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Calcium/calmodulin-dependent protein kinase II activity regulates the proliferative potential of growth plate chondrocytes

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SUMMARY

For tissues that develop throughout embryogenesis and into postnatal life, the generation of differentiated cells to promote tissue growth is at odds with the requirement to maintain the stem cell/progenitor cell population to preserve future growth potential. In the growth plate cartilage, this balance is achieved in part by establishing a proliferative phase that amplifies the number of progenitor cells prior to terminal differentiation into hypertrophic chondrocytes. Here, we show that endogenous calcium/calmodulin-dependent protein kinase II (CamkII, also known as Camk2) activity is upregulated prior to hypertrophy and that loss of CamkII function substantially blocks the transition from proliferation to hypertrophy. Wnt signaling and Pthrp-induced phosphatase activity negatively regulate CamkII activity. Release of this repression results in activation of multiple effector pathways, including Runx2- and β-catenin-dependent pathways. We present an integrated model for the regulation of proliferation potential by CamkII activity that has important implications for studies of growth control and adult progenitor/stem cell populations.

KEY WORDS: CamkII, Kinase, Growth plate, Cartilage, Chondrocyte maturation, Transit amplification, Adult stem cells, Chick, Mouse

INTRODUCTION

During development, the proper balance between progenitor cell proliferation and terminal differentiation must be achieved in order to generate functional tissues. The appropriate balance is especially important in organs that develop slowly, in particular those that continue to develop postnatally. Over long periods, small differences in rates of cell proliferation or cell differentiation can be amplified, resulting in defects in tissue formation and tissue growth.

Balancing cell proliferation and differentiation is crucial for proper growth of the skeleton. Elongation of the appendicular skeleton is regulated at the level of chondrocyte maturation in the growth plate cartilage that resides at the ends of the long bones (reviewed by Kronenberg, 2003). Within the growth plate cartilage, chondrocyte maturation is regulated both spatially and temporally resulting in morphologically and functionally distinct cellular zones (Fig. 1A). Under the articular surface resides a population of progenitor cells, the resting chondrocytes (Abad et al., 2002), which are progressively selected to enter a proliferative phase (Dodds, 1930). Within the proliferative zone, cells display aligned division planes and form columns composed of flattened chondrocytes by clonal expansion (Dodds, 1930; Li and Dudley, 2009). Subsequently, cell proliferation decreases and cells enlarge, forming prehypertrophic chondrocytes. Prehypertrophic chondrocytes terminally mature into hypertrophic chondrocytes that swell (hypertrophy) and deposit collagen X before undergoing cell death and replacement by blood vessels and bone-forming osteoblasts. It is this final process of cell hypertrophy that largely determines growth rate in long bones (Breur et al., 1991; Hunziker et al., 1987).

Previous studies in chick and mouse described a complex network of interacting signaling pathways that balances current requirements for growth (formation of hypertrophic chondrocytes) with the preservation of future growth potential (resting and proliferative chondrocytes). At the heart of this system lies the secreted signaling proteins indian hedgehog (Ihh) and parathyroid hormone-related peptide (Pthrp, also known as Pthlh) (reviewed by Kronenberg, 2003). The prehypertrophic chondrocytes produce Ihh, which acts to promote chondrocyte proliferation and to induce Pthrp expression in resting chondrocytes (Vortkamp et al., 1996). In turn, Pthrp secreted by resting chondrocytes antagonizes both *Ihh* function and the proliferative to prehypertrophic chondrocyte transition (Lanske et al., 1996). Overlaid on this central interaction are the actions of additional secreted factors, including Wnt5a and Wnt5b, which coordinately control the transitions from resting to proliferative and from proliferative to prehypertrophic chondrocyte (Yang et al., 2003); the bone morphogenetic proteins (Bmp) (De Luca et al., 2001; Lyons et al., 1991; Yoon and Lyons, 2004); and the fibroblast growth factors (Fgf) (Chen et al., 1999; Legeai-Mallet et al., 1998; Mancilla et al., 1998). Together, these factors provide positive and negative interactions that balance the number of progenitor cells with the rate of hypertrophy by defining a domain that is permissive for chondrocyte proliferation.

It is not known how information in the proposed gradients of secreted signaling molecules produces the sharp transition that terminates proliferation and induces hypertrophy. We, and others, previously demonstrated that expression of a constitutively activated form of calcium/calmodulin-dependent protein kinase II alpha (daCamkII) induces the morphological and molecular signatures of hypertrophy in chondrocytes of the proliferative zone

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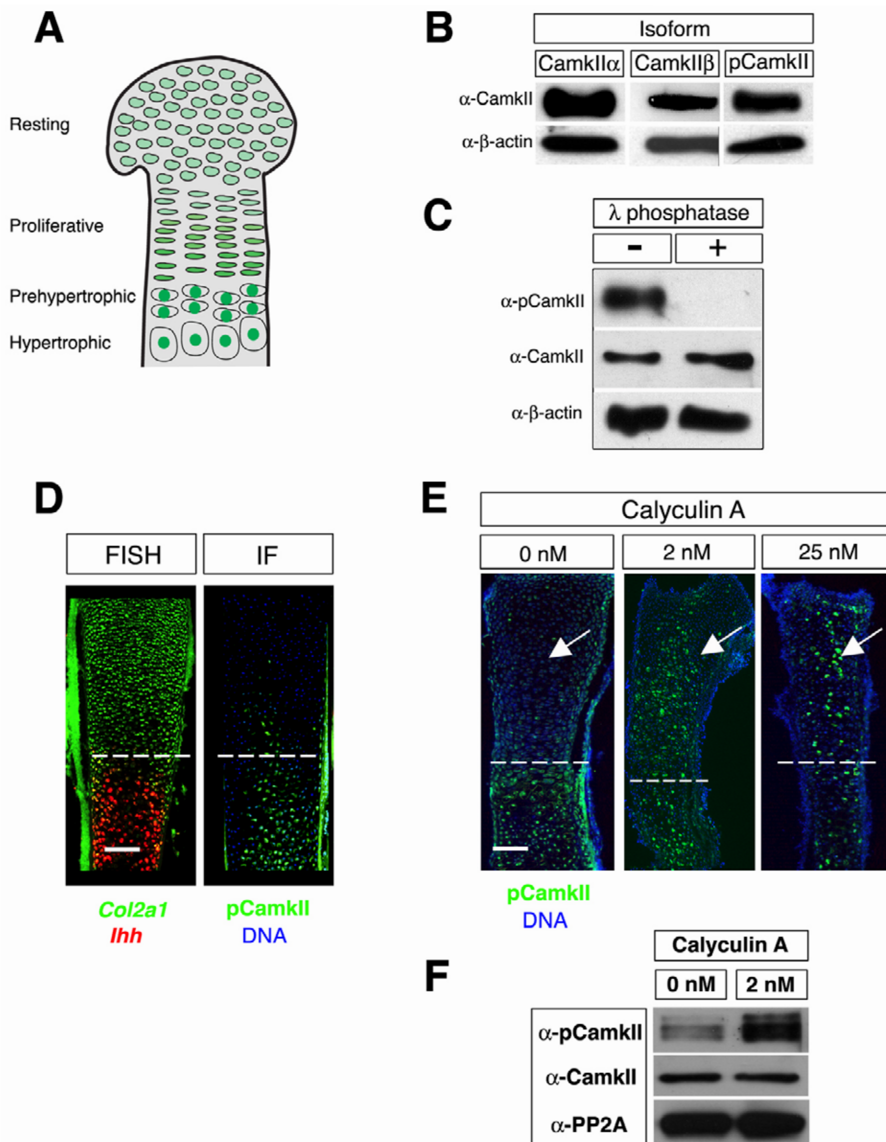

Fig. 1. CamkII is activated in the prehypertrophic zone. (**A**) The growth plate is composed of four zones: resting (RZ), proliferative (PZ), prehypertrophic (PHZ) and hypertrophic (HZ). (**B**) Western blot analysis of total chick growth plate confirms the presence of CamkIIα and CamkIIβ, including phosphorylated forms (pCamkII). (**C**) Lysates of total chick growth plates pretreated with λ protein phosphatase show substantial reduction in pCamkII without significantly altering CamkIIα (β-actin loading control), demonstrating phospho-specificity. (**D**) Analysis of adjacent sections of the chick growth plate (*n*=6) by fluorescent in situ hybridization (FISH) with probes for *Col2a1* (proliferative zone, green, left-hand column) and *Ihh* (prehypertrophic zone, red, left-hand column), or immunofluorescence (IF) with anti-pCamkII (green, right-hand column) revealed that nuclear pCamkII increases in chondrocytes at the PZ to PHZ transition (white dashed line). (**E**) Immunofluorescence of mouse metatarsals treated with calyculin A (*n*=6 per concentration) shows global activation of CamkII in chondrocytes (arrows), suggesting that negative regulators oppose CamkII activity. White dashed line indicates the border between the proliferative and the prehypertrophic zones. (**F**) Parallel western blot analysis (*n*=20 metatarsals; two experiments) shows increased phosphorylation of CamkII following calyculin A treatment that occurs without changes in total CamkII or PP2A levels. Scale bars: 500 μm in D; 100 μm in E.

