Runx2 to the *Ihh* promoter in chondrocytes was considered to play an essential role in chondrogenic differentiation and maturation,[26,27,51–53] our current data demonstrated that Ihh signaling transduction was induced by Nell-1 to promote chondrogenic maturation and differentiation, even in Runx2-deficient conditions where the DNA-binding functions of Runx2 were completely abolished. This finding suggests that Nell-1 recruits an alternative pathway, which bypasses the

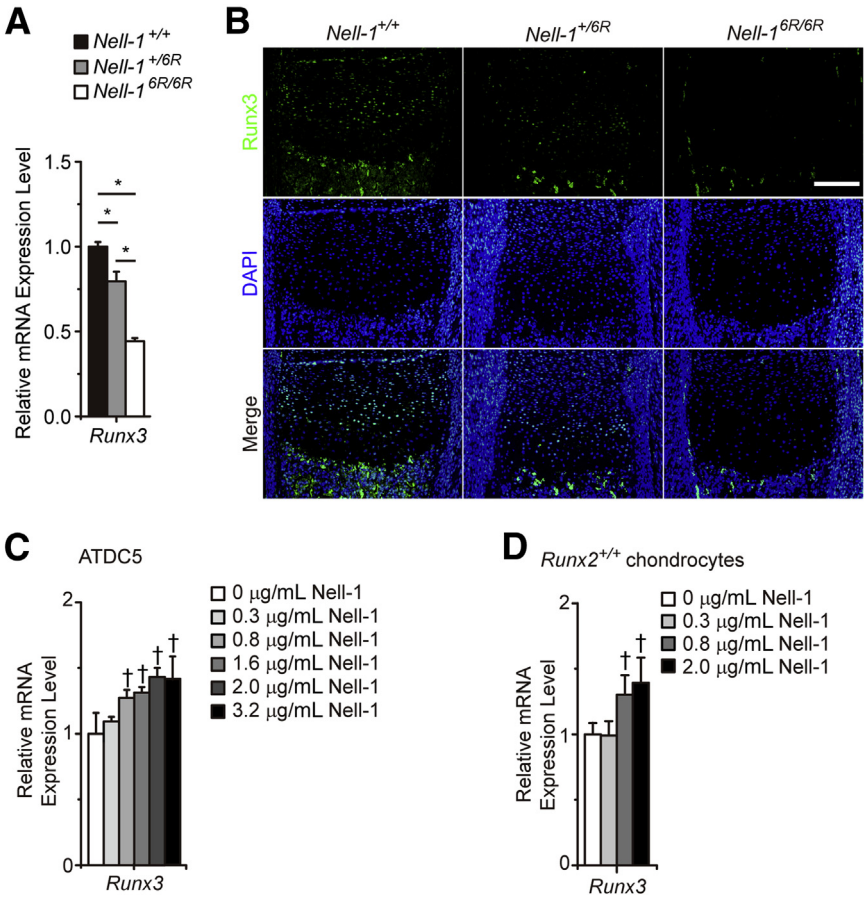

Figure 5 Nell-1 regulates Runx3 expression during chondrogenesis. **A:** Nell-1 deficiency reduces Runx3 expression in mouse chondrocytes. Data are normalized to *Nell1*$^{+/+}$ chondrocytes. **B:** Runx3 expression varies in the femurs of neonatal mice with different *Nell1* genotypes. Positive staining of Runx3 is found in the proliferating, prehypertrophic, and hypertrophic regions of *Nell1*$^{+/+}$ femurs, but barely detectable in the *Nell-1*$^{6R/6R}$ femurs. **C:** The *Runx3* expression levels in monolayer-cultured ATDC5 increase in a Nell-1 dose-dependent manner after 3 hour of stimulation, and reach peak expression levels with a 2.0 μg/mL Nell-1. **D:** Nell-1 enhances *Runx3* transcription in monolayer-cultured *Runx2*$^{+/+}$ chondrocytes after 12 hour of cultivation. **C** and **D:** Data are normalized to 0 μg/mL control groups. Data are expressed as means ± SD. $n = 6$ independent experiments performed in triplicate (**A**); $n = 3$ independent experiments performed in triplicate (**C** and **D**). $^{*}P < 0.05$; $^{\dagger}P < 0.05$ versus 0 μg/mL Nell-1 control group. Scale bar = 100 μm.

