

WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways

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Activation and disruption of Wnt/ β -catenin signaling both result in cartilage breakdown via unknown mechanisms. Here we show that both WNT-3A and the Wnt inhibitor DKK1 induced de-differentiation of human articular chondrocytes through simultaneous activation of β -catenin-dependent and independent responses. WNT-3A activates both the β -catenin-dependent canonical pathway and the Ca^{2+} /CaMKII noncanonical pathways, with distinct transcriptional targets. WNT-3A promotes cell proliferation and loss of expression of the chondrocyte markers *COL2A1*, *Aggrecan*, and *SOX9*; however, proliferation and *AXIN2*

up-regulation are downstream of the canonical pathway and are rescued by DKK1, whereas the loss of differentiation markers is CaMKII dependent. Finally, we showed that in chondrocytes, the Ca^{2+} /CaMKII-dependent and β -catenin-dependent pathways are reciprocally inhibitory, thereby explaining why DKK1 can induce loss of differentiation through de-repression of the CaMKII pathway. We propose a novel model in which a single WNT can simultaneously activate different pathways with distinct and independent outcomes and with reciprocal regulation. This offers an opportunity for selective pharmacological targeting.

Introduction

The articular cartilage covers the articulating surface of bone elements, ensuring frictionless joint motion. Osteoarthritis (OA) is a leading cause of disability worldwide, affecting up to one third of the population over the age of 50 (Lawrence et al., 1998; Lawrence et al., 2008) and costing \$185.5 million only in the US (Kotlarz et al., 2009). Cartilage breakdown is the hallmark of OA. Unfortunately, no treatment for OA is currently available, except for pain control and physiotherapy, until prosthetic joint replacement is required. Understanding the pathogenetic mechanisms leading to the disruption of homeostasis and cartilage breakdown is of paramount importance to the development of effective pharmacological treatments.

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Abbreviations used in this paper: AHAC, adult human articular chondrocyte; FZD, frizzled; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; LEF, lymphoid enhancer-binding factor; LRP, low-density lipoprotein receptor-related receptor; OA, osteoarthritis; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; Q-PCR, quantitative PCR; SO, safranin O; TCF, T cell factor.

Degeneration of the articular cartilage is the final outcome of an imbalance between catabolic processes triggered by injurious factors (biomechanical or inflammatory) and compensatory anabolic processes. Genetic studies in humans (Loughlin et al., 2004) and experimentation in animal models (Lories et al., 2007; Wu et al., 2010) have convincingly demonstrated the involvement of Wnt signaling in OA pathogenesis.

Wnts are a family of 19 morphogens involved in several processes from embryonic morphogenesis to homeostasis of adult tissues (Logan and Nusse, 2004). Wnts signal through multiple pathways (Macdonald et al., 2007; Semenov et al., 2007), the best characterized of which is the Wnt/ β -catenin signaling, also known as the “canonical” pathway. In this pathway, in the absence of ligands, the constitutively active kinase GSK-3 β

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phosphorylates β -catenin within a destruction complex, addressing it for degradation through the proteasome pathway (Logan and Nusse, 2004). The engagement of some members of the Wnt family, such as WNT-1 and WNT-3A (Hinck et al., 1994; Shimizu et al., 1997) with Frizzled (FZD) receptors and the coreceptors low-density lipoprotein receptor-related receptor 5/6 (LRP5/6), results in the disruption of the destruction complex, accumulation of β -catenin in the cytosol, and its migration in the nucleus, where β -catenin interacts with T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors and supports the transcription of target genes (Molenaar et al., 1996). Other Wnts, such as WNT-5A and WNT-11 (Kühl et al., 2000), can instead activate other signaling cascades, generally referred as “noncanonical” pathways, such as the planar cell polarity pathway (PCP) and the Ca^{2+} /CaMKII pathways (Sheldahl et al., 1999; Kühl et al., 2000; Semenov et al., 2007).

Activation of the canonical Wnt pathway is required and sufficient for embryonic joint specification (Hartmann and Tabin, 2001; Guo et al., 2004), and both canonical and noncanonical Wnt signaling regulate morphogenesis, proliferation, cell specification, and differentiation within the developing skeleton (Church et al., 2002; Enomoto-Iwamoto et al., 2002; Yang et al., 2003; Koyama et al., 2008).

In adult life, modulation of Wnt signaling has an important role in cartilage homeostasis. We demonstrated enhanced activation of the canonical Wnt pathway in osteoarthritic cartilage in humans (Dell’Accio et al., 2001) and after cartilage injury in mice (Dell’Accio et al., 2008; Eltawil et al., 2009). In addition, a loss of function polymorphism in the *SFRP3* gene, encoding for a soluble Wnt inhibitor (Leyns et al., 1997; Wang et al., 1997), has been linked to an increased susceptibility to develop OA (Loughlin et al., 2004; Lories et al., 2007). These data, together with other in vivo and in vitro experimental evidence, suggest that an over-activation of the β -catenin-dependent/Wnt pathway might be involved in OA pathogenesis (Schett et al., 2008; Luyten et al., 2009).

However, the results of functional studies using mouse genetics contrast with each other and are difficult to interpret. For instance, both repression and forced activation of the canonical Wnt signaling resulted in chondrodysplasia in embryonic life (Akiyama et al., 2004) and in OA development postnatally (Lories et al., 2007; Chen et al., 2008; Koyama et al., 2008; Zhu et al., 2008, 2009).

We demonstrated that the prototypical canonical activator WNT-3A and the inhibitor of the canonical pathway DKK1 both induce down-regulation of cartilage phenotypic markers and reduction of glycosaminoglycan (GAG)-rich extracellular matrix in vitro. Although the fact that an activator and an inhibitor of the canonical Wnt pathway both lead to the same outcome is very surprising, nevertheless it is in keeping with the in vivo data mentioned previously and provided a convenient system to dissect the underpinning molecular mechanisms. We therefore used this system to propose a new model according to which WNT-3A can simultaneously activate the β -catenin-dependent and CaMKII-dependent signaling, each with specific outcomes; reciprocal inhibition of these two pathways explains how differentiation is induced by WNT-3A

through activation of CaMKII, and by DKK1 through inhibition of the canonical pathway and consequent de-repression of the CaMKII pathway.

Results

Biological events associated with WNT-3A treatment of primary articular chondrocytes

WNT-3A is a prototypical, well-characterized activator of the β -catenin-dependent canonical pathway (Shimizu et al., 1997), which has an established role in arthritis (Nakamura et al., 2005), and it is expressed in articular cartilage (Fig. S1). Taking advantage of the availability of WNT-3A as recombinant protein, we investigated the effects of this ligand on the proliferation, differentiation, and extracellular matrix production of articular chondrocytes. Treatment of primary porcine articular chondrocytes with 100 ng/ml of recombinant WNT-3A over a 3-d time course resulted in a statistically significant increase of cell proliferation compared with vehicle control (Fig. 1 A). The proliferative effect of WNT-3A was confirmed in adult human articular chondrocytes (AHACs), where the proliferation marker *PCNA* was up-regulated 24 h after stimulation with WNT-3A (Fig. 1 B).

We then compared the expression of chondrocyte-lineage markers by confluent primary AHACs treated with either WNT-3A or vehicle control. *COL2A1*, *Aggrecan*, and *SOX9* mRNA were statistically significantly down-regulated after WNT-3A treatment (Fig. 1 C). The chondrocyte hypertrophy marker *MMP13* was also down-regulated. Other genes relevant to cartilage homeostasis/biology including *COL1A1*, *MMP-3*, and *ADAMTS5* (Goldring, 2006) were unchanged. The main function of articular chondrocytes is the production of a specialized extracellular matrix rich in highly sulphated GAGs, which provide the elastic biomechanical properties required for motion and weight bearing. Therefore, to test whether WNT-3A affected this important function of chondrocytes, we exploited the capacity of chondrocytes to produce large amounts of such matrix when cultured in 3D micromasses. Treatment of AHAC micromasses with 100 ng/ml of WNT-3A for 4 d induced a statistically significant decrease in the accumulation of highly sulphated GAGs, as evaluated by Alcian blue staining and spectrophotometric quantitation (Fig. 1 D; De Bari et al., 2001). Collectively, these findings indicate that WNT-3A stimulation activates proliferation and de-differentiation of articular chondrocytes.

