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Wnt signaling in human cartilage degeneration and chondrocytes de-differentiation

Zhe Xie

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Par Zhe XIE

**Wnt signaling in human cartilage degeneration and
chondrocytes de-differentiation**

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LIST OF ABBREVIATIONS

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ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AP-1	Activator protein 1
APC	Adenomatosis polyposis coli
ATF	Activating transcription factor
BMP	Bone morphogenetic protein
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CILP-1	Cartilage intermediate layer protein 1
CK1α	Casein kinase I α
CMV	Cytomegalovirus
COL2A1	Collagen type II alpha I
COMP	Cartilage Oligomeric Matrix Protein
CPC	Chlorure de cetylpyrimidium
CRD	Cys-rich domain
CS	Chondroitin sulfate
CTNNB1	Catenin beta-1
DAAM	DVL-associated activator of morphogenesis
DKK1	Dkkopfl
DMEM	Dulbecco's Modified Eagle's Medium
DVL	Dishevelled
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ERK	Extracellular signal-regulated kinase
FADD	Fas-Associated protein with Death Domain
FGF	Fibroblast Growth Factor
Fz	Frizzled

GAGs	Glycosaminoglycans
GSK-3β	Glycogen synthase kinase 3 beta
HA	Acide hyaluronique
IκB	Inhibitor of κ B
IKK	I κ B Kinase
IL-1β	Interleukine-1 beta
iNOS	Inducible nitric oxide synthase
JNK	Jun NH2-terminal Kinase
KS	Keratan sulfate
LRP5/6	Low density lipoprotein receptor-related receptor 5/6
LT-β	Lymphotoxin- β
LT-βRR	Receptor for lymphotoxin- β
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
NEMO	NF κ B essential modulator
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF κ B inducing kinase
OA	Osteoarthritis
PAM	Palmitoleic acid
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PDZ	Postsynaptic density-95/ disc large protein/zonula occludens-1
PG	Proteoglycan
PGE2	Prostaglandine E2
PRELP	Proline-arginine-rich end leucine-rich repeat protein

RAC1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras homolog gene family, member A
ROCK	RHO kinase
RPS29	Ribosomal protein S29
Runx2	Runt-related transcription factor 2
RYK	Receptor related to tyrosine kinase
SAPK/JNK	Stress-activated protein kinase/c-Jun NH(2)-terminal kinase
SYND	Syndecan
sFRP	Secreted Frizzled-related protein
si-RNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
TCF/LEF	T cell factor/lymphoid enhancer-binding factor
TGF-β	Transforming Growth Factor beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
WIF	Wnt inhibitory factor
Wnt	Wg (wingless) and Int

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Table 1	Role of Wnts and Wnt-related molecules in cartilage development
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INTRODUCTION

INTRODUCTION

I. Cartilage and osteoarthritis

A. Cartilage

Cartilage is a flexible connective tissue widely presents in different organs, such as joint, ear, intervertebral disk, rib, nose and bronchial tubes (Figure 1). Due to its special character, stiffer than muscle, flexibler than bone and with a smooth surface, cartilage is the perfect tissue to dissipate stress and maintain smooth and frictionless movement in the joint. Three types of cartilage were found in human body: elastic cartilage, hyaline cartilage and fibrocartilage. The articular cartilage is a typical hyaline cartilage that covers the ends of bones making up the joints in the body.

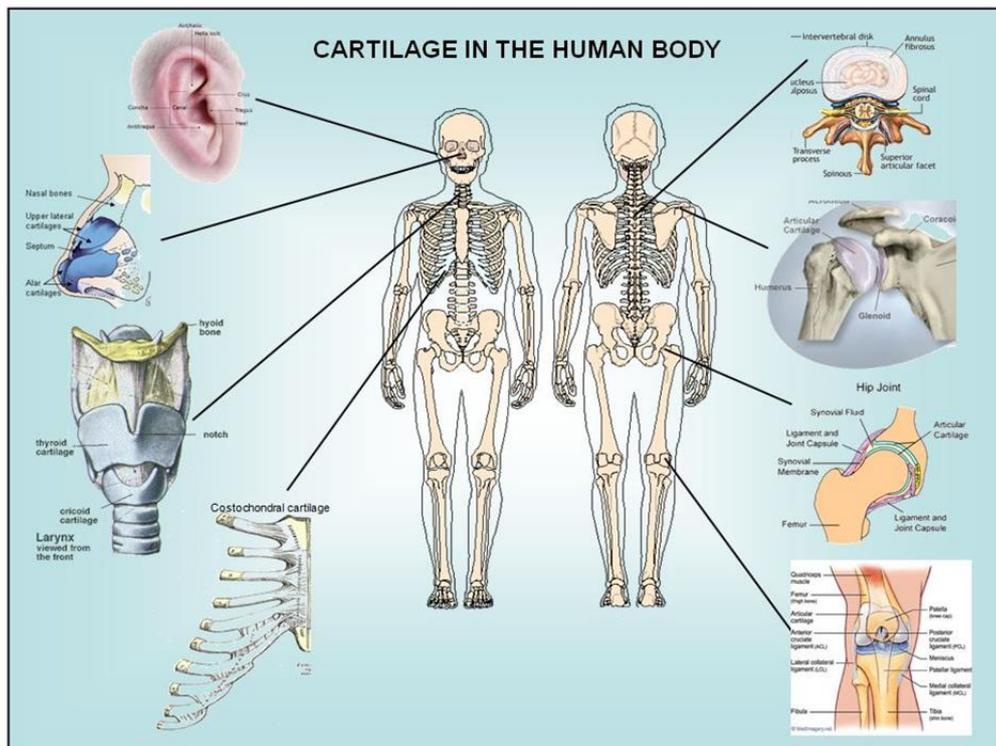


Figure 1. **Cartilage in the human body**

Cartilage widely exist in various organs of human body, like ear, nose, bronchial tubes, intervertebral disk, ribs and all the joints. (O. et al., 2011)

1. Composition

Specialized proteins and macromolecules compose the hyaline articular cartilage, that allow the tissue to function in the rigorous mechanical environments of articulating joints, such as shoulder, wrist, hip and knee. Besides fluid phase of water, the articular cartilage extracellular matrix (ECM) comprises three classes of proteins: collagens, proteoglycans, and other noncollagenous proteins, including link protein, fibronectin, cartilage oligomeric matrix protein, and the smaller proteoglycans, biglycan, decorin and fibromodulin (Figure 2).