(Taschner et al., 2008; Li and Dudley, 2009). However, at the time, the biological relevance of these findings was uncertain because localization of CamkII activity had not been demonstrated and loss-of-function experiments generated only very subtle phenotypes. Moreover, the regulation of CamkII activity by the integrated signaling environment had not been explored.

Here, we show, at single-cell resolution, that CamkII activity increases in cells during transition from the proliferative to the prehypertrophic state. Ectopic activation of CamkII in proliferative chondrocytes upregulates endogenous CamkII activity and promotes *Ihh* expression, a marker for definitive prehypertrophic chondrocytes. In vitro treatment of mouse metatarsals with chemical inhibitors of CamkII greatly reduces expression of both *Ihh* and *Col10a1* in hypertrophic chondrocytes, showing that endogenous CamkII activity is required for normal hypertrophy. We present evidence that CamkII activity in the growth plate cartilage is tightly regulated by a ubiquitous phosphatase activity and is opposed by both Wnt and Pthrp signaling. At the proliferative to prehypertrophic chondrocyte transition, CamkII activity induces cell hypertrophy through a branched set of effector pathways that include the transcriptional regulators β-catenin and Runx2. Together, our data suggest that induction of the hypertrophic program occurs when reduced inhibition elevates CamkII activity above threshold. We propose a model to explain how establishing the position of the proliferative-prehypertrophic border controls the replicative potential of the proliferative chondrocyte pool.

MATERIALS AND METHODS

Construction of recombinant retrovirus and processing of chick embryos

Retrovirus constructs and stocks were generated in RCAS(A) vectors (Hughes, 2004) and injected into chick embryos using standard methods (Logan and Tabin, 1998). RCAS-daCamkII was a gift from C. M. Chuong (University of Southern California, CA, USA), RCAS-Wnt5a and RCAS-Wnt5b were obtained from Addgene (deposited by Clifford J. Tabin, Harvard Medical School, MA, USA), and RCAS-dnFzd7 and RCAS-Fzd7 were previously described (Li and Dudley, 2009). HA-daCamkII and RCAS-Pthrp were generated by polymerase chain reaction using forward-primer: GCGTCTCCCATGTACCCATACGATGTTCCAGA, reverse-primer: TCCAGAAGATCCTCCAGATCCAGCGTAATCTGGAA and

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forward primer: GGAGAAGACCTCATGCTGCGGAGGCTGGTTCA reverse primer: GAAGCGGCCGCTCAATGCGTCCTTAAGCTGGGCT, respectively.

Mouse strains and animal care

Mouse strains *Ctnnb1^{tm2Kem}* (Brault et al., 2001) and *Col2a1::cre* (Ovchinnikov et al., 2000) were used to generate β-*catenin* mutants. Animal care and use was in accordance with Public Health Service guidelines and was approved by the Institutional Animal Care and Use Committee at Northwestern University. The morning after mating is designated as 0.5 days post coitum (dpc).

Isolation of chondrocytes and western analysis

Chondrocyte isolation was performed as described previously (Aszodi et al., 2003). Briefly, chondrocytes from tibiae and humeri were isolated from wild-type mice at 16.5 dpc or from wild-type chick embryos after 11 days of incubation (E11). Following removal of the periosteum and ossification center, the cartilage growth plate was cut into small pieces, homogenized in RIPA buffer containing protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA), and clarified by centrifugation. Where indicated, supernatant was dephosphorylated by λ protein phosphatase (New England Biolabs, Beverly, MA, USA). The following antibodies were used: anti-pCamkII (1:400; Cell Signaling, Beverly, MA, USA), anti-CamkIIα (1:500; Cell Signaling), anti-CamkIIβ (1:1000; Abcam, Cambridge, MA, USA), anti-HA (1:2000; Sigma-Aldrich, St Louis, MO, USA), anti-β-catenin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PP2A (1:500; R&D Systems, Minneapolis, MN, USA), anti-β-actin (1:1000; Cell Signaling) and HRP-conjugated secondary antibodies (1:5000; Cell Signaling).

Tissue preparation, immunofluorescence and in situ hybridization

Skeletal preparation was carried out as described previously (Li and Dudley, 2009). Limbs from mouse or 11-day chick embryos (E11) were dissected, fixed in 4% paraformaldehyde in PBS or in Bouin's Fixative (Ricca Chemical Company, TX, USA) overnight at 4°C. For standard histological analysis, tissue was dehydrated through an ethanol series followed by xylenes, embedded in paraffin (Paraplast X-tra; Richard-Allan Scientific, MI, USA), and sectioned. Sections were floated onto Superfrost Plus glass slides (VWR International, San Francisco, CA, USA) and stained with Masson's Trichrome using standard protocols.

For immunofluorescence (IF) and in situ hybridization (ISH) analysis, tissue was fixed in paraformaldehyde, equilibrated in 30% sucrose in PBS and frozen in Tissue-Tek O.C.T. Frozen sections (14 μm) were mounted on glass slides. For IF, sections were permeabilized in 1% Triton X-100 and blocked in 10% fetal bovine serum before incubation with primary and secondary antibodies. In addition to those described above, the antibodies used were: anti-pCamkII (1:50; Cell Signaling), anti-HA (1:500; Sigma-Aldrich), anti-Runx2 and anti-β-catenin (each at 1:50; Santa Cruz); and Cy3-conjugated anti-mouse, Alexa 488-conjugated anti-mouse, Cy3-conjugated anti-rabbit, Alexa 488-conjugated anti-rabbit, and Cy5-conjugated anti-mouse (each used at 1:500; Invitrogen, Carlsbad, CA, USA). Where indicated, tissue was counter-stained with Phalloidin 468 (1:200; Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI, at 0.1 μg/ml; Sigma-Aldrich). For ISH, RNA probes were produced using digoxigenin (DIG) or biotin-labeled ribonucleotide tri-phosophates (rNTPs; Roche Diagnostics), as described previously (Li and Dudley, 2009; Ahrens et al., 2009). Double-fluorescent in situ hybridization (FISH) was performed using TSA Plus Kits (Perkin-Elmer, Waltham, MA, USA). For combined FISH and IF, sections were treated with 2.5 μg/ml proteinase K (Roche) and hybridization was performed at 55°C.

Metatarsal cultures

The center three metatarsals were isolated from each hindlimb of 16.5 dpc mouse embryos, cleaned of soft tissue and cultured in α-MEM + 1% glutamine-penicillin-streptomycin (Invitrogen) containing 1% ITS+3 medium supplement, 10 mM β-glycerophosphate and 50 μM ascorbate (each from Sigma-Aldrich) in a humidified, 5% CO_2 environment at 37°C. In some experiments, isolated metatarsals were first incubated in α-MEM + 10% fetal bovine serum (Atlanta Biologicals) + 0.1% collagenase (Sigma-Aldrich) at 37°C for one hour to enable complete removal of the perichondrium. Cultures were supplemented with control diluent or the drugs KN-92 (cat. # 422709), KN-93 (#422708), AIP-II (#189485), NtideII (#208921) or calyculin A (#208851), obtained from EMD Calbiochem (Gibbstown, NJ, USA). Media and treatments were replaced every 24 hours.

Transfection assays

For nuclear localization experiments, cultured DF-1 fibroblast cells were transfected with 500 ng RCAS(A)-HA-daCamkII and 500 ng RCAS(A)-Runx2 in 24-well plates using Superfect (Qiagen, Valencia, CA, USA). After two days, immunofluorescence analysis was performed as described above.

To assay transcriptional activation of the *Ihh* promoter, collagenase-isolated chondrocytes were transfected (three wells per treatment; three experiments) with 250 ng *Ihh-luc*, 2 ng CMV-Renilla and 375 ng pCAG-HA-daCamkII and/or 375 ng pcDNA3.1-Runx2 in 24-well plates using Fugene (Roche Diagnostics). pCAG-GFP was added to maintain 1 μg total DNA per transfection. After two days, cell lysates were assayed with the Dual Luciferase Kit (Promega, Madison, WI, USA) and data were plotted as the fold increase in the firefly:renilla luciferase signal over control (no daCamkII or Runx2) and analyzed by Student's *t*-test.

