INTRODUCTION
Carboxypeptidase Z (CPZ) removes carboxyl-terminal basic amino acid residues, particularly arginine residues, from proteins. CPZ contains a cysteine-rich domain (CRD) similar to the CRD found in the frizzled family of Wnt receptors (1). The expression of CPZ overlaps with the expression pattern of several Wnt genes and persists in cartilage condensations throughout mouse gestation (2). Wnt signaling has recently been recognized as an important signal transduction pathway in regulating chondrocyte proliferation and differentiation during limb development. We have previously shown that thyroid hormone regulates terminal differentiation of growth plate chondrocytes through activation of Wnt-4 expression and Wnt/β-catenin signaling (3). Given that Wnt-4 contains a C-terminal arginine, that CPZ binds to Wnt-4 through its CRD, and that CPZ enhances the Wnt-4 dependent induction of the homeobox gene Cdx1 (4), it is reasonable to postulate that CPZ may enhance Wnt-4 activity through enzymatic removal of its terminal arginine. The objective of this study was to determine if CPZ modulates Wnt/β-catenin signaling and terminal differentiation of growth plate chondrocytes.

METHODS
Three-dimensional pellet cultures of 2-day old rat growth plate chondrocytes were maintained in serum-free medium supplemented with ITS+1. T3 (Tri-iodothyronine) was used at a concentration of 100 ng/ml. RNA knockdown experiments were performed using siRNA against Wnt-4 or CPZ. TCF/LEF transcriptional activity was evaluated using TOPFlash reporter. Protein levels of CPZ and beta-catenin were examined by Western blot. Homogenates of cell pellets were assayed for carboxypeptidase activity using dansyl-Phe-Ala-Arg as a substrate. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectroscopy was used to analyze the effect of CPZ on a synthetic peptide consisting of the carboxyl-terminal 16 amino acids of the Wnt-4 protein (VKCRQQRQRLVEMHTCR). A truncated Wnt-4 expression vector lacking the C-terminal arginine was constructed by site-directed mutagenesis. Wnt-4 conditioned medium was collected from HEK293 cells transfected with Wnt-4 plasmid.

RESULTS
CPZ and Wnt-4 mRNA were co-expressed throughout growth plate cartilage. T3 increased both Wnt-4 and CPZ expression, as well as CPZ enzymatic activity (Figure 1). Knockdown of either Wnt-4 or CPZ mRNA levels using an RNA interference technique, or blocking CPZ enzymatic activity with the carboxypeptidase inhibitor GEMSA reduced the thyroid hormone effect on both alkaline phosphatase activity and Col10a1 mRNA expression. Adenoviral overexpression of CPZ activated Wnt/β-catenin signaling and promoted the terminal differentiation of growth plate cells, and also enhanced the Wnt-4 activity in growth plate cells (Figure 3). Col-Immunoprecipitation and immunoblotting confirmed the physical association of Wnt-4 with CPZ. Overexpression of CPZ in growth plate chondrocytes removed the C-terminal arginine residue from a synthetic peptide consisting of the C-terminal 16 amino acids of the Wnt-4 protein (Figure 2). Compared to full-length Wnt-4, incubation of growth plate cells with the truncated Wnt-4 construct (∆Wnt-4) induced an increased activation of Wnt/β-catenin signaling, and was more potent in enhancing the positive effect of Wnt-4 on terminal differentiation (Figure 3).

DISCUSSION
This study identifies CPZ as an important enzyme that is able to remove the C-terminal arginine from Wnt-4 in growth plate chondrocytes. This removal not only increases Wnt-4 activity, but also further promotes terminal chondrocyte differentiation. These findings indicate that the potent and positive thyroid hormone effect on skeletal maturation may result in part from CPZ-mediated activation of Wnt signaling in the growth plate.

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