1.1 Water

Approximately 70-80% by weight, water primarily composes the mature articular cartilage. Articular cartilage is under the constant stressor of compressive loading, so without a high water fraction, the tissue would break down much more quickly under constant use. Due to the frictional resistance between the water and solid matrix, high pressure is required for compressive loading to decrease the total water fraction by forcing the fluid from the tissue. Functionally, mechanical compression of cartilage causes rapid pressurization of the fluid in the tissue, which in turn supports the load. This mechanism allows for the longevity of cartilage under repeated compression since loading is borne by fluid instead of a solid-solid interaction (Ateshian et al., 1994).

Water is also the main component in the synovial fluid that is present in the joint capsule. Since cartilage has no vascularity, the chondrocytes obtain nutrients through diffusion from the joint space. As the primary carrier, interstitial fluid plays an important role in transporting both nutrients and waste within the tissue (Linn and Sokoloff, 1965).

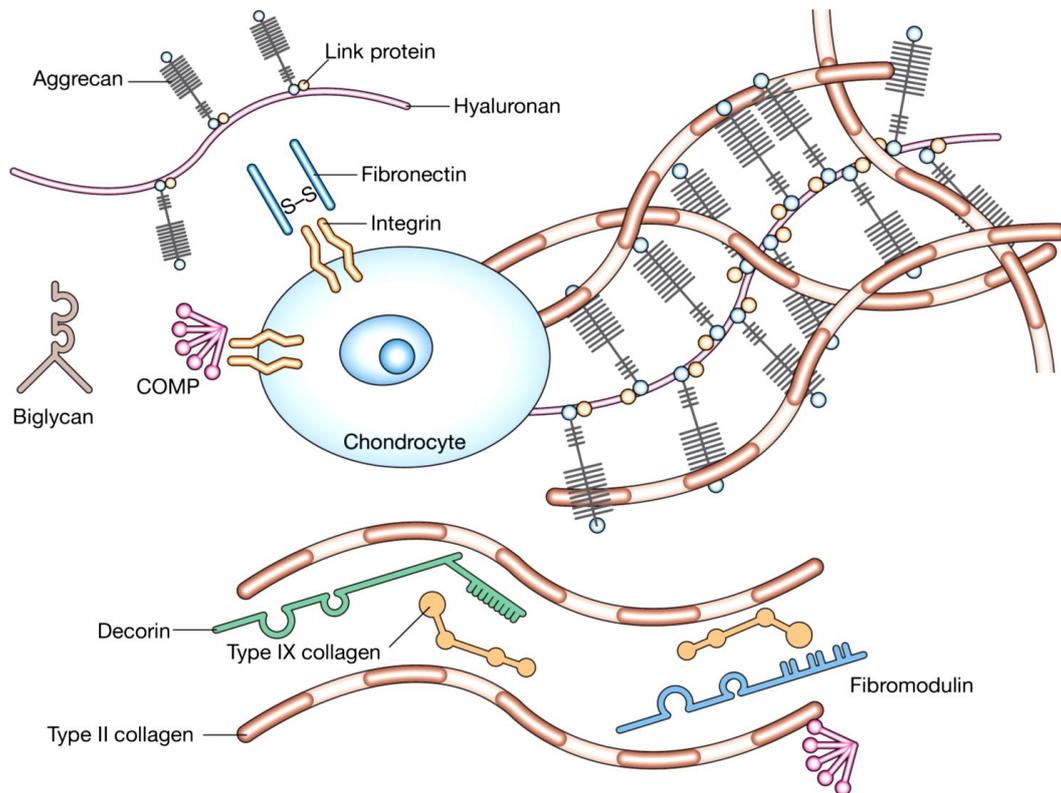


Figure 2. Extracellular matrix of cartilage

In articular cartilage there are three classes of proteins: collagens (mostly type II collagen); proteoglycans (primarily aggrecan); and other proteins (noncollagenous proteins, including link protein, fibronectin, cartilage oligomeric matrix protein; smaller proteoglycans, including biglycan, decorin and fibromodulin). The interaction between type II collagen and fibrilshighly negatively charged cartilage proteoglycans is responsible for the compressive and tensile strength of the tissue. Abbreviation: COMP, Cartilage oligomeric matrix protein. (Chen et al., 2006)

1.2 Collagens

The solid fraction of the cartilage is primarily collagens (50-75%) (Garrett et al., 2000), which serve a primary role in the structure of connective tissues throughout the body. There are at least 15 distinct collagen types composed of no fewer than 29 polypeptide chains. All members of the collagen family contain a region consisting of 3 polypeptide chains (α -chains) that form a unique triple helical structure. The composition of polypeptide chains is high numbers of the repeating peptide sequence glycine-X-Y,

where X and Y are frequently proline and hydroxyproline, with hydroxyproline providing stability via hydrogen bonds along the length of the molecule. The triple helix structure of the polypeptide chains provides articular cartilage with important shear and tensile properties, which help to stabilize the matrix (Sophia Fox et al., 2009).

Collagen type II is the predominant collagen in articular cartilage, representing 90-95% of the collagen in ECM, comprising over half the dry weight of the tissue. This collagen is comprised of three identical alpha-1(II) chains, they are encoded by the gene *Collagen, type II, alpha 1 (COL2A1)*. Not only found in articular cartilage, collagen II is also strongly expressed by proliferating human fetal columnar chondrocytes (Reichenberger et al., 1991). Forming fibrils and fibers intertwined with proteoglycan aggregates, collagen II is a primary indicator of hyaline cartilage differentiation. Furthermore, cartilage ECM lacking collagen II in transgenic mice cannot support the survival of chondrocytes (Yang et al., 1997), indicating the biological necessity of collagen II in articular cartilage.