RESULTS

CamkII is activated in prehypertrophic chondrocytes

Four CamkII isoforms (α, β, γ and δ) are expressed in growth plate chondrocytes (Taschner et al., 2008; Li and Dudley, 2009). We confirmed the presence of the alpha and beta isoforms by western blot analysis and also demonstrated that CamkII is activated in vivo using a phospho-specific antibody (anti-pCamkII) against the form of CamkII that is phosphorylated on threonine 286 (^{286}T; pCamkII), a modification that renders CamkII constitutively active (Fig. 1B,C) (Kameshita et al., 1999; Wang et al., 1998).

Standard immunofluorescence methods revealed nuclear localized pCamkII in prehypertrophic and hypertrophic chondrocytes, but not in resting and proliferative chondrocytes, of chick and mouse long bones (Fig. 1D,E; see Fig. S1 in the supplementary material) and vertebrae (see Fig. S2A in the supplementary material; data not shown). Using high-sensitivity methods, lower levels of nuclear pCamkII were detected in the other maturation zones (see Fig. S3 in the supplementary material), suggesting that CamkII activity is differentially regulated during chondrocyte maturation. Differential regulation could result from the action of phosphatases (Ishida et al., 1998a; Ishida et al., 1998b). Accordingly, pCamkII was upregulated in both the cytosol and the nucleus of resting and proliferative chondrocytes following treatment with calyculin A, a potent inhibitor of PP1 and PP2A phosphatase activity (Fig. 1E,F) (Fagerholm et al. 2010; Ishihara et al., 1989). Collectively, these results suggest that CamkII activity is tightly regulated during endochondral bone growth to limit high pCamkII levels to prehypertrophic and hypertrophic chondrocytes.

CamkII activates the hypertrophic program in proliferative chondrocytes

To investigate the relationship between CamkII activity and prehypertrophy, we used a previously generated phospho-mimetic (T286D) form of CamkII (daCamkII) that is constitutively active (Kuhl et al., 2000a) and we generated a novel form of daCamkII bearing an N-terminal hemagglutinin epitope tag (HA-daCamkII) to facilitate the analysis of mosaic cartilage at single cell resolution. This is advantageous for three reasons: first, it is difficult to obtain fully infected cartilage, presumably due to effects of daCamkII on the cell cycle (Taschner et al., 2008); second, performing analysis

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on affected cells in an otherwise wild-type environment demonstrates that the observed phenotype does not result from global disruption of the signaling network that regulates chondrocyte maturation; and third, the epitope tag permits distinction between cell-autonomous and non-cell-autonomous aspects of the phenotype. Except where specifically noted, both forms of daCamkII produced similar phenotypes in chick limb cartilage.

Ectopic expression of daCamkII (data not shown) or HA-daCamkII (Fig. 2A) in proliferative chondrocytes using RCAS(A) retroviral vectors (Hughes, 2004) increased nuclear pCamkII. Interestingly, we found that HA-daCamkII was not translocated to the nucleus (Fig. 2A). Therefore, the nuclear pCamkII detected results from activation of endogenous CamkII, consistent with phenotypic changes observed in gain-of-function experiments reflecting the endogenous functions of CamkII pathways.

Activation of CamkII in proliferative chondrocytes produced profound changes in cell morphology and resulted in upregulation of *Ihh*, consistent with induction of prehypertrophy (Fig. 2A,B,D) (Taschner et al., 2008). However, not every infected cell showed nuclear pCamkII (Fig. 2A) or expressed *Ihh* (Fig. 2B). We quantified these data and found that for both daCamkII and HA-daCamkII, induction of *Ihh* expression increased in a position-dependent manner from resting chondrocytes (0% for both constructs) to lower proliferative chondrocytes (31% and 11%, respectively) (Fig. 2B,C). The apparent difference in potency for tagged and untagged daCamkII was confirmed by co-infection (see Fig. S4 in the supplementary material). Together, these data suggest that full activation of the hypertrophic program requires threshold levels of CamkII activity and are consistent with the presence of an inhibitory gradient in the growth plate.

Although only a percentage of infected proliferative chondrocytes expressed *Ihh*, all displayed morphological changes and reorganization of the cortical actin cytoskeleton (Fig. 2D) that are consistent with chondrocyte hypertrophy (see Fig. S5 in the supplementary material). This phenotype was not observed in uninfected neighboring chondrocytes, demonstrating that ectopic *daCamkII* activity did not alter the global signaling environment or exhibit non-cell-autonomous effects. Furthermore, infected chondrocytes that failed to prematurely induce *Ihh* did so later in synchrony with neighboring wild-type cells (data not shown), demonstrating that *daCamkII*-expressing chondrocytes respond properly to endogenous maturation signals. Together, the phenotypic analysis suggests that CamkII activity promotes chondrocyte hypertrophy through separable events in the cytosol (actin reorganization) and nucleus (*Ihh* transcription).

CamkII activity is a key regulator of chondrocyte hypertrophy

We next asked whether the extent of hypertrophic maturation depends on the level of endogenous CamkII activity. Given the possibility of functional redundancy among isoforms (Fig. 1B), we designed an in vitro functional test using mouse metatarsals and defined chemical inhibitors. We chose metatarsals because they exhibit consistent growth rates (see Fig. S6 in the supplementary material), and we reasoned that chemical inhibitors would diffuse more freely into these small cartilage elements. In total, three different inhibitors were tested: KN-93, a small molecule competitive inhibitor of the CamkII-calmodulin interaction that leads to kinase activation (Ishida et al., 1995; Sumi et al., 1991); and AIP-II and NtideII, two cell permeable peptides that are specific and potent inhibitors of the kinase activity (Ishida and Fujisawa, 1995; Ishida et al., 1995). The more commonly used inhibitor KN-93 disrupted growth plate architecture at all doses (see Fig. S7 in the supplementary material). By contrast, AIP-II and NtideII showed specific effects on *Ihh* expression and on hypertrophic zone size, with AIP-II being more potent (Fig. 3). Presence of the perichondrium was not required for downregulation of pCamkII (see Fig. S8A in the supplementary material) or gene expression by AIP-II (see Fig. S8B in the supplementary material). Complete inhibition of hypertrophy could not be achieved because higher doses were toxic (data not shown). Together, our gain- and loss-of-function data demonstrate that CamkII activity is a primary cell autonomous regulator of chondrocyte hypertrophy.

Frizzled signaling regulates *CamkII* function

CamkII is one potential effector of frizzled (Fzd) signaling (Kuhl et al., 2000a; Kuhl et al., 2000b). To test this possibility, we first showed that RCAS(A)-*Wnt1*, an activator of the canonical Fzd/β-catenin pathway, did not alter nuclear pCamkII in prehypertrophic/hypertrophic chondrocytes of the chick (Fig. 4A). By contrast, RCAS(A)-*Wnt5a*, which is thought to activate noncanonical Fzd pathways (Hartmann and Tabin, 2000; Yang et al., 2003), strongly downregulated pCamkII even though the cells enlarged normally (Fig. 4A). Retroviral expression of dominant-negative Fzd7 (*dnFzd7*) (Li and Dudley, 2009), but not wild-type Fzd7, resulted in ectopic pCamkII in all maturation zones but not in every infected cell (Fig. 4A; data not shown). Many *dnFzd7*-expressing chondrocytes showed elevated nuclear and cytosolic pCamkII but were not overtly hypertrophic (Fig. 4A) and did not express *Ihh* (Li and Dudley, 2009), consistent with the known diverse actions of Wnt signaling in the growth plate cartilage (Hartmann and Tabin, 2000; Li and Dudley, 2009; Guo et al., 2009).

To explore potential epistasis between Wnt signaling and *CamkII* function, we investigated whether Wnt signaling could suppress the *daCamkII* phenotype in proliferative chondrocytes. Mosaic growth plates were generated by infecting limb buds with a 1:1 mixture of RCAS(A)-*HA-daCamkII* and either RCAS(A)-*Wnt5a* or RCAS(A)-*Wnt5b*. In this case, a common viral envelope protein was used because the non-cell-autonomous action of secreted Wnt ligands precludes the need for superinfection. We verified that the concentration of Wnt ligand was saturating by demonstrating global disruption of proliferative chondrocyte morphology (see Fig. S9A in the supplementary material) and by only analyzing limbs showing size reduction equivalent to fully infected tissue (data not shown) (Hartmann and Tabin, 2000). Under these conditions, neither Wnt ligand prevented induction of *Ihh* expression (Fig. 4B), affected actin reorganization or cell hypertrophy (see Fig. S9B in the supplementary material), or extinguished the endogenous pCamkII signal (Fig. 4C) in *HA-daCamkII*-expressing proliferative chondrocytes. Together, these data suggest that noncanonical Wnt signaling inhibits chondrocyte hypertrophy either upstream of, or in parallel to, CamkII phosphorylation.