We confirmed the validity of these data in vivo using a well-established model that measures the capacity of chondrocytes to form hyaline-like cartilage when implanted ectopically in nude mice (Dell’Accio et al., 2001). To provide prolonged WNT-3A stimulation of the chondrocytes in vivo, we co-injected freshly isolated porcine articular chondrocytes with 10% of growth-arrested L cells stably overexpressing WNT-3A (L-WNT-3A), or L cells as control. Growth arrest of L cells was successfully achieved by mitomycin C treatment (Fig. 2, A and B), without compromising their capacity to secrete biologically active WNT-3A for at least 14 d (Fig. 2 C). Indeed, conditioned medium from L-WNT-3A cells that had been

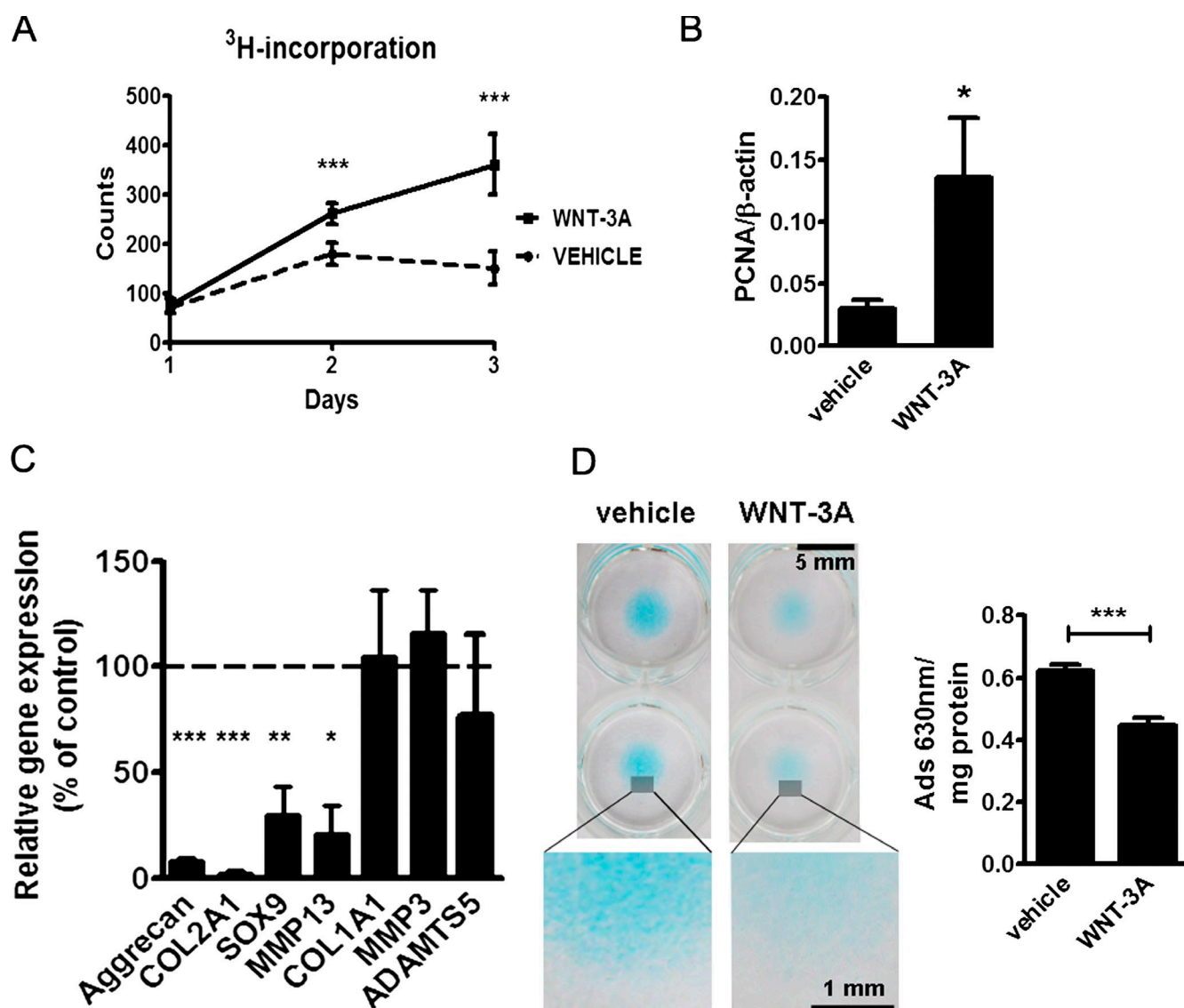


Figure 1. WNT-3A induces chondrocyte proliferation and de-differentiation in vitro. (A and B) 100 ng/ml WNT-3A stimulated proliferation of primary chondrocytes in vitro as evaluated by an [³H]thymidine incorporation assay in porcine articular chondrocytes (A; *n* = 6) and by up-regulation of the proliferation marker PCNA in primary AHACs by quantitative PCR (Q-PCR) (B; *n* = 4). (C and D) WNT-3A-induced down-regulation of the differentiation markers COL2A1, Aggrecan, SOX9, and MMP13, as evaluated by Q-PCR (C; *n* = 6), and reduced the accumulation of highly sulphated GAG in primary AHACs (D; by Alcian blue staining and spectrophotometric quantification; *n* = 4). Gene expression values are reported as a percentage of up- or down-regulation compared with control (100%, broken line in the graph). Statistical analysis was performed with an unpaired *t* test. Error bars indicate mean ± SEM. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005.

growth-arrested for 2 wk was able to activate the TCF/LEF1 reporter assay SUPER8XTOPFlash (Veeman et al., 2003) in porcine articular chondrocytes. 2 wk after the injection, the wet weight of the cartilage implants retrieved from mice injected with chondrocytes plus L-WNT-3A cells was statistically significantly higher compared with that of implants obtained from chondrocytes co-implanted with control L cells (Fig. 2, H and I) and statistically significantly less differentiated, as demonstrated by a weaker staining with safranin O (SO; Fig. 2, D–G, J, and K), which confirms that WNT-3A also mediated proliferation and loss of chondrocyte phenotype in an in vivo system. Similar results were obtained with primary AHACs (Fig. S2), thereby confirming that this biological outcome is conserved across mammals.

WNT-3A mediates proliferation of AHACs via the β-catenin-dependent pathway and their de-differentiation via a β-catenin-independent mechanism

WNT-3A is known to activate the canonical Wnt pathway in a variety of cells. We therefore set out to confirm the activation of this pathway by WNT-3A in AHACs. Treatment with 100 ng/ml recombinant WNT-3A resulted in accumulation of β-catenin (Fig. 3 A), activation of the SUPER8XTOPFlash reporter assay (Fig. 3 B), and a 250-fold up-regulation of the endogenous target gene *AXIN2* (Fig. 3 C), which is indicative of activation of the β-catenin-dependent canonical pathway (Yan et al., 2001).

To confirm that the observed AHAC phenotypic changes were caused by the activation of the canonical pathway, primary