Collagen types I, IV, V, VI, IX, and XI are also present but contribute only a minor proportion in articular cartilage. These minor collagens help to form and stabilize the type II collagen fibril network. Collagen XI molecules crosslink primarily with other collagen XI molecules within the heterofibril (Wu et al., 1992). It has also been shown to limit fibrillogenesis by inhibiting the growth of collagen II fibrils, which could be attributed to steric hindrance or interaction with other matrix molecules (Gregory et al., 2000); Collagen IX forms covalent links between other collagen IX molecules and also crosslinks collagen II molecules (Wu et al., 1992); Collagen VI is localized surrounding chondrocytes, it contains the sequence Arg-Gly-Asp that binds to chondrocyte receptors. (Bonaldo et al., 1990) It has been hypothesized that the close interactions between collagen VI and chondrocytes contribute to mechanotransduction (Guilak et al., 2006), so replicating the collagen VI structure may be important for creating an appropriate microenvironment for chondrocytes. Collagen I is not typically expressed in hyaline articular cartilage, but it appears in repair fibrocartilage that fills defects in damaged articular cartilage (Furukawa et al., 1980). Culturing chondrocytes can result

in de-differentiation and collagen I production (Darling and Athanasiou, 2005), thus, it is considered as a marker for the de-differentiation of chondrocytes. Like collagen I, collagen X is not natively expressed in hyaline cartilage, it can be found near the bone in the calcifying cartilage, in addition it is expressed by some cells at the articular surface of maturing cartilage (Kirsch and von der Mark, 1991) and in osteoarthritic cartilage (Walker et al., 1995), indicating the hypertrophy of chondrocytes.

1.3 Proteoglycans

Proteoglycans (PGs) are important bio-macromolecules composed of a core protein with attached glycosaminoglycan (GAG) chains. PGs represent the second-largest group of macromolecules in the ECM. Aggrecan, as the largest in size and the most abundant by weight of PGs, consists of a protein core with numerous GAG side chains, this macromolecule possesses more than 100 chondroitin and keratan sulfates chains. The protein core of aggrecan contains several distinct globular and extended domains where GAGs attach. Hyaluronan (HA) binds non-covalently through one such domain and is stabilized by a link protein (Kiani et al., 2002). The conglomeration of PGs into large macromolecules is essential for the proper functionality of cartilage.

PG networks in articular cartilage are like a mesh that is interlaced throughout, within the more organized collagen structure. The large size of this polymer mesh serves to immobilize and restrain it within the collagen network. The carboxyl and sulfate groups affords PGs a negative charge, which in turn gives cartilage ECM a net negative charge known as a “fixed charge density” (Maroudas et al., 1969). Due to this charge, the matrix imbibes fluid, swelling the tissue to maintain equilibrium. The swelling is balanced against the elastic restraint of the collagen network (Maroudas, 1976). As mentioned above, the functional properties of cartilage under compression are highly dependent on fluid pressurization within the tissue. Since the presence of PGs assists in the imbibition of water, it is apparent that a loss of PGs can result in a lack of fluid pressurization, and therefore, alters mechanical function. This breakdown in functionality is seen in advanced stages of osteoarthritis (OA).

Other proteoglycans within articular cartilage including biglycan, decorin, and fibromodulin, are also closely related in protein structure. And the proteoglycans are found not only within the ECM but also on the surface of chondrocytes. These membrane proteoglycans include the families of glypicans and syndecans, which will be furtherly introduced in another section.

1.4 Chondrocytes

As the sole cell type resident within articular cartilage, chondrocytes (Figure 3) arise in the embryo either from mesodermal origin such as the elements of the limb or from the neural crest which gives rise to the skeleton of the face. The chondrocyte is the basic metabolic unit of cartilage, and is responsible for limited matrix remodeling and the maintenance of the tissue. Since articular cartilage is not vascularized, chondrocytes obtain nutrients by diffusion from the synovial fluid, facilitated during joint movement (Archer et al., 1990). Consequently, the entire metabolism of the cell is geared towards operating at a low oxygen tension with the majority of the cell's energy requirements is sufficed by glycolysis. The characteristic gene and protein expressed in chondrocytes are closely associated with the matrix constituents of articular cartilage. Each chondrocyte establishes a specialized microenvironment and is responsible for the turnover of the ECM in its immediate vicinity. This microenvironment is indispensable to trap the chondrocyte within its own matrix and so prevents any migration to adjacent areas of cartilage. Maintenance of the surrounding matrix requires synthesis of proteoglycans and collagens as well as other small molecules. Disease and injury can change cartilage physiology as well as tissue turnover, which can progressively accelerate tissue breakdown.

Chondrocytes rarely form cell-to-cell contacts for direct signal transduction and communication between cells. They do, however, respond to a variety of stimuli, including growth factors, mechanical loads, piezoelectric forces, and hydrostatic pressures (Sophia Fox et al., 2009). Unfortunately, chondrocytes have limited potential for replication, a factor that contributes to the limited intrinsic healing capacity of cartilage in response to injury. Chondrocyte survival depends on an optimal chemical

and mechanical environment.