Runx2-*Ihh* promoter binding, to stimulate Ihh signaling and exhibit prochondrogenic activities.

Runx3 Functions as a Critical Mediator in the Nell-1—Stimulated Ihh Signaling Pathway in *Runx2*$^{-/-}$ Models

Nell-1 deficiency led to markedly decreased Runx3 mRNA expression in mouse chondrocytes (Figure 5A), and Runx3 protein expression in the proliferating, prehypertrophic, and hypertrophic regions of neonatal mice femurs (Figure 5B). Meanwhile, short-term Nell-1 administration, not only increased the expression of *Runx3* in the mouse chondrogenic ATDC5 cell line (Figure 5C) and *Runx2*$^{+/+}$ mouse primary chondrocytes (Figure 5D), but also induced Runx3 signaling transduction in *Runx2*$^{-/-}$ chondrocytes, as evidenced by an up-regulated transcription of both *Runx3* and a Runx3-downstream target gene, *Acan*[30] (Figure 6A), as well as by the increased nuclear accumulation of Runx3 (Figure 6B). In addition, CMV-driven Nell-1 overexpression reestablished the intense staining of Runx3 in neonatal *Runx2*$^{-/-}$ mouse femurs (Figure 6, C and D). Accordingly, Runx3 was determined to be a downstream target of Nell-1, at least in *Runx2*$^{-/-}$ models.

When *Runx3* was knocked down in *Runx2*$^{-/-}$ chondrocytes by shRNA (Supplemental Figure S2), the stimulating effects of Nell-1 on *Acan* and Ihh-associated genes were completely eliminated (Figure 7). These data collectively constitute strong evidence that Runx3 is critical in the Nell-1—responsive restoration of Ihh signaling transduction in *Runx2*$^{-/-}$ chondrocytes.

Discussion

Recently, a similar spatiotemporal expression pattern of Nell-1 and Runx2 in chondrocytes during long bone development was observed, and subsequently confirmed that Nell-1 is a downstream target of Runx2 in chondrocytes.[25] Ihh is recognized for its stimulation of chondrocyte proliferation and maturation, promotion of the transition from periarticular chondrocytes to proliferating chondrocytes, and regulation of columnar cell mass and chondrocyte hypertrophy.[52–56] Here, we showed that Runx2-induced Ihh expression was severely reduced in Nell-1—deficient circumstances, which indicates that Nell-1 is involved in Runx2-responsive Ihh up-regulation in chondrocytes. Moreover, Nell-1 significantly stimulated the expression of Ihh and its downstream molecules in