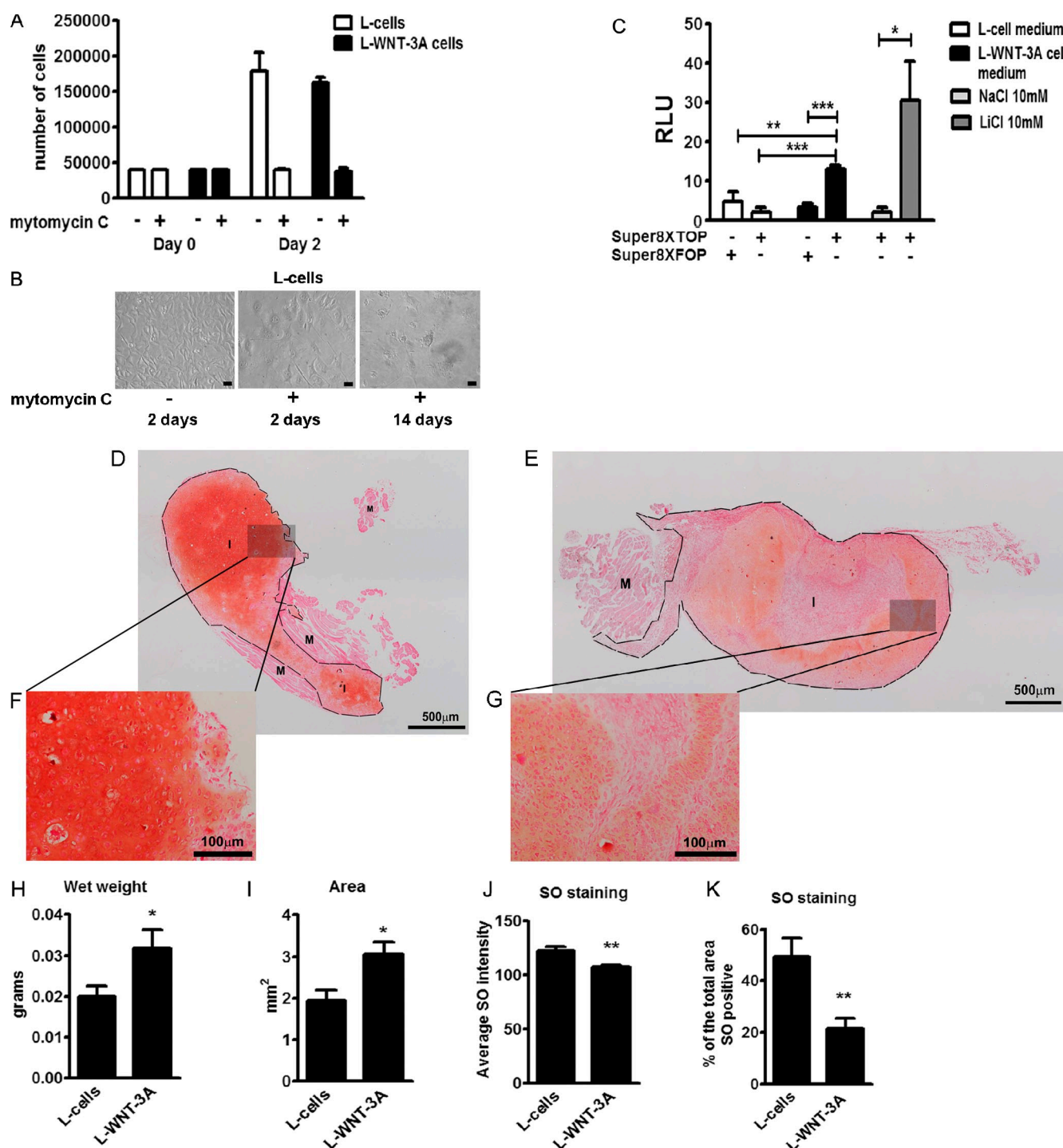


Figure 2. WNT-3A induces proliferation and de-differentiation of articular chondrocytes in vivo. (A and B) WNT-3A induced chondrocyte de-differentiation in vivo in an ectopic cartilage formation assay. Isolated porcine articular chondrocytes were co-injected intramuscularly in nude mice together with either L cells stably expressing WNT-3A (L-WNT3A) or control L cells (L cells; 12 injections per condition). Before injection, L cells and L-WNT-3A cells were growth-arrested by mitomycin C treatment. To confirm growth arrest, 2 d after treatment, a sample of cells with or without mitomycin C treatment was detached and counted (A; $n = 4$). Mitomycin C-treated cells stopped proliferating. One additional well was kept in culture for 14 d. The cells persisted but never reached confluency (B). Bar, 10 μ m. (C) To confirm that mitomycin C growth-arrested L-WNT-3A cells still produced biologically active WNT-3A, the conditioned medium (from the last change of medium) obtained 14 d after mitomycin C treatment was tested for the ability to activate the SUPER8XTOPFlash reporter assay in porcine articular chondrocytes. Indeed, conditioned medium from L-WNT-3A cells, but not from control cells, activated the SUPER8XTOPFlash reporter. The mutagenized SUPER8XFOPFlash vector was used to control for specificity ($n = 4$). (D–K) SO staining of cartilage implants obtained by the co-injection of porcine articular cartilage with L cells (D) or L-WNT-3A cells (E) in nude mice. Cartilage implants obtained from porcine articular chondrocytes co-injected with L-WNT-3A cells are on average larger (H and I) and less differentiated (J and K), as shown by a decreased SO staining in comparison with controls. Grayscale digital images from SO staining were used to calculate and compare the mean intensity of the staining and, after thresholding, the percentage of the total implant area that was positive for SO ($n = 12$ per condition). M, muscle; I, implant; RLU, relative luminescence unit. An unpaired t test was used for statistical evaluation. Error bars indicate mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

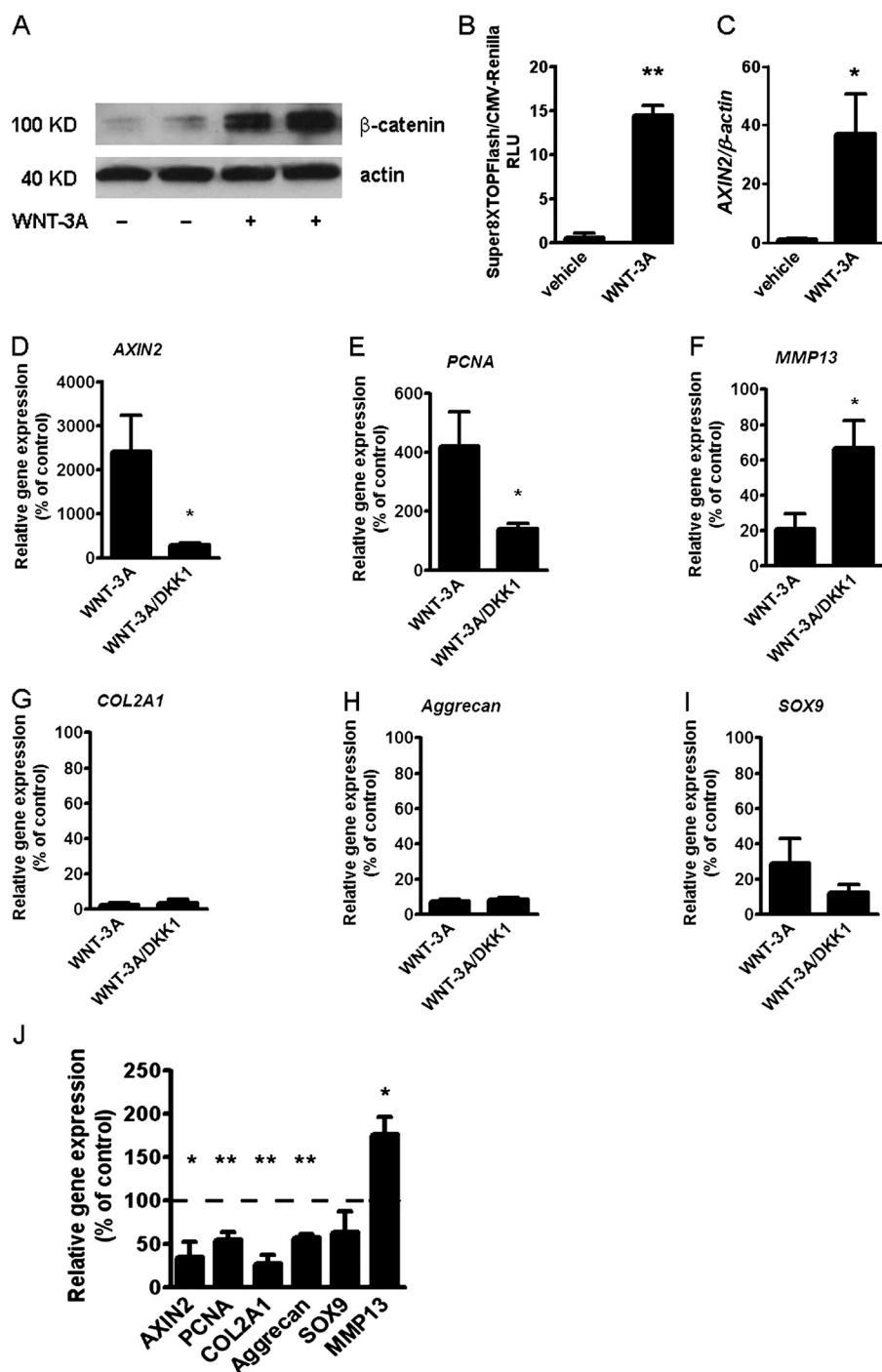


Figure 3. Inhibition of Wnt canonical pathway with DKK1 does not rescue the loss of the chondrocyte phenotype. (A–C) WNT-3A activates the Wnt canonical pathway in AHACs. (A) Western blotting for β -catenin in the cytoplasmic fraction of AHACs treated with 100 ng/ml WNT-3A or vehicle control for 24 h. (B) WNT-3A activated the SUPER8TOPFlash reporter assay in PO AHACs after 24 h of stimulation ($n = 3$). (C) WNT-3A up-regulated the expression of the endogenous Wnt canonical target gene *AXIN2* in AHACs as evaluated by Q-PCR. Values were normalized for the housekeeping gene β -actin. (D–I) Primary AHACs were treated for 24 h with 100 ng/ml of recombinant WNT-3A or DKK1, alone or in combination, and were subjected to gene expression analysis by PCR. Blockade of the Wnt canonical pathway by DKK1 rescued the modulation of *AXIN2*, *PCNA*, and *MMP13* mRNA (D–F) but not the down-regulation of *COL2A1*, *Aggrecan*, and *SOX9* (G–I). (J) In the absence of exogenous WNT-3A, treatment with 100 ng/ml DKK1 resulted, as expected, in down-regulation of the mRNA levels of *AXIN2* and *PCNA*, but also in a paradoxical down-regulation of *COL2A1* and *Aggrecan* similar to that induced by WNT-3A. Q-PCR data, $n = 6$. Gene expression values are reported as a percentage of up- or down-regulation compared with control (100% is indicated by the broken line in the graph). Data were analyzed with an unpaired t test. Error bars indicate mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.005$.

AHACs were treated with either 100 ng/ml WNT-3A alone or in combination with 100 ng/ml of recombinant DKK1, which specifically inhibits the β -catenin-dependent pathway by binding to LRP coreceptors and preventing their association with FZDs (Mao et al., 2001). Co-treatment with DKK1 reverted the WNT-3A-induced up-regulation of *AXIN2*, *PCNA*, and *MMP13* (Fig. 3, D–F), but, surprisingly failed to rescue the down-regulation of *COL2A1*, *Aggrecan*, and *SOX9* mRNA (Fig. 3, G–I). To investigate whether this was caused by excessive, exogenous WNT-3A stimulation, we investigated whether treatment with DKK1 alone, in the absence of exogenous WNT-3A, modified the phenotype of AHACs. Strikingly, treatment of AHACs with

DKK1, in the absence of WNT-3A, promoted by itself a down-regulation of *COL2A1* and *Aggrecan*, similar to that induced by WNT-3A, and up-regulation of *MMP13*, although, as expected, it did reduce *AXIN2* and *PCNA* mRNA expression (Fig. 3 J).