Although Fzd signaling might act via β-catenin to inhibit *CamkII* function (Kuhl et al., 2001), the observations that nuclear pCamkII is unaffected by Wnt1 signaling and that β-catenin and pCamkII are both upregulated in nuclei of prehypertrophic and hypertrophic chondrocytes (Fig. 5A) suggest that this is not the case. Moreover, we found that 42.5% (246/579) of proliferative chondrocytes that express *HA-daCamkII* also showed elevated nuclear β-catenin (Fig. 5B), unlike neighboring wild-type cells (0/311). Furthermore, expression of *Wnt5a* resulted in loss of both β-catenin and CamkII immunoreactivity in nuclei of prehypertrophic chondrocytes,

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whereas expression of *dnFzd7* enhanced nuclear signal for both proteins in the proliferative zone (Fig. 5C). Similarly, both pCamkII and β-catenin were downregulated in metatarsals treated with AIP-II (see Fig. S8A in the supplementary material). As β-catenin is constitutively degraded in the absence of signaling (Papkoff et al., 1996; Rubinfeld et al., 1997), these data suggest that CamkII activity substitutes for Fzd signaling to stabilize β-catenin in prehypertrophic and hypertrophic chondrocytes.

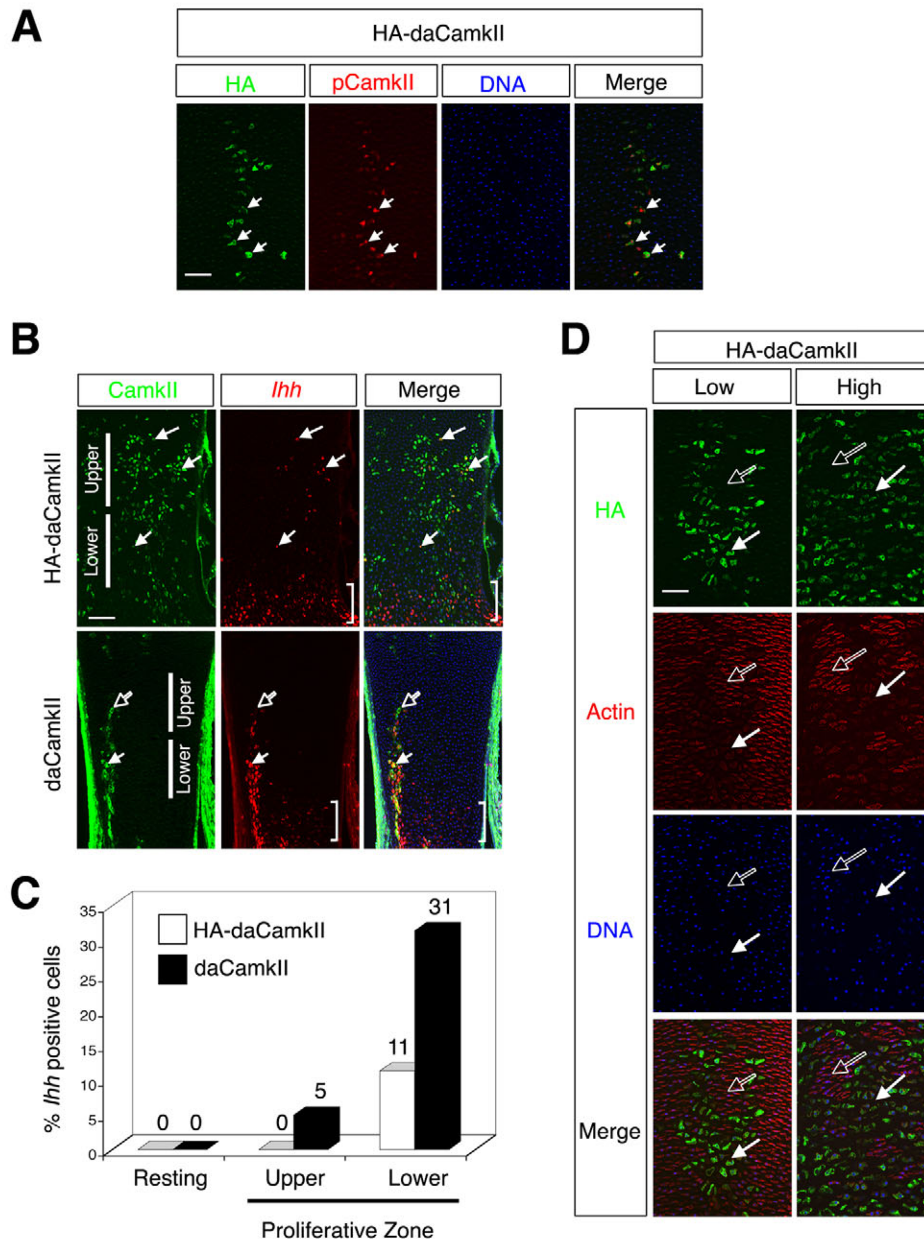

Fig. 2. daCamkII promotes prehypertrophy in the proliferative zone. Sections of chick growth plate cartilage infected with RCAS(A)-daCamkII or RCAS(A)-HA-daCamkII were analyzed by immunofluorescence (HA-daCamkII and pCamkII) and/or FISH (*daCamkII* and *Ihh*). Where indicated, DNA and actin were visualized using DAPI and phalloidin, respectively. (**A**) pCamkII (red) is present at high levels in the nucleus of proliferative chondrocytes that express HA-daCamkII (green), but not in neighboring wild-type cells (non-green), suggesting that daCamkII promotes CamkII activity in the growth plate (*n*=4). White arrows denote examples of double-positive cells. Note the absence of overlap within the cells for the HA and the nuclear pCamkII signals. (**B**) Expression of either *HA-daCamkII* (green, top panels) (*n*=7) or *daCamkII* (green, lower panels) (*n*=10) induces ectopic *Ihh* transcription (red) in proliferative chondrocytes. White brackets denote *Ihh* expression in the PHZ. White arrows indicate examples of double-positive cells, whereas open arrows highlight CamkII-positive, *Ihh*-negative cells. *Ihh* is induced with different efficiencies in the resting (not shown), the upper proliferative and the lower proliferative chondrocytes (indicated by solid bars) infected with the two daCamkII constructs, respectively. (**C**) Quantification of the data described in Fig. 2B. The number above each column is the percentage of infected cells that express *Ihh*. For each column, the number of infected cells examined was as follows: for *HA-daCamkII* infected cartilage, resting (*n*=570), upper proliferative (*n*=342) and lower proliferative (*n*=395) chondrocytes; for *daCamkII* infected cartilage, resting (*n*=1519), upper proliferative (*n*=495) and lower proliferative (*n*=1062). (**D**) At both high (*n*=3) and low (*n*=10) levels of infection, all *HA-daCamkII*-expressing cells (green, solid arrow) show hypertrophic morphology and low levels of cortical actin, whereas neighboring uninfected chondrocytes (non-green cells, open arrow) display the wild-type proliferative chondrocyte phenotype. Scale bars: 50 μm in A and D; 100 μm in B.

Co-regulation of CamkII activity and β-catenin stability could function to cooperatively effect hypertrophy (Dong et al., 2006) or to form a negative-feedback loop. To test these possibilities, we examined the loss-of-function phenotype in chondrocytes homozygous for a *Ctnnb1* conditional allele (Brault et al., 2001) that carry a *Col2a1::cre* transgene (Ovchinnikov et al., 2000). Mutant long bones were significantly shorter and showed reduced cell density compared with wild type (data not shown), as previously shown (Hill et al., 2005; Mak et al., 2006). Mutant growth plate chondrocytes matured (Fig. 5D) forming prehypertrophic (Fig. 5D,E) and hypertrophic chondrocytes (data not shown) that have normal levels of pCamkII positive nuclei (Fig. 5D,F). Thus, β-catenin is not an essential regulator of CamkII activity. Collectively, these data are most consistent with a minor role for β-catenin in hypertrophy (Guo et al., 2009) either downstream of or in parallel to *CamkII* function.