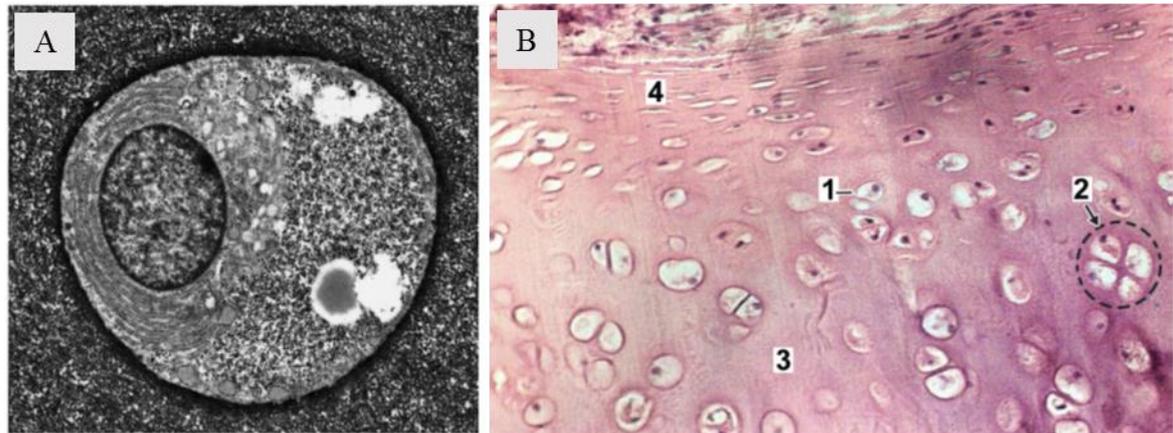


Figure 3. **Articular chondrocyte**

A. Electron micrograph of articular chondrocyte. The cell is typically rounded containing ordered rough endoplasmic reticulum, juxtanuclear Golgi apparatus and conspicuous deposits of glycogen including the remnants of lipid droplets. (Archer and Francis-West, 2003) B. Photomicrograph of hyaline cartilage. Key: 1.Chondrocyte; 2. Cell nest; 3. Basophilic matrix; 4. Perichondrium.(Vasudeva Neelam and Mishra Sqabita, 2014)

2. Structure

There are two primary reference frames for describing matrix organization of hyaline cartilage. The first respects depth within the tissue, structure zone varies from surface of the cartilage through to the bone (Figure 4). Variations exist in cell morphology, collagen fiber orientation, and biochemical composition. The second respects to distance from the chondrocyte membrane (Figure 5). Tissue surrounding the chondrocyte is named the pericellular matrix, in turn surrounded by the territorial and interterritorial matrices.

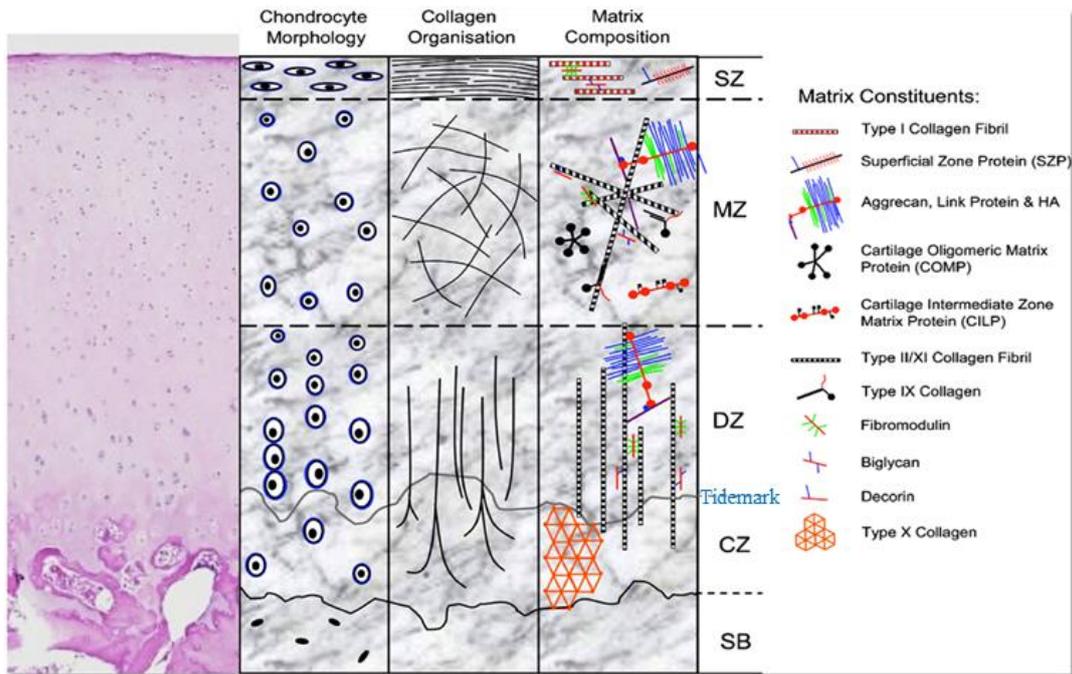


Figure 4. **H&E stain and schematic, cross-sectional diagram of healthy articular cartilage**

Cellular morphology, collagen organisation and matrix composition in the zones of articular cartilage. SZ, superficial zone; MZ, middle zone; DZ, deep zone; CZ, calcified zone; SB, subchondral bone. (Di Bella et al., 2015)

2.1 Zonal structure.

Hyaline cartilage from the articulating surface down to the subchondral bone has different characters on ECM structure and composition, as well as cell shape and arrangement within the tissue. Variations also exist in their mechanical characteristics, which tie directly into the overall functionality of cartilage.

The surface of articular cartilage is covered by a layer named lamina splendens, which was firstly described by MacConeil using phase contrasted microscope (MacCONAILL, 1951). This layer is an acellular, primarily non-fibrous region which has a thickness ranging from hundreds of nanometers to a few microns and contains unique collagen network.

The upper 10-20% of the cartilage often referred as the superficial zone. The collagen fibers of this zone (primarily, type II and IX collagen) have small diameter,

are densely packed and oriented parallel to the cartilage surface (Liverpool and Roy, 1969). This zone contains a relatively low proteoglycan content but relatively high number of chondrocytes which layer are densely packed and exhibit flattened, discoidal shapes that are oriented along the neighboring collagen fibers in a tangential direction (Stockwell, 1971). The superficial zone is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation.

