INTRODUCTION: The vast majority of cartilage abnormalities are associated with short stature and/or severe axial skeleton deformities. These phenotypes often result from both accelerated or repressed chondrogenesis and chondrocyte maturation. Despite sustained efforts towards understanding the mechanisms responsible for the commitment of progenitor cells into the chondrogenic lineage and their differentiation into hypertrophic chondrocytes, the molecular events that control the progression from immature to mature chondrocytes are still unclear. Although genetic evidence demonstrated a requirement for Wnt5a during cartilage development, little is known about the mechanisms underlying non-canonical Wnt5a-regulated chondrogenic growth and differentiation. We therefore investigated the signal transduction pathways induced by Wnt5a during chondrocyte differentiation and maturation.

METHODS: Cell and Micromass culture systems MLB13MYC Clone17 chondrogenic precursors (C17 cells) were cultured in DMEM, 10% FBS. For transfection assays, cells were cultured in DMEM, 2% FBS at a density of 1x10^5 cells/cm^2 overnight and treated with 50 ng/ml recombinant Wnt5a (R&D Systems). For micromass culture experiments, E11.5 limb bud cells or C17 cells were cultured as high density 10 µl drops containing 2x10^5 cells in DMEM, 2% FBS supplemented with 25 µg/ml ascorbic acid, 54 µg/ml beta-glycerol phosphate and 1X ITS (Invitrogen). Cultures were feed every three days for 21 days and either fixed or harvested as described below.

Alcian blue staining Cultures were fixed and stained with alcian blue (1% alcian blue, 3% acetic acid) for two hours.

Transfection Assays C17 cells were cultured as above and transfected with each indicated expression construct or multimerized response element using Fugene HD reagent (Roche).

DNA extraction and real time RT-PCR C17 cells or micromass cultures were differentiated as above and harvested for total RNA in TRIzol reagent (Invitrogen, Carlsbad, CA) and phenol/chloroform extraction. Specific primers for each gene were used in real-time RT-PCR analysis.

Western Blotting Cultures were fractionated into nuclear and cytoplasmic extracts. Following protein quantification, 20 µg of total protein was subjected to SDS-PAGE. Western blotting was carried out using antibodies directed against NFATc1 (Santa Cruz Biotechnology) p65 NF-κB (Cell Signaling Technologies), tubulin and actin (Sigma-Aldrich) at 1:1,000 dilution and corresponding secondary antibodies (Cell Signaling Technology) at a 1:10,000 dilution with chemiluminescent detection using the Pierce Femto reagent.

RESULTS: Wnt5a has been shown to stage-dependently regulate chondrocyte differentiation by promoting chondrogenic proliferation and inhibiting chondrocyte hypertrophy. The mechanism, however, through which Wnt5a temporally regulates chondrocyte differentiation and maturation has not been ascertained. We first established stage-dependent effects of Wnt5a using a C17 micromass culture system to simulate cartilage development. Expression of dnWnt5a at an immature stage decreased chondrocyte differentiation while dnWnt5a expression late during differentiation enhanced chondrocyte maturation. Thus, loss of Wnt5a function induces stage-dependent effects. In contrast, treatment with Wnt5a at an immature stage of chondrocyte development in both a primary limb bud-derived and the mouse limb-bud derived chondrogenic cell line C17 chondrocyte differentiation model enhanced chondrocyte maturation. Conversely, late Wnt5a treatment repressed chondrocyte maturation. In addition, while Wnt5a induced luciferase activity driven by a multimerized NFAT responsive element in chondrogenic progenitor cells, luciferase activity downstream of a NF-κB responsive element is repressed by Wnt5a. We therefore determined the signaling events mediated by Wnt5a during chondrogenesis and chondrocyte maturation. The increase in chondrocyte hypertrophy seen with early Wnt5a treatment was associated with an increase in NFAT activation and a decrease in NF-κB activation as assayed by nuclear localization. In contrast, the inhibitory effect of late Wnt5a treatment on chondrocyte maturation was associated with a decrease in NFκB activation and an increase in NF-κB activation. Furthermore, the increase in chondrocyte maturation with early Wnt5a treatment was blocked through CaMK or NFAT loss of function. However, the inhibitory effects of Wnt5a at late stages of chondrocyte differentiation were not blocked through inhibition of CaMK or NFAT. In addition, the repression of chondrocyte maturation observed with late Wnt5a treatment was abrogated by NF-κB loss of function. Mechanistically, Wnt5a acts to increase chondrocyte differentiation at an early stage through induction of Sox9, the master chondrogenic transcription factor. This Wnt5a-mediated induction of Sox9 is blocked through inhibition of CaMK/NFAT. Conversely, Wnt5a represses chondrocyte maturation via NF-κB-dependent inhibition of Runx2 expression. These data demonstrate for the first time that not only does Wnt5a inhibit chondrocyte hypertrophy through a novel IKK/NF-κB-dependent pathway, but that Wnt5a also promotes chondrogenesis through CaMK/CaN/NFAT signaling.

DISCUSSION: Previous reports concerning the effects on non-canonical Wnt signaling on chondrocyte differentiation have demonstrated that Wnt5a promotes chondrogenic proliferation and inhibits chondrocyte hypertrophy. In this study we describe the mechanism for the differential effects of Wnt5a on chondrogenesis and chondrocyte maturation. Our data demonstrate for the first time that not only does Wnt5a inhibit chondrocyte hypertrophy through a novel IKK/NF-κB-dependent pathway, but that Wnt5a also promotes chondrogenesis through CaMK/CaN/NFAT signaling. Future work will be aimed at determining how the switch cell program elicited by this single growth factor is achieved.