The finding that DKK1 not only did not rescue WNT-3A-induced loss of phenotypic markers but, on its own, promoted it, suggests that although proliferation and *AXIN2* up-regulation are driven by activation of the canonical Wnt pathway, the loss of phenotypic markers and of extracellular matrix is regulated through a different pathway that is antagonized by the canonical one. To explain the apparent paradox that WNT-3A and DKK1 have the same effect, we hypothesized that, under our experimental

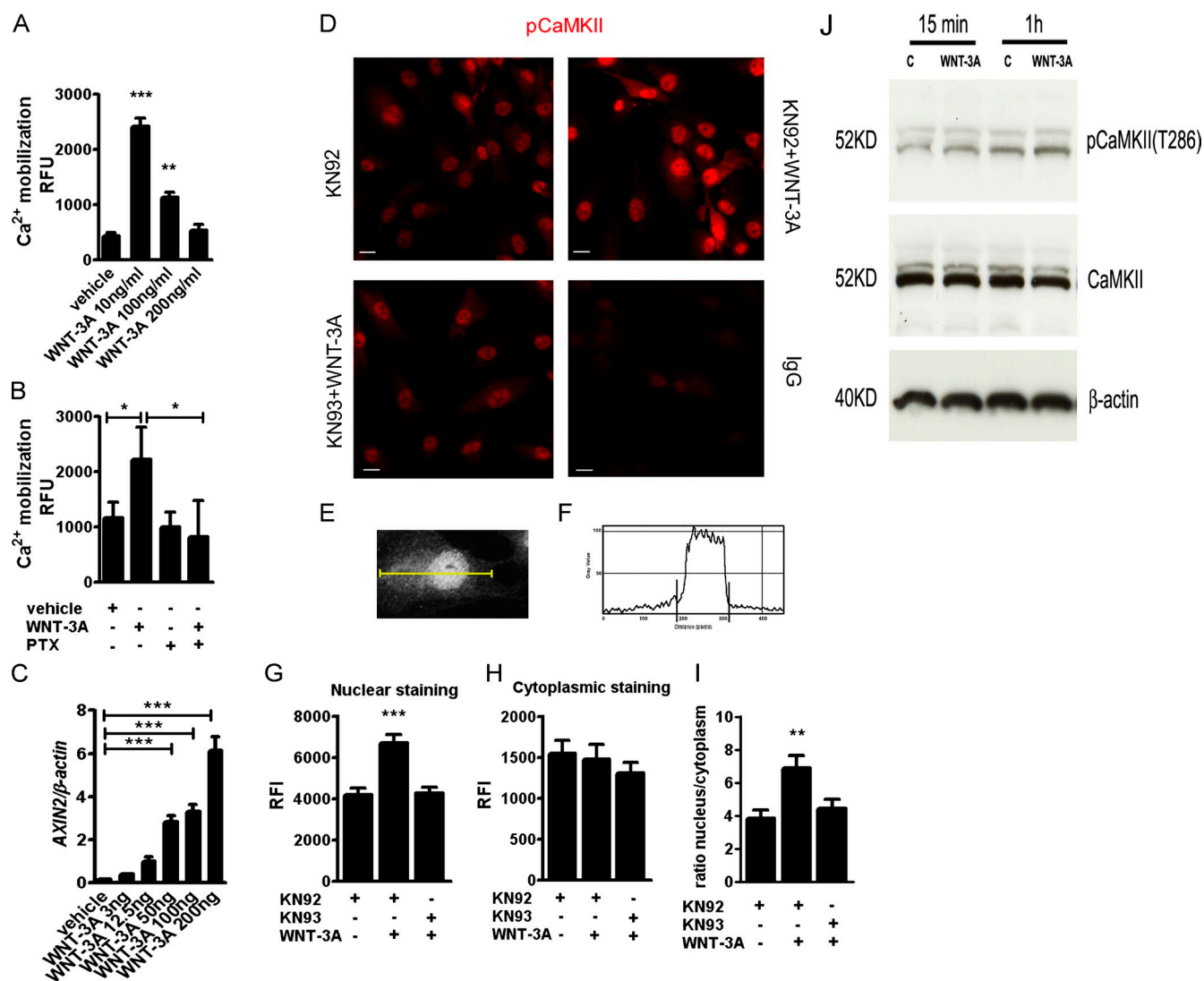


Figure 4. WNT-3A promotes intracellular Ca²⁺-mediated phosphorylation of CaMKII and its nuclear translocation. (A) WNT-3A caused calcium mobilization in AHACs, particularly at low doses. Primary AHACs were treated with different amounts of recombinant WNT-3A or vehicle control for 5 min and then subjected to fluorometric determination of calcium mobilization ($n = 9$). (B) WNT3A-induced calcium mobilization assay in primary AHACs is G protein dependent. AHACs were preincubated overnight at 37°C with 1 μg/ml of the G protein inhibitor PTX and then treated for 5 min with 100 ng/ml of WNT-3A or vehicle control. PTX treatment blocked calcium mobilization induced by WNT-3A ($n = 6$). (C) Q-PCR for *AXIN2* mRNA in primary chondrocytes exposed for 24 h to different doses of recombinant WNT-3A. WNT-3A induced dose-dependent up-regulation of *AXIN2* in primary AHACs ($n = 3$). (D) WNT-3A induced nuclear accumulation of the phosphorylated form of CaMKII. Immunofluorescence staining for pCaMKII in PO AHACs stimulated for 24 h with WNT-3A in combination with either the CaMKII inhibitor KN93 or its inactive analogue KN92 (both 10 μM). Bar, 20 μm. (E–I). Quantification of the pCaMKII fluorescence intensity in the nuclear and in the cytoplasmic fractions of stimulated AHACs. The pixel intensity profile was plotted over a linear section of a whole cell (E and F). The nuclear and cytoplasmic fluorescence intensity was then calculated as the area under the curve (G–I). (J) WNT3A promotes phosphorylation of CaMKII in T286 at 15 min and 1 h from WNT-3A stimulation. RFU, relative fluorescence unit; RFI, relative fluorescence intensity. Data were analyzed with an unpaired t test except in C, where a Kruskal Wallis with a Dunn's post-test was used. Error bars indicate mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

conditions, the modulation of *AXIN2*, *PCNA*, and *MMP13* and chondrocyte proliferation are dependent on the Wnt-β-catenin canonical pathway, whereas the WNT-3A-mediated down-regulation of *COL2A1* and *Aggrecan* are β-catenin independent.

WNT-3A simultaneously activates the canonical and the Ca²⁺/CaMKII-dependent Wnt pathways in chondrocytes in a dose-dependent manner

We therefore hypothesized that WNT-3A induces loss of chondrocyte differentiation through activation of a noncanonical

Wnt pathway. We focused on the Wnt-Ca²⁺/CaMKII pathway because it has been shown to be active in chicken epiphyseal cartilage (Taschner et al., 2008) and to mediate Wnt signaling in zebrafish and *Xenopus* embryos (Slusarski et al., 1997a,b; Sheldahl et al., 1999; Kühl et al., 2000) antagonizing β-catenin. Therefore, to test whether WNT-3A activates the noncanonical Ca²⁺/CaMKII-dependent pathway, we treated primary AHAC cultures with increasing doses of WNT-3A while monitoring calcium mobilization (as a readout of the activation of the Ca²⁺-dependent pathway) and *AXIN2* expression (as a readout for the activation of the canonical pathway).

In keeping with our hypothesis, WNT-3A elicited activation of both pathways; notably, Ca^{2+} mobilization occurred preferably at low doses and *AXIN2* up-regulation at higher doses (Fig. 4, A and C).

In zebrafish and *Xenopus* embryos, it was shown that the Ca^{2+} /CaMKII pathway signals in a G protein-dependent manner (Slusarski et al., 1997a; Sheldahl et al., 1999; Kühl et al., 2000). In our experimental system, pertussis toxin (PTX), a $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha t}$ protein inhibitor (Slusarski et al., 1997a; Sheldahl et al., 1999), efficiently blocked WNT-3A-induced calcium mobilization (Fig. 4 B). Next, to evaluate the involvement of CaMKII, we treated AHACs with 100 ng/ml WNT-3A or vehicle control for 24 h and stained the monolayers with an antibody to detect the activated, phosphorylated form of CaMKII (Soderling, 1999). PhosphoCaMKII staining was increased in WNT-3A-treated samples with increased nuclear localization, confirming the activation of this pathway (Fig. 4, D–I). Such phosphorylation was abrogated in the presence of the selective CaMKII inhibitor KN93 (Fig. 4 D). CaMKII phosphorylation was also detected by Western blotting analysis, after 15 min and 1 h from the addition of WNT-3A to the cells (Fig. 4 J). Altogether, our data indicate that exogenous application of WNT-3A is sufficient to dose dependently activate both the canonical β -catenin-dependent and noncanonical Ca^{2+} /CaMKII-dependent pathways (see Fig. 7, A and B).