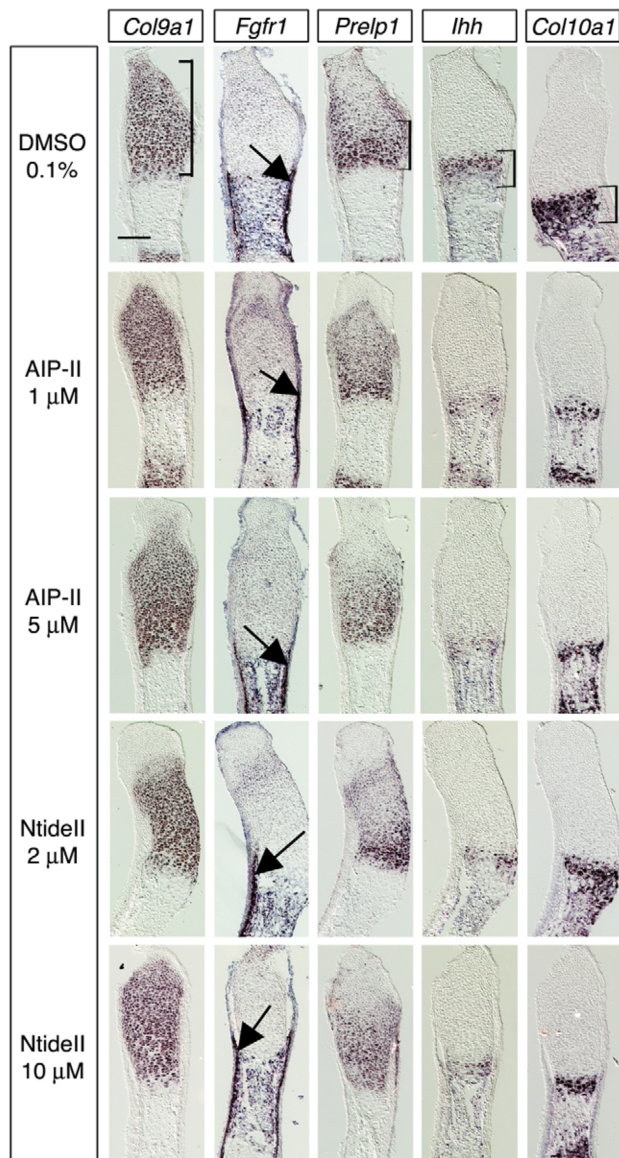

Fig. 3. CamkII activity is required for prehypertrophic and hypertrophic maturation. In situ hybridization analysis of mouse metatarsal cultures treated with inhibitors of CamkII activity (AIP-II and NtideII at the concentrations indicated). Relevant cell types and maturation states were identified as follows: *Col9a1* (immature chondrocytes), *Fgfr1* (osteoblasts, perichondrium, resting chondrocytes), *Prelp1* (proliferative chondrocytes), *Ihh* (prehypertrophic chondrocytes) and *Col10a1* (hypertrophic chondrocytes). The correct size and/or location of these expression domains are indicated by arrows (*Fgfr1*) or brackets (all others) presented in the solvent control (top panels). AIP-II treatment consistently reduced expression of hypertrophic markers (*Ihh* and *Col110a1*) without substantially affecting expression of markers for other maturation zones (*Col9a1*, *Fgfr1* and *Prelp1*) or non-chondrocyte cell types (*Fgfr1*). Similar results were observed for NtideII. For all treatments, *n*=8-12 metatarsals. Scale bar: 200 μm in all panels.

Pthrp antagonizes CamkII activity

The finding that noncanonical Wnt signaling antagonizes endogenous CamkII activation fails to explain one key observation – that *daCamkII* expression in resting chondrocytes did not activate the hypertrophic program (Fig. 2C and Fig. 6A). We reasoned that Pthrp signaling might inhibit *CamkII* function because Pthrp is the primary factor produced by resting chondrocytes and is known to oppose chondrocyte hypertrophy (Lanske et al., 1996; Vortkamp et al., 1996). Accordingly, RCAS(A)-Pthrp promoted global loss of proliferative and hypertrophic chondrocyte morphology (see Fig. S8A in the supplementary material). In cartilage co-infected (1:1) with RCAS(A)-HA-daCamkII, RCAS(A)-Pthrp prevented expression of *Ihh* (Fig. 6B), activation of endogenous CamkII (Fig. 6C) and reorganization of cortical actin and induction of hypertrophic morphology (see Fig. S10 in the supplementary material). These findings, together with results from the calyculin A studies (Fig. 1D,E), suggested that Pthrp acts via phosphatases to regulate CamkII activity. Consistent with this possibility, pCamkII signal was rescued in PTH-treated metatarsals by the addition of calyculin A (Fig. 6D,E). Thus, unlike Wnt signaling, Pthrp signaling is a dominant repressor of *daCamkII* function that acts by regulating the state of CamkII phosphorylation.

CamkII activity synergizes with Runx2

We noted that Pthrp signaling also inhibits hypertrophy via PKA-dependent phosphorylation of Runx2 (Franceschi et al., 2003; Guo et al., 2006; Li et al., 2004; Swarthout et al., 2002), a transcription factor required for chondrocyte hypertrophy (Sato et al., 2008). Therefore, we investigated whether the opposing outputs from Pthrp signaling and CamkII activity might converge at Runx2. We found that in cultured fibroblast cells Runx2 showed preferential localization to the nucleus only in cells that also expressed HA-daCamkII (Fig. 7A). Moreover, in chondrocytes, CamkII and Runx2 together increased transcription from the *Ihh* promoter over Runx2 alone (Fig. 7B). Together, these data suggest that CamkII promotes chondrocyte hypertrophy in part via regulation of Runx2 activity.

DISCUSSION

Endogenous CamkII activity is required for chondrocyte hypertrophy

Previous studies showed that ectopic activation of CamkII induces premature hypertrophy in proliferative chondrocytes (Li and Dudley, 2009; Taschner et al., 2008). However, the absence of data concerning the activity profile of CamkII in the growth plate and the subtle loss-of-function phenotype made the relevance of these findings to the normal physiological process uncertain. By combining three powerful tools – epitope-tagged daCamkII, a phospho-specific antibody and potent chemical inhibitors – we