The next is the middle (transitional) zone, which occupies approximately 40%-60% of the total cartilage thickness. In this zone the chondrocyte density is much lower and the cell shape is more spherical, but it contains more proteoglycans and thicker collagen fibrils (Stockwell, 1971). The middle zone provides an anatomic and functional bridge between the superficial and deep zones and is the first line of resistance to compressive forces.

Representing approximately 30% of articular cartilage thickness, the deep zone is the last region of purely-hyaline tissue before reaching bone. The chondrocytes in this zone often arranged in columnar orientation, slightly elongated and oriented in the direction of collagen fibers, and perpendicular to the articular surface (Egglı et al., 1988). Not only the cell density is the lowest of the three cartilaginous zones, in the layer, but also proteoglycan content is much lower than in the middle zone. Its collagen structure is characterized by large fibers that form bundles oriented perpendicular to the articular surface and are anchored in the underlying subchondral bone. The deep zone is responsible for providing the greatest resistance to compressive forces.

A thin line termed the “tidemark” is present between the deep zone and calcified zone of articular cartilage (Redler et al., 1975). In the calcified layer, the cell population is scarce and chondrocytes are hypertrophic. In this region collagen fibrils from the deep zone anchor to subchondral bone, which minimizes the stiffness gradient between the rigid bone and more pliable cartilage (Radin and Rose, 1986). Underlying this region of the cartilage is the subchondral bone, which is the ultimate anchorage point for cartilage tissue as a whole.

2.2 Territorial structure.

In addition to the zonal organization associated with cartilage ECM, the tissue also has a micro-scale structure oriented with respect to distance from the chondrocyte cell membrane (Figure 5). The region immediately surrounding a chondrocyte is termed the pericellular matrix. A number of matrix molecules, most notably type VI collagen, perlecan, aggrecan monomers and small aggregates, hyaluronan, biglycan, and type IX collagen, are found either exclusively or at higher concentrations in the pericellular matrix as compared to the surrounding ECM (Wilusz et al., 2014). The exact function of the pericellular matrix is not fully understood, studies suggest that it plays a critical role in controlling the mechanical environment and mechanobiology of cells in cartilage. The region immediately surrounding the pericellular matrix is termed the territorial matrix. The territorial matrix exhibits a higher concentration of proteoglycans than the surrounding ECM, as well as having a finer collagen structure (Hunziker et al., 1997). The interterritorial matrix, which contains large collagen type IV fibers and different concentrations of proteoglycans, comprises the bulk of articular cartilage, providing the tissue with its mechanical properties. Loading of articular cartilage involves force transmission through the interterritorial, territorial, and pericellular matrices before reaching the chondrocytes. This structure can serve as either a protective layer for the chondrocyte or a transducer that amplifies strain, such that cellular-level strains are more homogenous throughout the tissue depth despite large inhomogeneities in local ECM strains (Choi et al., 2007).

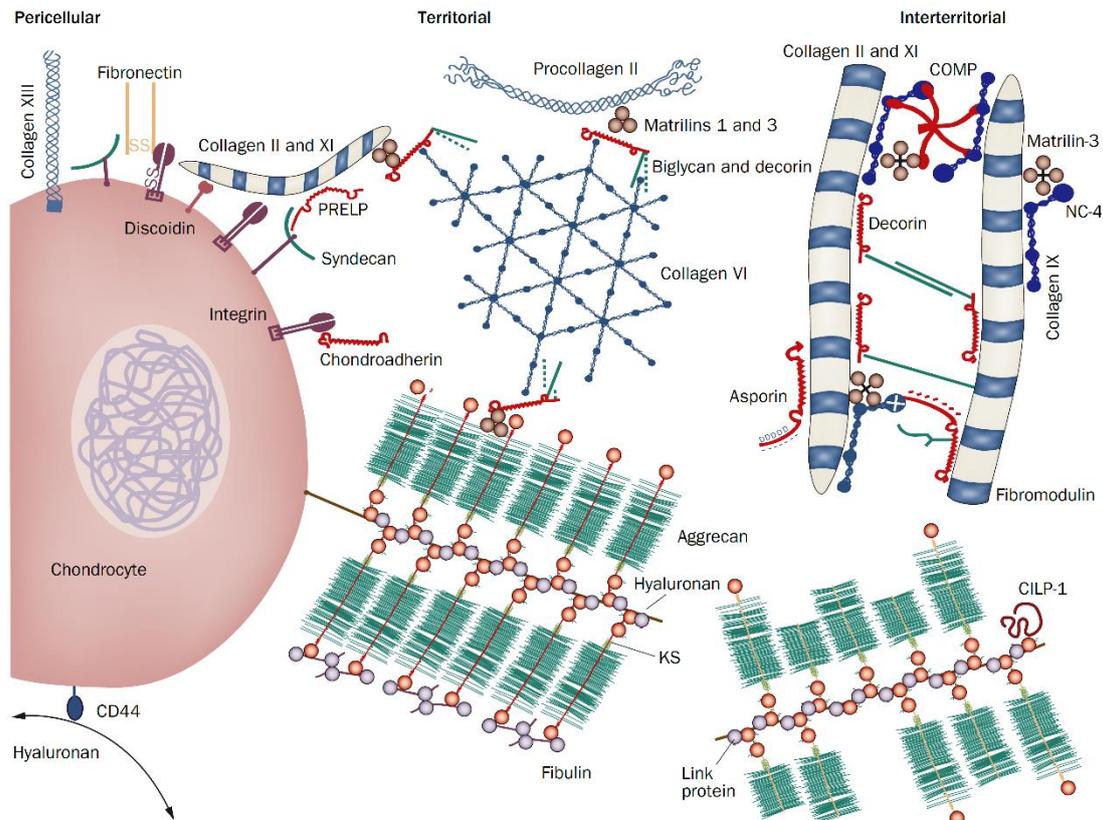


Figure 5. The molecular organization of normal articular cartilage

In the articular cartilage, cartilage matrix surrounding chondrocytes is arranged into zones defined by their distance from the cell. Immediately around the cell is the pericellular matrix where molecules that interact with cell surface receptors are located. Next to the pericellular matrix, slightly further from the cell, lies the territorial matrix. At largest distance from the cell is the interterritorial matrix. The types of collagens and the collagen-binding proteins in the matrices are different in each zone. Abbreviations: CILP -1, cartilage intermediate layer protein 1; COMP, cartilage oligomeric matrix protein; CS, chondroitin sulfate; KS, keratan sulfate; PRELP, proline-arginine-rich end leucine-rich repeat protein. (Heinegård and Saxne, 2011)