Wnt canonical and Wnt-CaMKII noncanonical pathways are reciprocally inhibitory in AHACs

In *Xenopus*, the Wnt Ca^{2+} /CaMKII pathway has been shown to inhibit the Wnt- β -catenin-dependent pathway (Sheldahl et al., 1999; Kühl et al., 2000). To investigate the interaction between the CaMKII pathway with the Wnt canonical pathway after WNT-3A stimulation, we treated confluent passage 0 (P0) AHACs with 100 ng/ml WNT-3A and either the selective CaMKII inhibitor KN93 or its inactive analogue KN92 (both 10 μM) for 24 h. CaMKII inhibition by KN93 significantly increased the WNT-3A-induced up-regulation of *AXIN2* mRNA (Fig. 5 A). In contrast, KN92, which is structurally similar to KN93 but does not inhibit CaMKII (Marley and Thomson, 1996), did not alter the expression of *AXIN2* (Fig. 5 A). To study if the Wnt/ β -catenin pathway modulates the Wnt- Ca^{2+} /CaMKII pathway, we treated AHACs with WNT-3A and DKK1, alone or in combination. Both WNT-3A and DKK1 stimulation resulted in statistically significant activation of Ca^{2+} mobilization, which was further increased when DKK1 and WNT-3A were used together, thereby demonstrating that the selective inhibition of the canonical pathway enhances the activation of the Ca^{2+} mobilization (Fig. 5 B).

Collectively, our data demonstrate reciprocal inhibition of the Wnt- β -catenin and Wnt- Ca^{2+} /CaMKII pathways after WNT-3A stimulation in articular chondrocytes, and that the inhibition of one causes de-repression of the other (see Fig. 7, C–E).

To gain a better insight into this mechanism and specifically examine whether the selectivity of the blockade for the canonical pathway was essential for Ca^{2+} mobilization or if general out-titration of Wnts also induced Ca^{2+} mobilization,

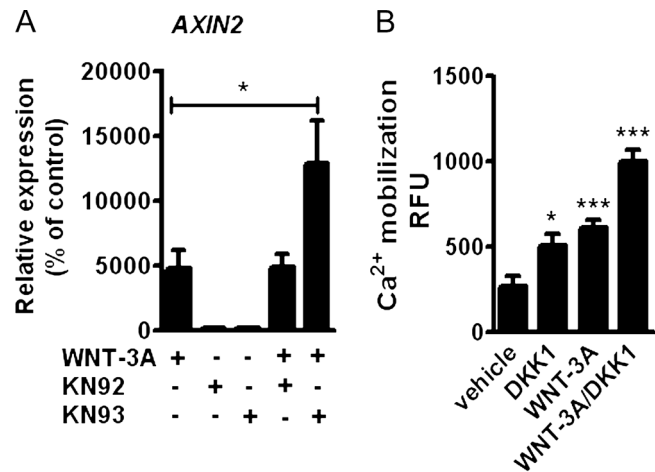


Figure 5. Wnt canonical and Wnt-CaMKII noncanonical pathways are reciprocally inhibitory in AHACs. (A) Q-PCR for *AXIN2* of primary AHACs treated with 100 ng/ml WNT-3A in the presence of the CaMKII inhibitor KN93 or its inactive analogue KN92 (10 μM). (B) DKK1 promoted intracellular calcium accumulation in P0 AHACs. The inhibition of the canonical pathway with DKK1 enhanced intracellular calcium accumulation induced by WNT-3A. In all the experiments, $n = 4$. Data in A were analyzed with ANOVA; data in B were analyzed with an unpaired t test. Error bars indicate mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.0005$.

we repeated the same experiment using sFRP1 as a Wnt inhibitor. We chose sFRP1 because it is known to bind and inhibit several Wnts, including WNT-3A (Galli et al., 2006; Wawrzak et al., 2007), preventing their interaction with FZD receptors. sFRP1 treatment prevented WNT-3A-induced intracellular calcium accumulation, but, in contrast to DKK1, did not induce Ca^{2+} mobilization on its own, thereby confirming that Ca^{2+} mobilization induced by DKK1 is caused by selective inhibition of the canonical pathway and consequent de-repression of the Ca^{2+} /CaMKII pathway (Fig. S3).

Distinct effects in chondrocytes of the WNT-3A-mediated activation of β -catenin and CaMKII pathways

Having demonstrated that WNT-3A can signal simultaneously through two intracellular pathways, we set out to discriminate CaMKII-dependent from β -catenin-dependent transcriptional targets. To this end, we treated chondrocytes with WNT-3A in the presence of either the CaMKII inhibitor KN93 or the inactive compound KN92. As expected, WNT-3A induced a significant down-regulation of *COL2A1*, *Aggrecan*, and *SOX9* mRNA compared with control, but CaMKII blockade with KN93 resulted in a statistically significant rescue of *COL2A1* and *SOX9* expression (Fig. 6, B and D), which demonstrated that WNT-3A-induced chondrocyte de-differentiation is at least in part CaMKII dependent. In contrast, KN93 did not rescue the down-regulation of *MMP13* mRNA (Fig. 6 E). Therefore WNT-3A up-regulates *AXIN2* and *PCNA* through the canonical pathway and down-regulates *SOX9* and *COL2A1* in a CaMKII-dependent manner (Fig. 7, A and B).

In contrast, nonspecific forced Ca^{2+} mobilization using phorbol 12-myristate 13-acetate (PMA) resulted in down-regulation of *AGGRECAN* but not *SOX9* or *COL2A1* (Fig. S4).

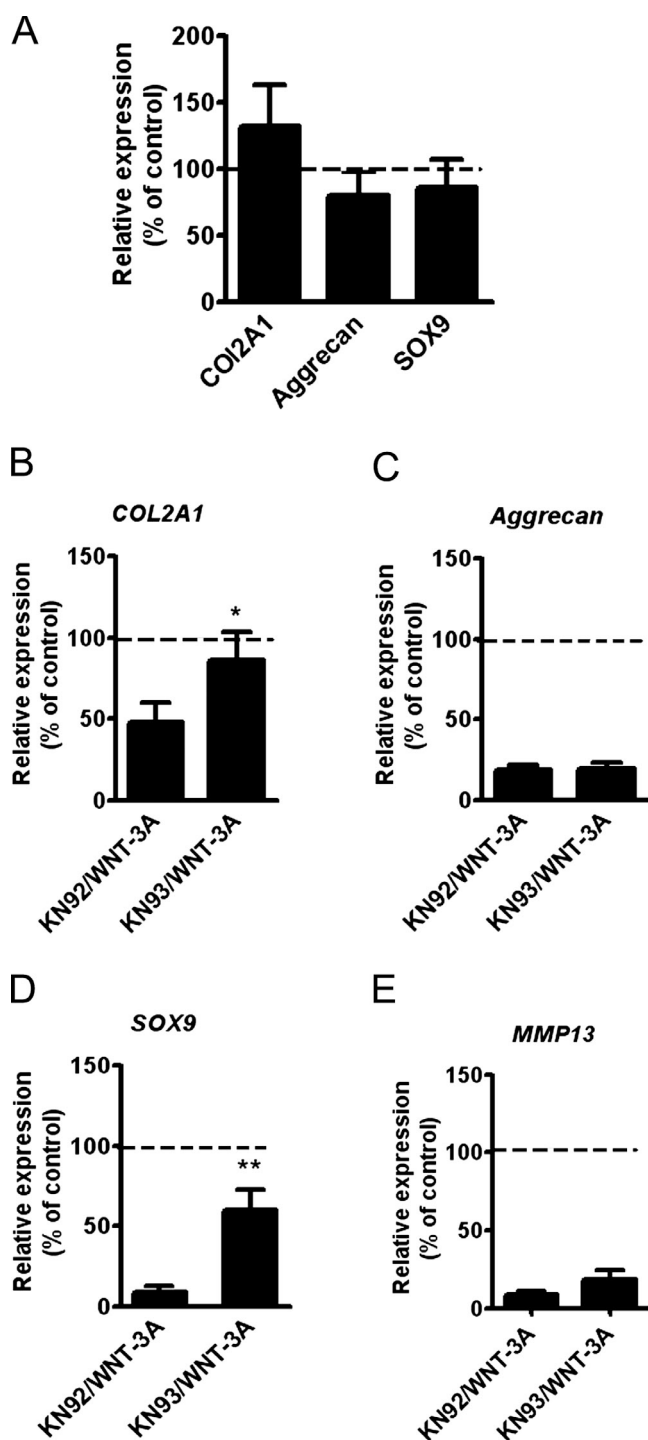


Figure 6. Inhibition of the Ca^{2+} /CaMKII pathway rescued the loss of *COL2A1* and *SOX9* mRNA expression in AHACs. (A) Blockade of CaMKII with KN93 did not alter the basal levels of *Aggrecan*, *COL2A1*, and *SOX9*. Primary AHACs were treated for 24 h with 10 μM KN93 or its inactive analogue KN92. (B–E) CaMKII blockade rescues WNT-3A–induced down-regulation of *COL2A1* and *SOX9* mRNA. Primary AHACs were cultured for 24 h in the presence of WNT-3A or vehicle control, and either the CaMKII inhibitor KN93 or the inactive control KN92. Gene expression was evaluated by Q-PCR. The values were normalized for β -actin and expressed as a percentage of the KN92-treated group (100% is indicated by the broken line in the graphs; $n = 6$). Statistical analysis was performed with an unpaired t test. Error bars indicate mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.005$.

This, and the fact that instead CaMKII blockade rescued the expression of *COL2A1* as well as *SOX9*, suggests that the WNT-3A–induced effects on these three genes rely specifically on the CaMKII-dependent Ca^{2+} -pathway.