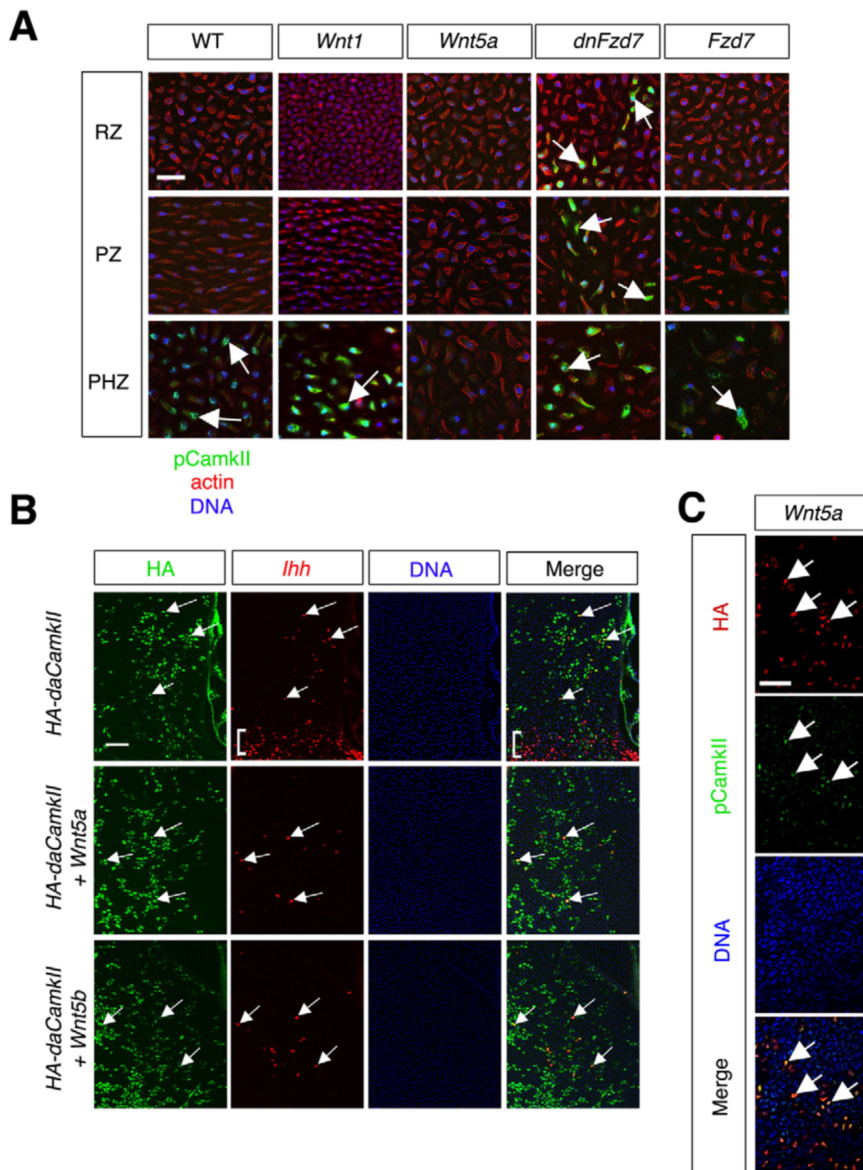

Fig. 4. Fzd signaling inhibits CamkII activity. Sections of growth plate cartilage from wild-type (WT) chick embryos or from embryos infected with the indicated viruses were processed for immunofluorescence (pCamkII and/or HA in A and C) or both immunofluorescence and in situ hybridization (HA and *Ihh*, respectively, in B). Where indicated, DNA (blue) was stained with DAPI and actin (red) was visualized with phalloidin. (**A**) In the growth plate, high levels of pCamkII are normally restricted to the PHZ/HZ. White arrows indicate pCamkII signal. Infection with RCAS(A)-Wnt1 (*n*=6) increases cell density in the growth plate (data not shown) but does not alter the CamkII activation domain. Infection with either RCAS(A)-Wnt5a or RCAS(A)-Fzd7 decreases nuclear pCamkII levels. By contrast, inhibition of Fzd signaling by RCAS(A)-*dnFzd7* infection promotes nuclear pCamkII in both the RZ and the PZ. Phalloidin staining shows that all the cells are disorganized, indicative of highly infected cartilage. *n*=8 for each condition. (**B**) Images of the PZ and PHZ (bracket) chondrocytes expressing *HA-daCamkII* either alone (*n*=5) or in combination with *Wnt5a* (*n*=5) or *Wnt5b* (*n*=5) show that Wnt signaling does not interfere with induction of *Ihh* by activated CamkII. Arrows indicate examples of cells expressing *Ihh* and brackets denote *Ihh* expression in the PHZ. (**C**) Immunofluorescence shows that co-infection with RCAS(A)-Wnt5a signaling does not block activation of endogenous CamkII (pCamkII) by RCAS(A)-HA-daCamkII (*n*=10). Scale bars: 50 μm in A; 200 μm in B; 100 μm in C. RZ, resting zone; PZ, proliferative zone; PHZ, prehypertrophic zone.

demonstrate that CamkII activity is upregulated in prehypertrophic and hypertrophic chondrocytes, that daCamkII activates endogenous CamkII and that inhibition of endogenous CamkII activity specifically downregulates the hypertrophic program. Our data suggest that CamkII activity induces hypertrophy in part by enhancing Runx2 transcriptional activity and by stabilizing nuclear β-catenin. In this context, the distinct phenotypes of *Runx2* (Guo et al., 2006) and *Ctnnb1* (Hill et al., 2005; Mak et al., 2006) mutant mice are most consistent with β-catenin acting downstream or in parallel to Runx2 activity to regulate late stages of hypertrophy.

CamkII is a node in the hypertrophic program

Although CamkII is a key regulator of chondrocyte hypertrophy, expression of *daCamkII* only weakly induced hypertrophy in upper proliferative chondrocytes and showed no activity in resting chondrocytes. These position-specific differences in daCamkII potency inhibitory interactions of signaling pathways are the main regulators of endogenous CamkII activity in the growth plate cartilage. Specifically, inhibition of ectopic *Ihh* expression and nuclear pCamkII by Pthrp signaling provides a molecular mechanism for the robustness of the resting chondrocyte phenotype and strongly suggests the pre-eminence of Pthrp in the regulation of chondrocyte hypertrophy in the growth plate (Fig. 7C).

How Pthrp signaling regulates *CamkII* function has only been partially elucidated. Our finding that calyculin A treatment globally upregulates pCamkII in chondrocytes, even in the presence of Pthrp signaling, supports a model in which phosphatase activity is a primary effector of Pthrp regulation of CamkII (Kozhemyakina et al., 2009). Although seeming paradoxical because expressed daCamkII activity does not depend on phosphorylation, increased phosphatase activity would dephosphorylate endogenous pCamkII on which activation of pathway effectors probably depends. Whether dephosphorylation of pCamkII is the primary mechanism of Pthrp-dependent inhibition of hypertrophy in a physiological context is uncertain because treatment with calyculin A failed to restore hypertrophy in the presence of Pthrp signaling despite upregulating pCamkII, presumably due to pleiotropic effects of blocking the ubiquitous functions of PP1 and PP2A phosphatases.

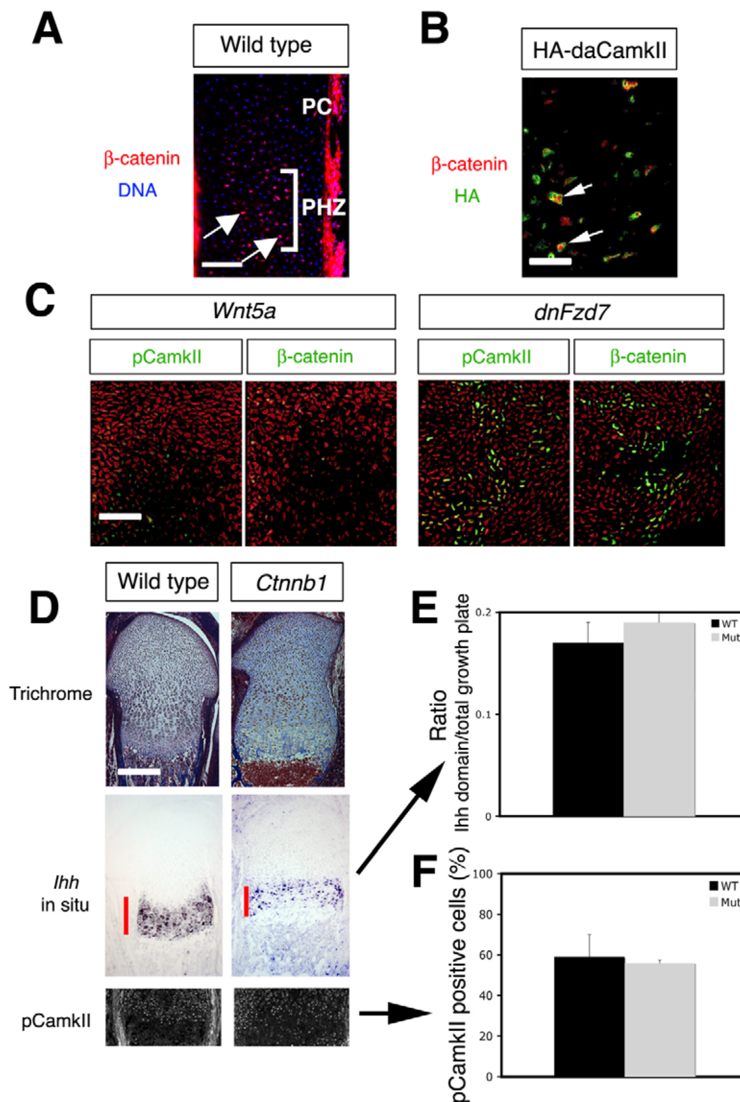

Fig. 5. CamkII acts partly through β-catenin. (**A-C**) Sections of chick growth plate cartilage either uninfected (A) or infected with RCAS(A)-HA-daCamkII (B), RCAS(A)-Wnt5a or RCAS(A)-dnFzd7 (C) were analyzed by immunofluorescence for HA-daCamkII (B), pCamkII (C) and β-catenin (A-C). (A) β-catenin is readily detected in nuclei (arrows) of wild-type prehypertrophic and hypertrophic chondrocytes (*n*=3). Bracket indicates prehypertrophic zone (PHZ). PC, proliferative chondrocytes. (B) Many RCAS(A)-HA-daCamkII-infected proliferative chondrocytes (white arrows; *n*=8) show high levels of nuclear β-catenin (red). (C) Ectopic *Wnt5a* expression reduces both pCamkII (green) and nuclear β-catenin (green) signals (*n*=3), whereas ectopic expression of *dnFzd7* promotes both pCamkII (green) and nuclear β-catenin (*n*=3), consistent with co-regulation of these proteins by Wnt signaling. Red signal is the actin cytoskeleton, which is reduced in hypertrophic chondrocytes. (**D-F**) The effect of loss of β-*catenin* function on pCamkII and chondrocyte maturation was examined in *Ctnnb1* mutant embryos. Trichrome staining revealed similar organization of growth plate chondrocytes in wild-type and *Ctnnb1* mutant samples, although a lower cell density is observed in mutants (data not shown). The size of the prehypertrophic zone (*Ihh*; red line) relative to the length of the growth plate is similar (P>0.05) in wild-type (0.17 ± 0.02 mm; *n*=10) and mutant (0.19 ± 0.009 mm; *n*=10) samples (E). No obvious differences (P>0.05) in nuclear enrichment of pCamkII were detected between wild-type (59 ± 11%; *n*=8) and mutant (56 ± 8.9%; *n*=3) growth plates (F). Error bars indicate s.d. Scale bars: 100 μm in A; 50 μm in B; 200 μm in C; 300 μm in D.