3. The turnover of cartilage matrix

Collagens and proteoglycans interact with a charged fluid environment to give articular cartilage its unique mechanical properties. Like many other metabolically active tissues, in articular cartilage the ECM is maintained in a state of constant turnover (Garrett et al., 2000), however different molecular components are reconstituted at different rates,

and variations even exist among the same components which have distinct spatial location. The turnover of collagen is estimated to be very slow (>100 years), whereas aggrecan turnover is more rapid, with a half-life of 8-300 days in rabbit (Mankin and Lippiello, 1969). Cartilage biomechanical function is rely on this normal tissue structure in homeostasis, which is maintained by the existence of an appropriate number of chondrocytes with normal biosynthetic function. Many proteinases responsible for the cartilage catabolism exist in the extracellular space. Of these proteinases that degrade cartilage collagens and proteoglycans in joint disease, matrix metalloproteinases (MMPs) and aggrecanases have been given the greatest attention because they degrade native collagens and proteoglycans.

3.1 MMPs

MMPs are the main enzymes involved in ECM catabolism, but their activity is low in normal conditions. MMP activity was firstly discovered as a collagenase in the resorbing tadpole tail during metamorphosis in 1962 (Gross and Lapiere, 1962). The first purified human collagenase MMP-1 was isolated from rheumatoid synovium, which suggested its role in cartilage destruction (Woolley et al., 1975). Then 24 MMP genes including two duplicated MMP23 genes were discovered in human genome. The extracellular multidomain metalloproteinases encoded by these genes mostly comprised a signal peptide, a propeptide, a catalytic metalloproteinase domain, a linker (hinge) peptide and a hemopexin domain. (Nagase et al., 2006) Most MMPs are secreted as zymogens and are subsequently activated in the extracellular space. MMP activation primarily occurs through proteolytic cleavage (by Serine proteases or other MMPs) or by modifying the thiol group by oxidation (Bonnans et al., 2014). They include the collagenases (MMP-1, MMP-8, and MMP-13), the gelatinases (MMP-2 and MMP-9), stromelysin-1 (MMP-3), and membrane type I (MT1) MMP (MMP-14). Of the three collagenases, MMP-13 is probably the one which plays the greatest part in the pathology of OA degrading the “resident” collagen fibrils more remote from the cell in the territorial and interterritorial matrix (Billingham et al., 1997). And the MMP-3, which can activate pro-collagenases is detectable in OA and rheumatoid arthritis

synovial fluids and joint tissues, it is produced *in vitro* by chondrocytes in response to inflammatory cytokines (Burrage et al., 2006).

3.2 Aggrecanase

Although several of the MMPs, including MMP-3, MMP-8, and MMP-14, are capable of degrading proteoglycans, aggrecanase are still regarded as the principal aggrecan degrading enzymes in cartilage. The later activity was first reported in 1991 describing the cleavage of aggrecan core protein at the Glu³⁷³-Ala³⁷⁴ bond, a distinct site from the MMP cleavage site of the Asn³⁴¹-Phe³⁴² bond (Sandy et al., 1991). The first aggrecanase was identified as a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and designated as ADAMTS-4 (aggrecanase 1). Comprising 19 members in human, ADAMTS are secreted multidomain metalloproteinases, consisting of a signal peptide, a prodomain, a metalloproteinase domain, a disintegrin domain, a thrombospondin type I motif, a cysteine-rich domain, a spacer domain, and a second thrombospondin motif of variable numbers of repeats at the C-terminal region (Kelwick et al., 2015). Lines of evidence indicate that ADAMTS-4 and -5 (aggrecanase 2) are the principal enzymes involved in the OA (Verma and Dalal, 2011). *In vitro* ADAMTS-5 is about 1,000 times more potent than ADAMTS-4 (Gendron et al., 2007), knockdown of either ADAMTS-4 or ADAMTS-5 in human cartilage explants and chondrocytes attenuated aggrecan breakdown (Song et al., 2007). Moreover, It has been shown that ADAMTS-4 mRNA was induced by catabolic cytokines such as interleukin-1 β (IL-1 β) in human cartilage and chondrocytes but ADAMTS-5 mRNA was not regulated by the cytokine (Koshy et al., 2002). So in human the ADAMTS-4 may be the more promising target for OA treatment, compared to ADAMTS-5.

3.3 Factors involved in cartilage homeostasis

How chondrocytes well maintains the synthesis and degradation rates of cartilage ECM in balance has remained somewhat of a mystery, however, several factors are confirmed to be critical for the cartilage homeostasis: mechanical loading within a physiological range, hypoxic environment, cellular homeostasis, and inflammatory factors.

Several *in vitro* studies have examined the effect of physiological mechanical loading on chondrocyte function and matrix synthesis. A decrease of proteoglycan content associated with increased MMP-3 and ADAMTS-5 expression is observed in rodents after hind limb immobilization, whereas joint movement prevents this cartilage destruction (Leong et al., 2010). Moreover, in human normal chondrocytes, by increasing aggrecan and decreasing MMP-3 expression, mechanical stimulation has opposite effects catabolism. (Millward-Sadler et al., 2000) However, information regarding the importance of defining the optimal mechanical parameters required for mechanical conditioning and biosynthesis of anabolic proteins still needs to be established.