Discussion

We have demonstrated that chondrocyte exposure to exogenous WNT-3A can simultaneously activate both the β -catenin–dependent and the G protein–mediated Ca^{2+} /CaMKII pathways, which are in equilibrium because of reciprocal inhibition. These two pathways modulate the transcription of distinct target genes involved in different biological processes: WNT-3A promotes chondrocyte proliferation through β -catenin and differentiation through CaMKII. According to our model, blockade of the canonical/ β -catenin pathway will also cause articular-chondrocyte de-differentiation through de-repression of the CaMKII pathway (Fig. 7, A and B).

This model may help explaining contradictions in the current literature. In particular, both activation and disruption of the canonical Wnt signaling result in chondrodysplasia with delayed maturation of chondrocytes (Enomoto-Iwamoto et al., 2002; Akiyama et al., 2004) and in cartilage breakdown in adulthood (Chen et al., 2008; Koyama et al., 2008; Zhu et al., 2008, 2009). Similarly, in the mouse embryo (Yamaguchi et al., 1999; Yang et al., 2003), both the overexpression and the knockout of WNT-5A, which in zebrafish and *Xenopus* has been shown to activate the Ca^{2+} /CaMKII pathway (Slusarski et al., 1997b; Kühl et al., 2000), result in a similar phenotype of chondrodysplasia, in both cases with reduced chondrocyte proliferation and hypertrophic maturation (Yamaguchi et al., 1999; Yang et al., 2003). Although this phenotype, in vivo, may be partially determined by compensatory mechanisms involving modulation of the *Ihh*–PTHrP pathway, *COL2A1* expression was up-regulated in the growth plate of WNT-5A knockout mice and down-regulated in mice overexpressing WNT-5A under the transcriptional control of the collagen type 2 promoter (Yang et al., 2003). In addition, in vitro, WNT-5A dose-dependently down-regulated the activity of a *COL2A1* reporter in mouse chondrocytes. Importantly, this study showed that WNT-1, a well validated canonical Wnt, could also decrease *COL2A1* reporter activity, though in a TCF-independent manner, because overexpression of TCF1 failed to achieve the same result (Yang et al., 2003). This may suggest that WNT-3A may not be the only canonical Wnt to signal through multiple pathways.

In keeping with our data, Taschner et al. (2008) found that forced activation of CaMKII in the chick epiphyseal cartilage resulted in down-regulation of cell-cycle regulators and acceleration of hypertrophic differentiation, which is notoriously associated with down-regulation of *COL2A1* and *SOX9* (Lefebvre et al., 1998; Taschner et al., 2008). However, care must be applied in comparing our results with Taschner et al. (2008) because of the intrinsically different biology of epiphyseal chondrocytes, which are destined to undergo hypertrophic differentiation and eventually be replaced by bone, and the articular chondrocytes, which are resistant to hypertrophy and to endochondral bone formation. In addition, in Taschner et al. (2008), proliferation is

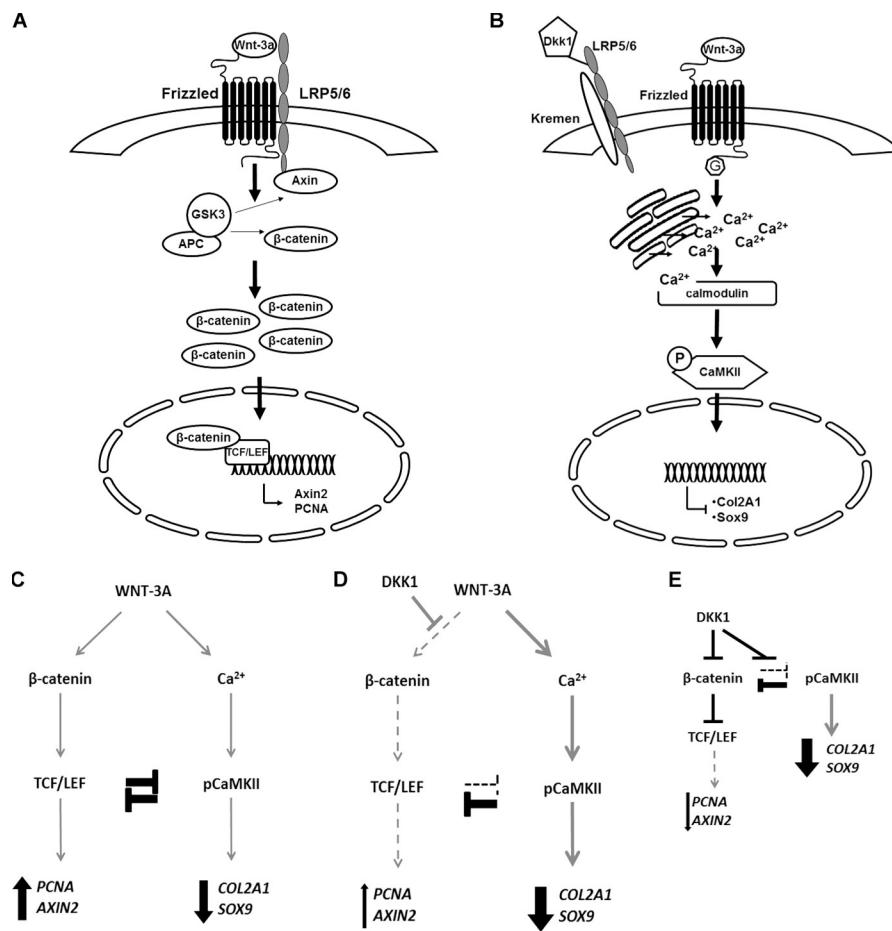


Figure 7. Simultaneous activation and reciprocal inhibition of β -catenin- and CaMKII-dependent pathways in AHACs. (A and B) In AHACs, WNT-3A up-regulates AXIN2 expression and proliferation through the Wnt canonical pathway (A), but induces loss of differentiation via a G protein-mediated Ca²⁺/CaMKII-dependent pathway (B). (C–E) These two pathways are reciprocally inhibitory and in equilibrium. Therefore, either exogenous WNT-3A (C) or blockade of the canonical pathway with DKK1 (D–E) will both result in loss of chondrocyte phenotype.

driven by noncell autonomous mechanisms including secretion of Ihh and consequent PTHrP signaling. With all these limitations in comparing the experimental setups, this paper and a recent similar paper from Li et al. (2011), in which overexpression of constitutively active CaMKII was complemented by loss of function experiments, show the role of this kinase in the initiation of hypertrophy, a process that is associated with the loss of stable chondrocyte markers including *SOX9* and *COL2A1* (Lefebvre et al., 1998).

The reciprocal inhibition of the canonical and CaMKII-dependent pathways activated by WNT-3A may represent a hub through which different stimuli active in cartilage and known to influence CaMKII can dramatically influence the outcome of Wnt signaling by switching between β -catenin and CaMKII-dependent target genes. Such stimuli include inflammatory molecules (Pritchard and Guilak, 2006; Racioppi and Means, 2008), biomechanics (Valhmu and Raia, 2002; Shimazaki et al., 2006), and PTHrP activity (Taschner et al., 2008).

Although we and others (Slusarski et al., 1997a; Sheldahl et al., 1999; Kühl et al., 2000) have shown that Ca²⁺/CaMKII activation is G protein-coupled receptor (GPCR) dependent, it is still unclear how Wnts activate G proteins. Based on the similarity between FZD receptors and other GPCR (Wang et al., 2006), it is tempting to postulate that, in the absence of DKK-engaged LRP, FZD receptors may function as GPCRs (Wang et al., 2006; Koval and Katanaev, 2011). However, we cannot

exclude that in our system, G protein activation may be indirect, e.g., through the activation of a different GPCR.

A separate issue is as to whether, in our system, individual FZD receptors can activate both pathways, or if the simultaneous activation of canonical and CaMKII pathways is caused by the coexpression, on chondrocytes, of FZD receptors with different signaling specificity. Slusarski et al. (1997a) demonstrated that in zebrafish, expression of rat frizzled 2 receptor (Rfz-2), but not Rfz-1, increased intracellular calcium accumulation in a way that was enhanced by the coexpression of *Xenopus* Wnt-5a (Xwnt-5a). In addition, in a mammalian cell line, Xwnt-5a but not Xwnt-8 stimulated calcium accumulation via Rfz-2 in a PTX-sensitive way (Liu et al., 1999). Subsequently, Kühl et al. (2000) demonstrated that in *Xenopus*, WNT-5A-induced Ca²⁺ mobilization resulted in CaMKII activation in a FZD-specific manner. Only some FZD receptors were able to signal through CaMKII, and such signal was also strictly ligand-specific.

We have demonstrated that AHACs express different FZD receptors (Fig. S5) known to mediate both the canonical (Gazit et al., 1999; Terasaki et al., 2002; Karasawa et al., 2002; Kemp et al., 2007) and the Ca²⁺-dependent pathways (Kühl et al., 2000; Robitaille et al., 2002; Nishita et al., 2010). Therefore, it is possible that the receptor repertoire of chondrocytes may determine the balance of the WNT-3A signaling.

Alternatively we cannot exclude the possibility that mechanisms downstream of FZD receptors may activate CaMKII:

GSK3 inhibitor LiCl also down-regulated *COL2A1* expression (Ryu et al., 2002; Ryu and Chun, 2006) in different systems, with the caveat that lithium is also an inhibitor of the inositol pathway (Berridge and Irvine, 1989). Hence, these data are difficult to interpret.