Wnt signaling also inhibits CamkII activation – as shown by expression of *dnFzd7*, *Wnt5a* and *Wnt5b* – but neither Wnt ligand interferes substantially with *daCamkII* function, suggesting that Pthrp and Wnt signaling repress CamkII activity by distinct mechanisms. How Wnt signaling inhibits CamkII activity is not known, but our data suggest that repression occurs via a noncanonical Wnt pathway because Wnt1 signaling failed to downregulate pCamkII. Moreover, pCamkII is highest in cells showing elevated nuclear β-catenin, an effector of canonical Wnt signaling. In this context, β-catenin does not appear to be an effector of canonical Wnt signaling, because nuclear β-catenin is upregulated in proliferative chondrocytes that express *dnFzd* or *daCamkII*. These observations suggest that CamkII acts directly or indirectly to stabilize nuclear β-catenin in chondrocytes and, as a result, brings into question the use of nuclear β-catenin as a definitive marker of canonical Wnt signaling in the absence of functional studies. Together, these data support a model in which CamkII activity is determined by the integration of information from multiple signaling pathways by distinct mechanisms. Collectively, the diverse inputs and multiple independent outputs in combination with the sufficiency and importance of CamkII activity for hypertrophy suggests that CamkII is a key decision-making node in a pathway that regulates entry to the hypertrophic program in chondrocytes.

Activity-dependent output from the CamkII activity node

One curious result of these studies is that a low percentage of chondrocytes expressing *daCamkII* displays a definitive prehypertrophic phenotype. Thus, many *daCamkII*-expressing proliferative chondrocytes show altered cell morphology, and downregulation of cortical actin (this study) and the cell cycle (Taschner et al., 2008), but only a proportion of cells activate *Ihh* expression and stabilize β-catenin. The fact that hypertrophic morphology, loss of cortical actin and downregulation of the cell cycle form a stable phenotype independent of *Ihh* or *Col10a1* expression (this study) (Taschner et al., 2008) suggests that the hypertrophic program is composed of at least two modules that act coordinately but are not absolutely co-regulated. Thus, chondrocyte hypertrophy might be a multi-step process that occurs in a narrow temporal window.

How might activation of CamkII result in distinct phenotypic states? The different penetrance of ectopic hypertrophy in *daCamkII*- and *HA-daCamkII*-expressing proliferative chondrocytes suggests that the effector pathways are sensitive to pCamkII levels. This result was unexpected because the allosteric activation mechanism of oligomeric CamkII would predict a bi-stable switch. However, in neurons, the addition of phosphatase activity has been

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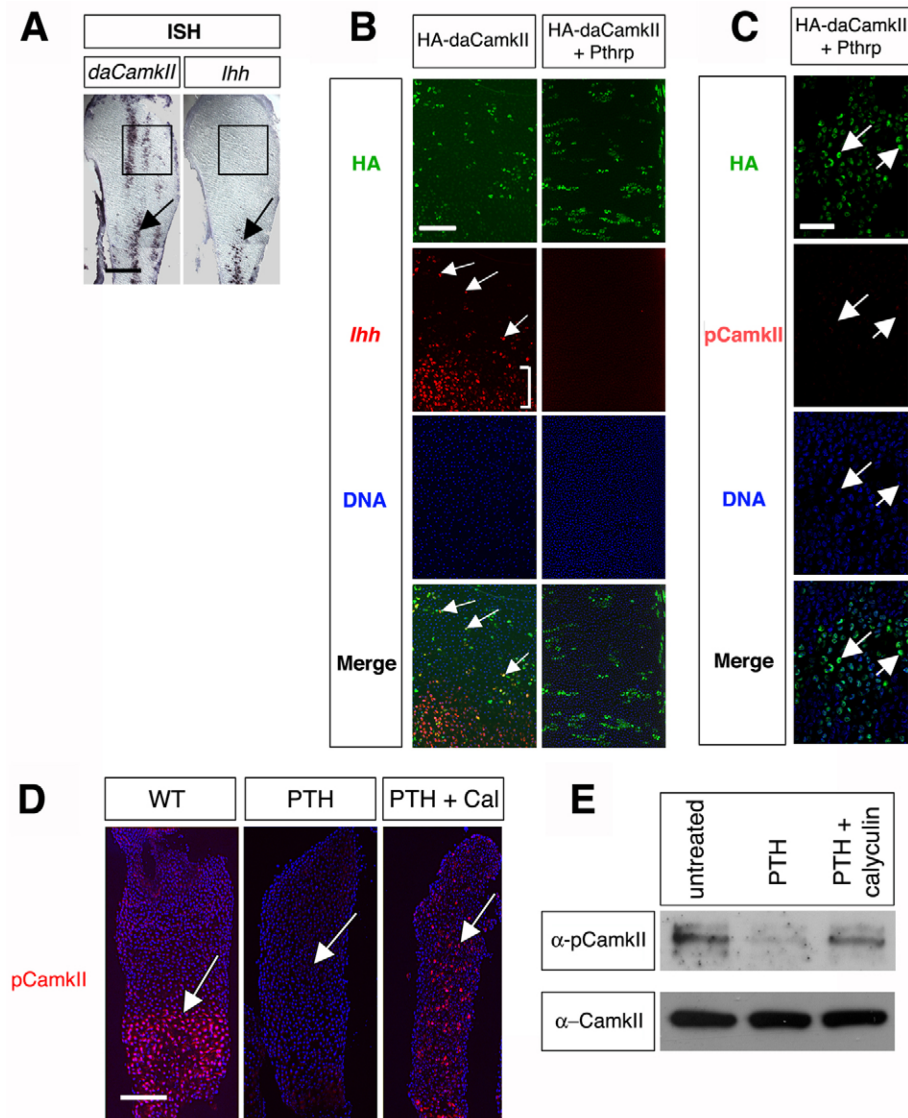

Fig. 6. Pthrp signaling inhibits hypertrophy by regulation of CamkII activity. (**A**) In situ hybridization analysis for *daCamkII* (infected cells) and *Ihh* revealed that RCAS(A)-daCamkII does not induce *Ihh* expression in resting chondrocytes (box) Arrows indicate ectopic expression in the proliferative zone. (**B**,**C**) To test whether Pthrp signaling antagonizes *CamkII* function in the resting zone, chick growth plates infected with either RCAS(A)-*HA-daCamkII* (green, immunofluorescence) alone (*n*=5), or with both RCAS(A)-*Pthrp* and RCAS(A)-*HA-daCamkII* (*n*=5) were analyzed for *Ihh* expression (red) by FISH. HA-daCamkII induced *Ihh* expression (white arrows) in the absence (B, left panels), but not in the presence (B, right panels), of Pthrp (white arrows). Pthrp signaling blocks activation of endogenous CamkII (pCamkII) by HA-daCamkII in the proliferative zone (C, arrows). (**D**) To test if Pthrp signaling acts on pCamkII through phosphatases, mouse metatarsals lacking the perichondrium were cultured in the presence of diluent (WT), 10 nM PTH1-108 (PTH) or 10 nM PTH1-108 + 2 nM calyculin A (PTH+Cal) (*n*=20 metatarsals per treatment). WT samples display pCamkII (red) in the prehypertrophic and hypertrophic chondrocytes (white arrow), but PTH treated metatarsals lack pCamkII (white arrow). The addition of calyculin A results in ectopic pCamkII similar to Fig. 1D. (**E**) Parallel experiments analyzed by western blot show similar changes in pCamkII without affecting total CamkII levels. Scale bars: 1 mm in A; 200 μm in B; 100 μm in C; 250 μm in D.

shown to generate a tri-stable CamkII system (Pi and Lisman, 2008) that could explain the distinct phenotypic states. Whether CamkII activity is the sole gatekeeper for one or both hypertrophic modules remains to be determined because upregulation of pCamkII in *dnFzd7*-expressing and calyculin A-treated chondrocytes fails to promote any aspect of the hypertrophic phenotype. In these contexts, absence of a hypertrophic phenotype might reflect the pleiotropic effects of these treatments. In particular, the high levels of cytosolic pCamkII in affected chondrocytes compared with wild-type and HA-daCamkII-induced prehypertrophic chondrocytes could indicate additional regulation by nucleocytoplasmic shuttling. The strong correspondence between pCamkII and β-catenin levels in the nucleus further suggests that regulated nuclear entry or retention might underlie different signaling states. Moreover, interactions with other proteins, such as cell surface receptors and cytoskeletal components that modulate CamkII activity (Lisman and Zhabotinsky, 2001; Robison et al., 2005), could produce distinct activity dependent effects in different subcellular compartments. Accordingly, activity sensitive outputs could permit a gradient of CamkII activity to temporally regulate sequential steps in the hypertrophic program.