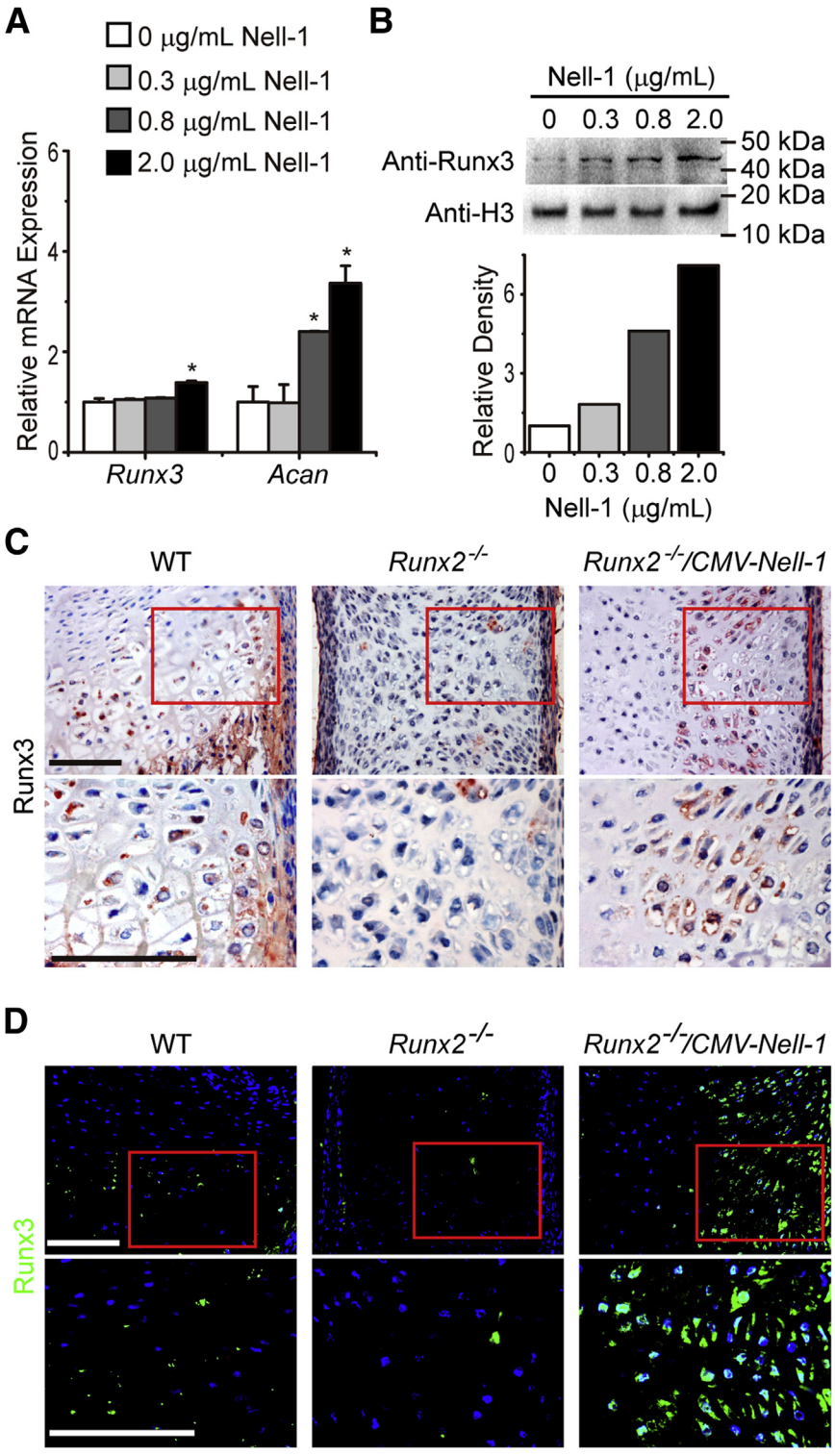

Figure 6 Nell-1 promotes Runx3-associated molecule expression and nuclear accumulation of Runx3 in *Runx2*-deficient chondrocytes. **A:** The transcription of *Runx3* and the Runx3 direct downstream target *Acan* in $Runx2^{-/-}$ chondrocytes increase with the Nell-1 treatment. **B:** The nuclear accumulation of Runx3 in $Runx2^{-/-}$ chondrocytes is also increased in a Nell-1 dose-dependent manner. Data are collected after 12-hour Nell-1 stimulation. CMV-driven Nell-1 overexpression re-establishes Runx3 expression in neonatal $Runx2^{-/-}$ mouse femurs. **C** and **D:** Both (**C**) and immunofluorescent (**D**) staining are used. The **boxed areas** are shown at higher magnification below. Data are expressed as means ± SD. $n = 3$ independent experiments performed in triplicate (**A**). $^*P < 0.05$ versus 0 μg/mL Nell-1 control group. Scale bars = 100 μm.

chondrocytes, and blocking Ihh signaling transduction, at least partially, diminished the prochondrogenic activities of Nell-1. Thus, Ihh signaling transduction appears to be critical for Nell-1's promotion of chondrogenesis. Furthermore, Nell-1's capacity to enhance the expression of Ihh-associated genes and promote chondrogenic differentiation[25] even extended to $Runx2^{-/-}$ conditions, demonstrating that there is another undescribed pathway by which Nell-1 activates Ihh signaling and promotes chondrogenesis.