Whichever the mechanism, it is unlikely that, in our system, the activation of the Ca^{2+} /CaMKII pathway is indirect, as Ca^{2+} mobilization occurred as early as 5 min after the addition of WNT-3A.

Equally, although it cannot be completely excluded, it is unlikely that exogenous WNT-3A may have caused promiscuous activation of receptors, first because Ca^{2+} release took place particularly at low doses of WNT-3A, whereas β -catenin activation was observed only at the higher doses; and second, because DKK1 also caused Ca^{2+} release and chondrocyte de-differentiation in the absence of exogenous WNT-3A (Figs. 3 J, 5 B, and 7, D and E), which cannot be explained by promiscuous receptor activation by supraphysiological levels of exogenous WNT-3A. Intriguingly, in addition, direct activation of the PKC pathway by WNT-3A was found in the ST2 cell line mediating osteogenesis (Tu et al., 2007).

In our system, activation of CaMKII was associated with its nuclear localization, which could be blocked with the CaMKII inhibitor KN93. A similar activation-dependent nuclear localization of phosphoCaMKII has been described in smooth muscle cells (Cipolletta et al., 2010) and very recently in epiphyseal chondrocytes (Li et al., 2011) in vivo. Nuclear localization of CaMKII is regulated by several factors including splicing of an NLS, phosphorylation of serines downstream of the NLS, and binding to anchoring and interacting molecules (Griffith et al., 2003). The specific function of the nuclear localization in this context is not known; however, subcellular localization of CaMKII has been shown to restrict/regulate substrate specificity in different cell types or in response to other signals (Griffith et al., 2003; Tsui et al., 2005). Therefore, it is tempting to speculate that CaMKII may have a nuclear function, either in mediating chondrocyte de-differentiation or in suppression of the canonical pathway. In this regard, it has been shown that CaMKII activation in colon cancer cells mediates nuclear export of TCF isoforms (Najdi et al., 2009).

One novel and important aspect of our study is that the β -catenin-dependent pathway and the Ca^{2+} /CaMKII noncanonical pathway have different transcriptional targets: *AXIN2* and cell proliferation are specifically regulated by the canonical pathway, whereas the loss of chondrocyte phenotype (*COL2A1* and *SOX9*) are mediated by the Ca^{2+} /CaMKII arm of WNT-3A stimulation. This has important implications from the pharmacological point of view. In fact, for its role on cartilage and joint homeostasis, the Wnt signaling is an attractive therapeutic target. Unfortunately, the multitude of processes regulated by Wnts and the complexity of their regulation has long been a hurdle. Understanding how downstream biological events are individually regulated by Wnts in different cells represents an opportunity to achieve a higher degree of target specificity. Indeed, the availability of molecules and drugs that can selectively interfere with either CaMKII or the canonical Wnt pathway offers the possibility of targeting individual outcomes of

Wnt stimulation and avoiding concerns related, for instance, to the role of β -catenin in cancer development (Morin et al., 1997; Sunaga et al., 2001).

Materials and methods

Cartilage harvest and chondrocyte isolation

Adult human articular cartilage was obtained from patients who underwent joint replacement for knee OA after obtaining informed consent. The cartilage samples were provided by P. Achan (Barts and the London National Health Service Trust, London, England, UK). All procedures were approved by the East London and The City Research Ethics Committee 3.

Cartilage tissue was dissected from preserved areas of the femoral condyles and the patellar groove. The cartilage was sliced full thickness, excluding the mineralized cartilage and the subchondral bone. From each sample, one full thickness specimen was processed for histological grading, and the rest was used for chondrocyte isolation. The aliquot destined to chondrocyte isolation was washed twice in high-glucose DME (DME/F-12 1:1 plus GlutaMax; Invitrogen) containing 10% FBS, 1 mM sodium pyruvate (NaPyr), and 2% antibiotic antimycotic solution (Invitrogen). Cartilage was then digested for 30 min at 37°C in 1 mg/ml pronase (Roche) and then overnight at 37°C in 1 mg/ml collagenase P (Roche) prepared in complete medium (same composition as above, with 1% antibiotic/antimycotic solution) under agitation. The AHACs recovered from the digestion were then resuspended in complete medium and seeded at a density of 10,000 cells/cm². The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. The aliquot destined for histological scoring was fixed in 4% paraformaldehyde in PBS at pH 7.4, paraffin embedded, sectioned, stained, and scored for features of OA as described previously (Mankin et al., 1971; Dell'Accio et al., 2008). Only cells obtained from samples with a Mankin score <4 were used for subsequent experiments. All experiments were performed using freshly isolated or confluent P0 cells.

Porcine chondrocytes were isolated from the metatarsal joints of adult pigs obtained within 24 h of death from a local abattoir. The articular cartilage was dissected in sterile conditions and processed for cell isolation as described for human chondrocytes.

Titred thymidine incorporation

Freshly isolated porcine articular chondrocytes were seeded at a density of 6,000 cells/cm² and left to attach overnight in standard culture conditions. The day after seeding and at all the time points analyzed, the cells were treated for 6 h with 100 ng/ml of recombinant WNT-3A (R&D Systems) or vehicle (0.1% BSA in PBS) in complete medium at 37°C. 2 $\mu\text{Ci}/\mu\text{l}/\text{cm}^2$ of [³H]thymidine was then added, and after 12 h of incubation, the cells were lysed in trichloroacetic acid. The radioactivity (recorded as counts per minute) was measured using a 1900 Liquid Scintillation analyzer (Packard).

Gene expression analysis

Total RNA was extracted from AHACs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 500 ng of total RNA from each sample was reverse transcribed using ThermoScript Reverse transcription kit (Invitrogen) with oligo dT primer. Quantitative and semiquantitative PCR was performed with hot-start DNA polymerase (QIAGEN) in the presence of 0.1 $\times$ SYBR green (Sigma-Aldrich) and 0.2 $\times$ ROX dye (Invitrogen) using, respectively, a T7900 HD or a 96 well GeneAmp PCR system 9700 machine (Applied Biosystems). Primer sequences are listed in Table I.

SDS-PAGE and Western blotting

After treatment, cells were washed once in ice-cold PBS containing phosphatase and protease inhibitors (Roche). Total cell extracts were obtained by scraping the cells in extraction buffer (10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.05% NP-40, pH 7.9) and leaving the lysates on ice for 30 min. Protein concentration of the samples was determined by a bicinchoninic acid assay (Thermo Fisher Scientific).

The extracts were resolved by SDS-PAGE, and transferred to a nitrocellulose membrane (GE Healthcare). Human β -catenin was detected with anti- β -catenin rabbit monoclonal (diluted 1:1,000; Cell Signaling Technology), whereas β -actin was detected with anti- β -actin mouse polyclonal antibody (diluted 1:10,000; Sigma-Aldrich), followed by incubation with HRP-conjugated secondary antibodies (diluted 1:2,000; Santa Cruz Biotechnology, Inc.). Antibodies were diluted in 5% nonfat milk/0.01% Tween 20 in PBS. In the case of CaMKII and phospho-CaMKII analysis, the membranes were blocked in 5% BSA/0.01%

Table 1. Primer sequences used for quantitative and semiquantitative gene expression analysis

Gene	Sense	Antisense	Length
<i>β-actin</i>	5'-CACGGCTGCTTCCAGCTC-3'	5'-CACAGGACTCCATGCCAG-3'	134 bp
<i>ADAMTS-5</i>	5'-GACCGATGGCACTGAATGTA-3'	5'-TGACAGCTGGAGTTGTCTCT-3'	145 bp
<i>Aggrecan</i>	5'-GTTGTCATCAGCACCAGCATC-3'	5'-ACCACACAGTCCTCTCCAGC-3'	509 bp
<i>Axin-2</i>	5'-TACCGGAGGATGCTGAAGGC-3'	5'-CCACTGGCCGATTCTCTCT-3'	345 bp
<i>Col1A1</i>	5'-GCCCTGTCTGCTTCTGTAA-3'	5'-GGTTCAGTTGGGTGCTTG-3'	104 bp
<i>Col2A1</i>	5'-CTGCTCGTCGCCGCTGCTCT-3'	5'-AAGGGTCCCAGGTCTCCATC-3'	432 bp
<i>MMP-3</i>	5'-CAACCGTGAGGAAAATCGATGAG-3'	5'-CGGCAAGATACAGATTACGCTCAA-3'	440 bp
<i>MMP-13</i>	5'-ACGGACCCATACAGTTGAATACAGC-3'	5'-CCATTGTGGTGTGGGAAGTATCATC-3'	360 bp
<i>PCNA</i>	5'-GGAGAAGCTGGAAATGGAAAC-3'	5'-CTGCATTAGAGTCAAGACCC-3'	548 bp
<i>Sox9</i>	5'-GAACGCACATCAAGACGGAG-3'	5'-TCTCGTTGATTCTGCTGCTC-3'	631 bp
<i>WNT-3A</i>	5'-CCATCCTCTGCCTCAAAATC-3'	5'-TGGACAGTGGATATAGCAGCA-3'	70 bp
<i>FZD1</i>	5'-TTCAGCAGCACATTCTGAGG-3'	5'-CCTGCACACATTTCCCTTT-3'	154 bp
<i>FZD2</i>	5'-TCACGGTCTACATGATCAA-3'	5'-GCAACCTAAAAGTGAAATGG-3'	266 bp
<i>FZD3</i>	5'-GGATGATCAAGAAGCAAG-3'	5'-TTGAGCCGATGAGAAGTACT-3'	186 bp
<i>FZD4</i>	5'-GACTTTGGAAGGAACCTTTT-3'	5'-TGAAACCCCGTCTCTACTAA-3'	238 bp
<i>FZD5</i>	5'-GGTTTGGTGCAGGTGAATTT-3'	5'-CTACAGCATGGGATAGGCACT-3'	125 bp
<i>FZD6</i>	5'-TTCGGCAGCTCACTAGGATT-3'	5'-CATCAGAAAATCTTGCCCAA-3'	190 bp
<i>FZD7</i>	5'-CTGGAGTTCTTTGAAATGTGCT-3'	5'-AAGGTTAGTCTCCATGATTCTC-3'	133 bp
<i>FZD8</i>	5'-CGGTTTGGGTATTCTTAATG-3'	5'-ACAGGGGTAAGCCTCTAAAC-3'	215 bp
<i>FZD9</i>	5'-AACACAGAGAAGCTGGAGAA-3'	5'-ACCACAGTGACATGAAAAT-3'	251 bp
<i>FZD10</i>	5'-AGAAACCCTTCAGTGCTACA-3'	5'-AAAGTGCTCTGCCAACCTA-3'	205 bp