Oxygen and nutrients coming from the vascular supply in the articular capsule, synovium and subchondral bone are arduously to reach the cells, so hypoxia is the normal environment for chondrocytes which synthesize and accumulate higher amounts of type II collagen and aggrecan when cultured under hypoxia rather than normoxia (Ströbel et al., 2010). Additionally, the application of more physiologic oxygen percentage reduced the activity of catabolic enzymes implicated in cartilage breakdown, the basal synthesis and release of MMP-1 and MMP-13, as well as generation of type II collagen cleavage fragments, are lower under hypoxia than in normoxia (Ströbel et al., 2010).

Cartilage is a postmitotic tissue with very low, barely detectable rates of cell replication, so the cellular homeostasis of chondrocytes critically impact the tissue structure. Cellular homeostasis requires defense mechanisms that protect cells against oxidants and prevent DNA mutations and telomeric DNA damage. For example, the unfolded protein response is activated when unfolded or misfolded proteins are detected, to prevent protein dysfunction and aggregation. The autophagy is the principal mechanism for the removal of damaged and dysfunctional organelles and macromolecules for chondrocytes (Lotz and Caramés, 2011). Normal functioning of these cellular homeostasis mechanisms maintains the function of chondrocytes in cartilage.

Secreted inflammatory factors are critical mediators of the disturbed metabolism and enhanced catabolism of joint tissue involved in disease. Among them Interleukin-1 β (IL- β) and Tumor necrosis factor (TNF) are the best studied main proinflammatory cytokines (Kapoor et al., 2011). As described above, these factors can down-regulate the major component of ECM type II collagen and aggrecan, and stimulate chondrocytes to release several proteolytic enzymes like MMPs and ADAMTSs. More details will be showed in the next section.

B. Osteoarthritis

OA is a disease characterized by the destruction of articular cartilage and alterations of underlying subchondral bone. Unfortunately, no curative therapy which can halt disease progress or reverse the cartilage damage is available for instant. The main goals of current OA therapy are the control of pain and the improvement of articular function. For many patients, the lack of disease-modifying OA drugs results in irreversible cartilage damage which eventually necessitates surgical intervention. Thus, as the most common form of arthritis, OA forces more than one million patients per year to go to the hospital for the total knee or total hip replacement, which poses great costs both financially and to the patient's quality of life.

Many factors contribute to the originating of OA, like ageing, obesity, trauma, genetics, amongst others, the underpinning molecular mechanisms of OA are not fully understood. Secreted inflammatory factors such as proinflammatory cytokines, are critical mediators of the disturbed metabolism and enhanced catabolism of joint tissue involved in OA (Kapoor et al., 2011). The breakdown of the cartilage matrix caused by the mechanical and inflammatory stimulations leads to the development of fibrillation and fissures, the appearance of gross ulcerations, and the disappearance of the full thickness surface of the joint. This is accompanied by bone changes with osteophyte formation and thickening of the subchondral plate (Figure 6). Nowadays, OA is not regarded as a single disease, but rather as a common final stage of joint failure, of which the initial stages are not well elucidated. Therefore, the understanding of the early stage

of OA and the alteration in the cartilage seems to be critical to find a cure for this disease.

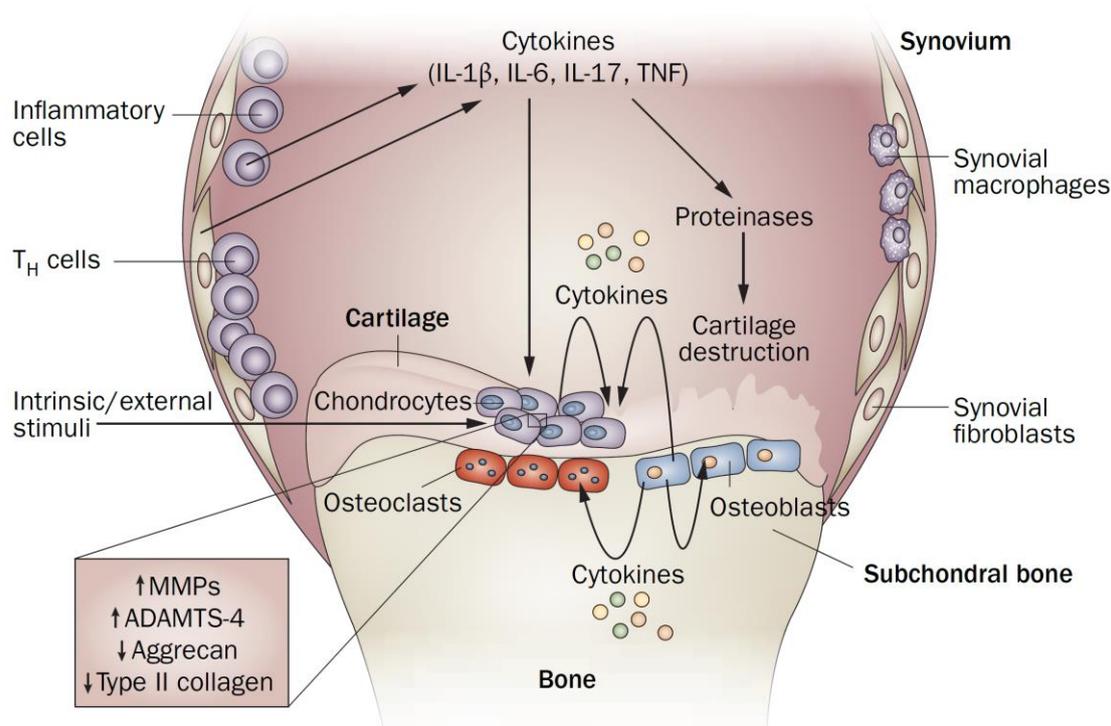


Figure 6. **The role of proinflammatory cytokines in the pathophysiology of OA.**

The levels of proinflammatory cytokines, including IL-1 β , TNF and IL-6, are elevated in OA. Several mechanisms including downregulation of anabolism and upregulation of catabolism, and inflammatory responses are triggered. Abbreviations: ADAMTS, a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs; IL, interleukin; MMP, matrix metalloproteinase; TNF, tumor necrosis factor. (Kapoor et al., 2011)

1. Osteoarthritis and extracellular matrix

During the progression of OA, the integrity of the ECM homeostatic is affected: the aggrecan content is decreased, while the collagen content is increased but composition is changed.