How does the integrated signaling environment shape the pCamkII activity landscape? Central to our model (Fig. 7C) is the premise that the distribution of extracellular Pthrp generates a gradient of intracellular phosphatase activity, resulting in an inverse gradient of intracellular CamkII activity. Overlaid on this gradient are more broadly distributed factors, such as Wnt ligands, that serve to establish a signaling threshold for the high-level activation of CamkII (Kitagawa et al., 2009) that is necessary for full activation of the hypertrophic program. In this model, Wnt signaling could function either by generating a consistent level of inhibition across the growth plate or by establishing region-specific thresholds through localized expression of different ligands to produce step-wise transitions from a gradient of pCamkII activity. Conspicuously absent from this model is an activator of CamkII. In part, this reflects the failure of these studies to identify a signaling molecule that upregulates pCamkII activity. However, it is also not apparent from these data that activation is a specific event because global upregulation of pCamkII following treatment with calyculin A suggests that the pathway upstream of CamkII is activated in most chondrocytes. These data support a model in which

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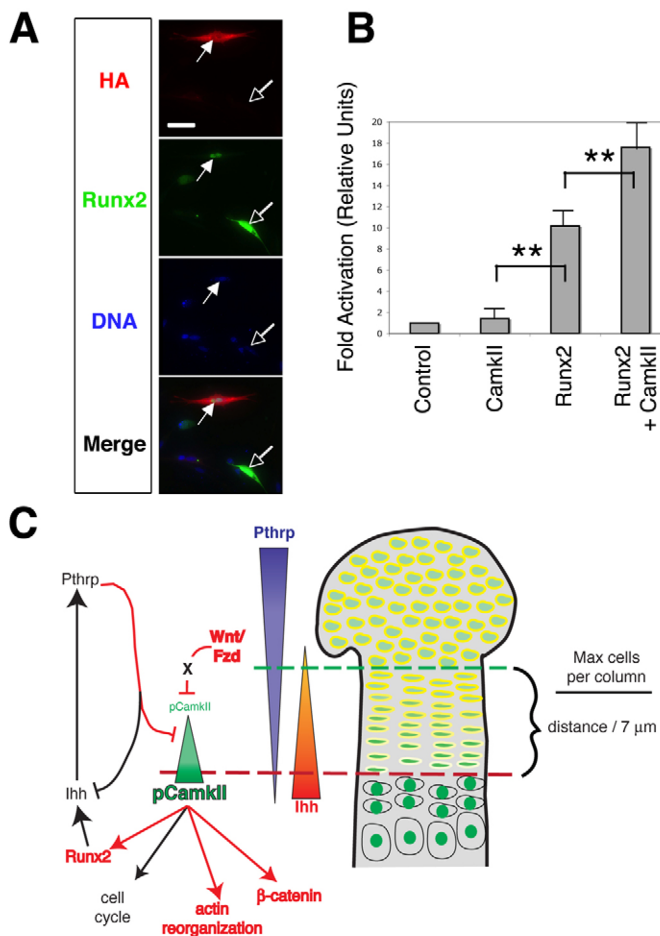

Fig. 7. Proliferative potential of growth plate chondrocytes is regulated by CamkII activity. pCamkII and Pthrp might exert opposing effects on hypertrophy through the common target Runx2. (**A**) To test this possibility, DF-1 fibroblast cultures were transiently transfected with RCAS(A)-HA-daCamkII (red) and RCAS(A)-Runx2 (green). In two experiments, an average of 58% of co-transfected cells (40/76 and 31/46 in experiments 1 and 2, respectively) showed strong, predominantly nuclear localization of Runx2 (white arrows). By contrast, in cells that displayed only Runx2 expression, only 8% (5/59 and 2/25 in experiments 1 and 2, respectively) showed predominantly nuclear Runx2 (open arrows show a cell with both nuclear and cytosolic signal). (**B**) Luciferase reporter experiments (three experiments in triplicate per condition) show that co-transfection with pCAG-HA-daCamkII upregulates pcDNA3.1-Runx2-induced transcription from the *Ihh* promoter. **Differences between compared data sets (brackets) are statistically significant, $P<0.001$. Data are expressed as mean ± s.d. (**C**) A model for regulation of proliferation and hypertrophy by CamkII. Left: a summary of the genetic pathway that regulates chondrocyte maturation and CamkII activity in the growth plate. The green triangle represents a hypothetical gradient of CamkII activation across the PZ. Red indicates the novel interactions described in this paper. The 'X' represents hypothetical intermediates regulated by the Fzd pathway that act upstream to repress CamkII activation. Right: we propose that a gradient of Pthrp signaling (purple triangle) generates a gradient of phosphatase activity (yellow outline on chondrocytes) that results in an inverse gradient of CamkII activity (green in chondrocytes). CamkII activity reaches a threshold at the PZ/PHZ boundary necessary for high-level nuclear enrichment (green circles) of pCamkII. Thus, antagonistic interactions between opposing gradients of Pthrp and Ihh (orange triangle) signaling define the distal end of the PZ (green dashed line) and the inhibitory action of Pthrp on CamkII activity defines the proximal boundary of the PZ (red dashed line). This distance, which is dependent on the relative strengths of Pthrp and Ihh signals, determines the maximum number of proliferative chondrocytes that can be generated from a single mother row cell because each daughter cell occupies ~7 μm along the longitudinal axis (Hunziker, 1994). Scale bar: 25 μm in A. PZ, proliferative zone; PHZ, prehypertrophic zone.

inhibitory, not activating, mechanisms are the primary determinants of pCamkII activity that in turn promotes exit from the proliferative phase and entry into hypertrophy.

Regulating the proliferative potential of growth plate chondrocytes

Interestingly, the process that generates hypertrophic chondrocytes in the growth plate exhibits parallels with the development of adult stem cells. For example, in *Drosophila* germ stem cells, daughter cells enter a special proliferative phase known as 'transit amplification' (de Haan et al., 1996; Izergina et al., 2009; Schulz et al., 2004) that, like proliferative chondrocytes, generates a large number of progenitor cells for terminal differentiation. One important issue is whether exit from the amplification phase involves progressive dilution of a proliferation factor or accumulation of a differentiation factor (Insco et al., 2009). In growth plate chondrocytes, accumulation of CamkII activity triggers a dose-dependent onset of terminal differentiation (by upregulation of Runx2 and β-catenin) that is the direct result of progressive reduction of inhibitory factors, including phosphatase activity, which could result from the dilution of regulatory factors. Thus, dilution and accumulation mechanisms might work in concert to facilitate the transition from proliferation to terminal differentiation in growth plate chondrocytes. How similar chondrocyte behavior conforms to stem cell models remains to be determined. However, one interesting possibility is that growth plate cartilage might prove to be a useful model for mechanistic studies of stem cell proliferation because the complete range of maturation states is present simultaneously, and yet, as a result of the precise architecture, these states are arrayed in spatially separate domains showing unique morphologies.

Determining the precise onset of terminal differentiation limits the proliferative potential of stem/progenitor cell progeny. Our data suggest that the range of action of secreted signaling molecules robustly defines the proliferative space, which, when cell size (proliferative chondrocytes are 7-8 μm tall) (Breur et al., 1994; Hunziker, 1994) and cell packing (columns) are tightly regulated, limits the number of chondrocytes that can be generated from an individual progenitor cell in a unit of time. Restricting the number of cells generated, in turn, determines growth potential by limiting the number of cells available for hypertrophy. Furthermore, displacement of the entire developing column from the source of Pthrp, as a result of continual recruitment of new mother row cells that initiate formation of new columns (Dodds, 1930), establishes a time window for column formation that sets a limit on total cell amplification from a single progenitor cell. It is intriguing to think, therefore, that precise regulation of CamkII activity by modulation of signaling pathway output could provide a mechanism for age-dependent and growth plate-dependent differences (Breur et al., 1991; Hunziker et al., 1987; Wilsman et al., 1996) in growth rates of developing long bones.

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Competing interests statement
The authors declare no competing financial interests.

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