Tween 20 in PBS, and the antibodies (1:2,000 anti-CaMKII [Abcam]; 1:1000 anti-pT286CaMKII [Cell Signaling Technology]) were diluted in the same buffer.

Transfections and reporter assay

Subconfluent AHACs and porcine articular chondrocytes were cotransfected with SUPER8XTOP/FOPFlash TCF/LEF–firefly luciferase reporter vector (gift of R. Moon, University of Washington, Seattle, WA) and CMV-Renilla luciferase vector (in a ratio 1:10) by using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. 24 h after transfection, the medium was replaced and the cells were stimulated for 24 h with recombinant WNT-3A 100 ng/ml (R&D Systems) or WNT-3A conditioned medium at 37°C, 5% CO₂. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and a TD-20/20 Luminometer (Turner Designs). Firefly luciferase activity was normalized by Renilla luciferase activity and expressed as relative luciferase units.

Ectopic cartilage formation assay in vivo

The ectopic cartilage formation assay was performed as described previously (Dell'Accio et al., 2001). In brief, with a few modifications, for each injection 5 million freshly isolated porcine chondrocytes were mixed with either 500,000 growth-arrested (treated with 7.5 µg/ml of mitomycin C [Sigma-Aldrich] diluted in complete medium for 2 h at 37°C) L cells overexpressing WNT-3A (L-WNT-3A cells, American Type Culture Collection) or with the same amount of mitomycin C–treated control cells (L cells, American Type Culture Collection).

Each sample was resuspended in 50 µl of sterile PBS and injected intramuscularly in the posterior compartment of the thigh of 4-wk-old female CD1^{nu/nu} mice (Dell'Accio et al., 2001). We performed 12 injections per condition (24 in total). The same experiment was replicated with human articular chondrocytes (*n* = 3 implants for each condition). Animals were maintained in isolator cages under an unrestricted diet. The animals were then killed after 2 wk and the cartilage implants were retrieved. All procedures were approved by the Local Ethics committee and the UK Home Office.

The implants were paraffin embedded for histological analysis.

Histology

Images of L cells treated with mitomycin C were captured with an inverted microscope (Eclipse TS100; Nikon) using a Plan-Fluor 10× 0.30 NA objective lens at room temperature. Images were acquired with a camera (Coolpix 4500; Nikon) and analyzed with Photoshop 7.0 (Adobe). 5-µm-thick sections were obtained from the center of the cartilage implants isolated from the nude mice, thereby representing the maximum sectional area, and

stained with SO according to standard protocols. All the sections were mounted in DPX (BDH chemicals). Images of the sections were taken with a microscope (BX61; Olympus) by using UPlan-Apochromat 4× NA 0.16 and 20× NA 0.70 objective lenses. Images were acquired at room temperature, with an F-View II (SIS; Olympus) camera and Cell P software. Three different parameters for each section were assessed by using ImageJ software: the area covered by the cartilage tissue, the mean of the intensity of the staining, and the percentage of the total area of the section with an intensity of staining equal to a fixed, thresholded value (130). The values were then averaged. In the case of implants obtained from porcine articular chondrocytes, one section per implant was analyzed. For the implants obtained by human chondrocytes, five sections for each implant were analyzed, covering a total thickness of 350 µm.

The contrast of the images was enhanced using Photoshop 7.0 for better rendering without altering the relationship of the target to the control images.

Calcium mobilization assay

Calcium mobilization was evaluated using Fluo-4-Direct kit (Invitrogen) in accordance to manufacturer's specifications. In brief, P0 chondrocytes were labeled for 1 h (30 min at room temperature and 30 min at 37°C) with the Fluo-4-Direct calcium assay buffer containing Probenecid (Invitrogen), and were subjected to treatments with growth factors or inhibitors as indicated for individual experiments. After treatments, the fluorescence emitted by the cells (excitation wavelength 494 nm, emission wavelength 516 nm) was recorded using a fluorometer (Galaxy; Fluo-Star). The fluorescence was expressed as relative fluorescence units.

Micromass culture and Alcian blue staining

Freshly isolated human articular chondrocytes were resuspended at a density of 2.0×10^7 cells/ml in complete medium, and micromass cultures were obtained by pipetting 20-µl drops of cell suspension into each well of a 24-well plate. The cells were left to attach for 3 h and then 1 ml of complete medium was added. 24 h later, micromasses were exposed to 100 ng/ml of WNT-3A or vehicle for 4 d, changing medium every 48 h. Finally, micromasses were fixed and whole-mount stained with Alcian blue as described previously (De Bari et al., 2001). After proteoglycan extraction with 6 M guanidine HCl, the absorbance was read at 630 nm with a GENios spectrophotometer (Tecan). Images of the micromasses were acquired at room temperature with a camera (Coolpix 4500; Nikon), with the macro setting and daylight.

The contrast of the pictures was then modified with Photoshop 7.0 for best graphic rendering, equally for the vehicle-treated and for the WNT-3A treated micromasses.

Phospho CaMKII immunofluorescence

AHACs used for immunofluorescence were seeded on chamber slides (Lab-Tek) and treated for 24 h with 100 ng/ml of recombinant WNT-3A in combination with 10 μ M KN92 or KN93 (EMD). The cells were then washed in PBS and fixed in 4% buffered PFA, pH 7.4, for 5 min at room temperature. Autofluorescence was quenched in 50 mM NH₄Cl for 10 min. After 1 h of blocking in FBS diluted 1:5 in PBS/0.2% Triton X-100 (blocking buffer), sections were incubated overnight at 4°C with anti-phospho-CaMKII rabbit polyclonal antibody (Cell Signaling Technology) or rabbit IgG (Dako) as a negative control, diluted 1:200 in blocking buffer. After washing, cells were incubated with cy3-conjugated goat anti-rabbit antibody 1:300 (Jackson ImmunoResearch Laboratories, Inc.) for 1 h. Slides were mounted in Mowiol (EMD), and images were acquired with a fluorescence microscope (BX61; Olympus) using a Uplan-Fluor 40x NA 0.85 objective lens. Images were acquired at room temperature, by using an F-View II (SIS) camera and Cell P software. After acquisition and densitometric analysis (see the next paragraph), the contrast of the images was enhanced for best graphic rendering using Photoshop 7.0, all with the same parameters, without altering the relationship of the target to control the images. The fluorescence intensity was measured by using ImageJ software on the original photographs. The average pixel intensity profile (y axis: 0 = black, 255 = white) was plotted over a linear section of a whole cell. The mean nuclear and cytoplasmic fluorescence intensity, obtained by the plot profile as shown in Fig. 4 E, were generated by analyzing 40 cells per field in duplicate for each condition.

Statistical analysis

Parametric data were compared with a *t* test. For multiple comparisons, we used analysis of variance (ANOVA) analysis, with a Dunnett post-test. Nonparametric values were analyzed by a Kruskal-Wallis test, with a Dunn's post-test. *P*-values <0.05 were considered significant. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005. All the data in the graphs are expressed as mean $\pm$ SEM.

Online supplemental material

Fig. S1 shows the expression of WNT-3A in human articular cartilage. Fig. S2 shows that cartilage implants obtained by the co-injection of human articular chondrocytes with L cells overexpressing WNT-3A are bigger and less differentiated than controls. Fig. S3 shows that sFRP1 does not promote intracellular calcium mobilization and partially blocks the ability of WNT-3A to increase intracellular calcium levels. Fig. S4 shows that PMA promotes intracellular calcium accumulation in articular chondrocytes and induces down-regulation of *Aggrecan*. Fig. S5 shows the gene expression levels of FZD receptors in human articular chondrocytes cultured in monolayer (PO) and in human cartilage explants. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201011051/DC1>.

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