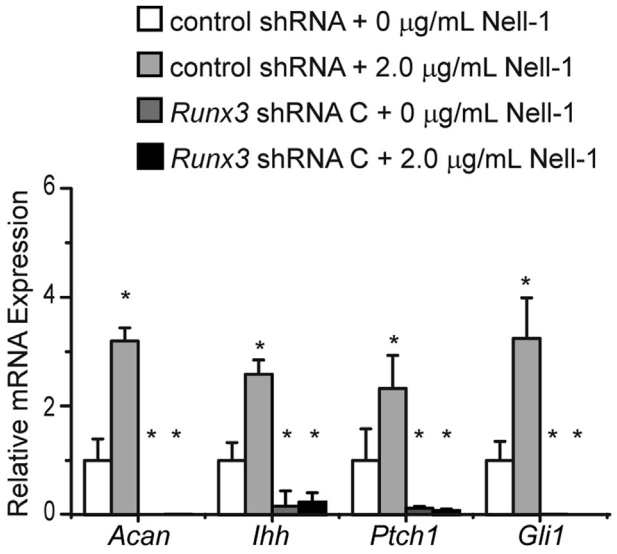

Figure 7 Runx3 is required for Nell-1 to stimulate Ihh signaling in *Runx2*$^{-/-}$chondrocytes. The transcription of the Runx3 direct downstream target *Acan* and Ihh-associated genes are inhibited in *Runx3* knockdown *Runx2*$^{-/-}$ chondrocytes, even with Nell-1 stimulation. Data are collected after 12-hour of Nell-1 stimulation, and normalized to the shRNA +0 μg/mL Nell-1—treated control group. Data are expressed as means ± SD. $n = 3$ independent experiments performed in triplicate. *$P < 0.05$ versus shRNA + 0 μg/mL Nell-1 control group by two-sample *t*-test.

As described in our previous study,[25] the partial rescue of neonatal *Runx2*$^{-/-}$*/CMV-Nell1* mice chondrogenesis in the mid shaft of the femur led to the investigation of a possible involvement of Runx3, another transcription factor required for complete chondrogenic differentiation and maturation,[27,29] in Nell-1—mediated compensation. In this study, a correlation between Nell-1 and Runx3 expression levels in chondrocytes was demonstrated. Particularly, Nell-1 stimulated Runx3 expression in both the chondrogenic cell line and in primary chondrocytes. At the same time, *Runx3* knockdown in *Runx2*$^{-/-}$ chondrocytes completely blocked the Nell-1—stimulated expression of Ihh-associated molecules. Therefore, Runx3 can be recognized as an important mediator for Nell-1 → Ihh signaling transduction, and for subsequent chondrogenic differentiation.

To control chondrogenic differentiation and maturation, the N-terminus of Runx2 directly binds to the *Ihh* promoter,[28,31,51] whereas the C-terminus of Runx2 presents a context-dependent transcriptional activation/suppression function.[57,58] For the first time, this study identified an alternative Ihh-activation pathway that is mediated by Nell-1, and does not rely on the presence of Runx2. The identification of the alternative Runx2 → Nell-1 → Runx3 → Ihh cascade (Figure 8) may explain previous observations that either Runx2 or Runx3 deficiency does not completely block chondrocyte terminal differentiation, but that a double knockout of Runx2/Runx3 results in the absolute elimination of chondrocyte maturation.[27]

It is well known that Ihh, which is secreted by newly formed hypertrophic chondrocytes, stimulates parathyroid hormone-related protein (PTHrP) expression, whereas PTHrP slows the formation of these cells.[59] Huang et al[60] demonstrated that the master chondrogenic factor, Sox9, is a downstream target of PTHrP signaling in the growth plates of endochondral bones. Tavella et al[61] also showed that sonic hedgehog, and other members of the hedgehog family, can up-regulate the expression of Sox9 in the chondrocytes both *in vitro* and *in vivo*. In addition, when ablating Ihh in condylar cartilage of neonatal mice, the expression of Sox9 was lower than that in wild-type mice.[62] The current data that Nell-1 induced *Sox9*, which was blocked by an Ihh signal inhibitor, provide new support of the hypothesis that there is potential crosstalk between Sox9 and Ihh, even though Ihh is mainly expressed in the prehypertrophic and hypertrophic chondrocytes,[63] whereas Sox9 is an early chondrogenic marker.[64]

The current findings demonstrate the complexity of the interaction between Nell-1 and Runx2. Besides the Runx3-Ihh pathway, the precise interactions of Runx2 and Nell-1 that regulate chondrogenic differentiation remain largely unknown. For instance, although our previous studies demonstrated that Nell-1 cannot regulate Runx2 expression in chondrocytes,[25] whether Nell-1 modulates post-translational modifications of Runx2, such as phosphorylation,[65,66] has not been determined. In addition, previous studies showed that the LEF/TCF/β-catenin complex promotes Runx2 expression and induces chondrocyte hypertrophy.[67] Although the noncanonical Wnts (eg, Wnt5a) induce early-stage chondrogenesis through intracellular calcium release via a G-protein coupled receptor activation, Wnt5a can also act as an inhibitor of chondrocyte hypertrophy by stimulating the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt)-dependent pathway that activates NF-κB to inhibit Runx2 expression.[68] Evidently, Runx2 is also a key molecule of the canonical and noncanonical Wnt pathways in the context of chondrogenic regulation, which suggests that it may function as a convergent point for multiple signaling pathways in the functional regulation of chondrogenesis. Given that Nell-1 has been suggested to be a novel activator of the

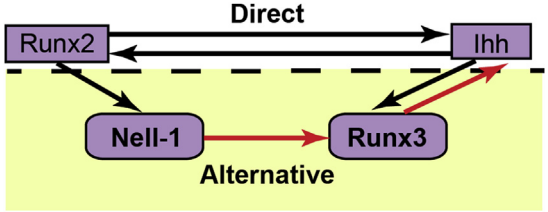

Figure 8 Diagram of direct and alternative Runx2-Ihh regulation in chondrogenesis. **Black arrows** present established knowledge: Runx2 is known to directly bind the Ihh promoter and stimulate Ihh expression in chondrocytes, and Ihh up-regulates the expression of Runx2 and Runx3 during chondrogenesis. **Red arrows** present conclusions determined in the current study: As an alternative pathway, Nell-1 acts as a downstream Runx2 mediator that induces Ihh signaling transduction via Runx3, which does not rely on Runx2 expression.

canonical Wnt signaling pathway in osteogenesis,[10] it is speculated that the effects of Nell-1 in modulating chondrogenesis downstream of Runx2 may also occur through a complex crosstalk among the Ihh and Wnt signaling pathways. Clearly, future studies are required for the investigation of such complex interactions to gain a comprehensive understanding of Nell-1's important role in chondrogenesis.

In summary, we revealed that Nell-1's prochondrogenic bioactivities can occur through a Runx3-mediated Ihh signal activation. This is the first report to describe Nell-1, a downstream molecule of Runx2 in chondrocytes, as triggering a signaling pathway for chondrogenesis regulation that is not dependent on the expression, nuclear import, and DNA binding functions of Runx2. This finding, not only highlights the essential modulatory feature of Nell-1 in chondrogenic differentiation and maturation, but also enriches our knowledge of the complex interactions between a diverse group of growth factors and extracellular matrix molecules during chondrogenesis.

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C.L., Z.Z., J.J., X.Z., K.T., and C.S. designed the study; C.L., Z.Z., J.J., W.J., C.T.C., and X.Z. conducted the study; C.L., J.J., W.J., and X.Z. collected data; C.L., Z.Z., J.J., X.Z., and K.T. analyzed data; C.L., Z.Z., X.Z., and K.T. interpreted data; Y.-H.Z., K.T., and C.S. provided financial support; C.L., Z.Z., K.L., E.A.B., and E.C.C. wrote the manuscript; Z.Z., X.Z., K.T., and C.S. revised the manuscript; X.Z., K.T., and C.S. approved the final version of the manuscript.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.ajpath.2017.09.020*.

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