The negatively charged proteoglycan, aggrecan, can attract water molecules and provide the compressive resistant and shock absorbing capability to cartilage under loading. In the initial stages of OA in a rat model, the aggrecan gene expression is significantly down-regulated, while the aggrecanase ADAMTS-4 is significantly

upregulated (Nam et al., 2011). This finding agrees with those observations in human early OA samples; proteoglycan synthesis is markedly lower in the normal appearance OA cartilage (Lorenzo et al., 2004). However, when minor cartilage surface fibrillation occurs, the tissue level of aggrecan does not seem to decrease. During this phase, the fragments of the protein that contain a binding site for hyaluronan are retained in the tissue, demonstrating that although there is no overall loss of the proteoglycan, its turnover is altered (Maroudas et al., 1998; Saxne and Heinegård, 1992). When the disease progresses, this fragile balance will be broken and end in an overall loss of the aggrecan in all the zones of the cartilage (Lahm et al., 2010). The relationship between proteoglycan aggregates and interstitial fluid provides compressive resilience to cartilage through negative electrostatic repulsion forces (Maldonado and Nam, 2013). So the reduction of proteoglycan content will decrease the compressive modulus of cartilage and consequently expose the tissue to greater strains when receiving mechanical stress. On the other hand, aggrecan degradation products have been shown to promote further joint degeneration. The recent research demonstrated that stimulation of human chondrocytes with human aggrecan 32-mer peptide increased the expression of catabolic markers like MMP3 and MMP13 at the mRNA and protein levels (Lees et al., 2015). So the loss of aggrecan not only makes cartilage more fragile to the mechanical stress but also promotes the pathological process of OA.

Unlike the decreased aggrecan, collagen synthesis rate increases in the early stages of OA and remains raised (Videman et al., 1981). Along with the increased ratio of collagen/aggrecan synthesis, the major collagen composition collagen type II has been shown to be replaced by type I, which is mainly found in subchondral bone tissue in healthy joints (Lahm et al., 2010). The compositional change affects the mechanical stability of the ECM. Despite they are both fibrillar collagens, compared to collagen type I, type II chains contain a higher content of hydroxylysine as well as glucosyl and galactosyl residues which mediate the interaction with proteoglycans (Gelse et al., 2003). Therefore, the decreased collagen type II content during OA inevitably undermines the integrity of ECM networks. Moreover, Silver et al. (2002) showed that

the type II collagen parallel to the cleavage line pattern in the superficial zone stores elastic energy. A reduced ability to store elastic energy caused by enzymatic cleavage of cartilage collagen in OA may lead to fibrillation and fissure formation (Silver et al., 2002). These changes in the structure and composition of extracellular progressively alter the biological and mechanical microenvironments of the cartilage, and impair its physiological function, even modulate cellular activities as described in the next section.

2. Osteoarthritis and chondrocytes

Chondrocytes are responsible for synthesizing matrix components, the maintenance and repair of the ECM are achieved via a group of degradative enzymes. One notable alteration of chondrocytes in arthritic joints is their production of nonchondrocytic ECM. In addition to the increase in the production of collagen type I replacing type II as previously described, chondrocytes isolated from OA cartilage have shown to produce more collagen type X, a marker of hypertrophic chondrocytes, comparing to undetectable expression of the protein in healthy cartilage (Yang et al., 2006). Inflammatory cytokines like IL-1 β inhibits the transcription factor SOX9 (SRY (sex determining region Y)-box 9), in turn, decreases the expression of collagen type II and aggrecan in articular chondrocytes (Dai et al., 2012). Additionally, this persistent inflammatory stimulation directly induces the catabolic activities of chondrocytes by upregulating the expression of matrix degrading enzymes such as MMP-1, MMP-3, MMP-13, and ADAMTS-4 (Koshy et al., 2002).

On the other hand, the altered microenvironment by ECM changes, further drives dysfunction of chondrocytes. The increase in local matrix stiffness caused by the dynamically altered ECM appears to suppress chondrocytic activities of the cells. Studies showed that chondrocytes can sense the stiffness of matrix and respond to it by altering their phenotype, resulting in changing of the ration of collagen type II to type I. In an optimal stiffness, the COL2A1 and aggrecan gene expression is highest, softer or stiffer will reduce these normal matrix components expression (Allen et al., 2012). Chondrocytes can sense mechanical stress and changes in the pericellular matrix largely

through receptors for ECM components. The receptors on the resting chondrocyte are protected from interacting with certain matrix components by the unique composition of the pericellular matrix. So the changes in matrix composition during OA not only affect the mechanical environments of chondrocytes but also alter interactions of matrix proteins with the cells.

According to the research of Tew and colleagues, the majority of chondrocyte-expressed transcripts were similar between chondrocytes from intact OA cartilage and those from fibrillated OA cartilage, but the later exhibited rapid decay with, interestingly, a clear bias toward shortening of mRNA half-life in chondrocytes. Short-lived transcripts included genes involved in transcriptional regulation, localized to the nucleus, or involved in the regulation of programmed cell death (Tew et al., 2014). Not only the cell function but also cell number is decreased in OA cartilage. Chondrocytes near the superficial layer form clusters, whereas in the deep and calcified layers, they undergo apoptotic death (Del Carlo and Loeser, 2008). Chondrocyte proliferation is somewhat activated, but cannot resist the predominant catabolic activity. In brief, at the initiation stage of the OA, the dysfunctional and less-number chondrocytes induce the alteration of the cartilage.

Another fascinating biologic event that has gathered much attention over the past years is autophagy. Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis. In articular cartilage, a postmitotic tissue characterized by a very low rate of cell turnover, autophagy would seem to be essential to the maintenance of normal cell function and survival as it removes dysfunctional organelles, including mitochondria, peroxisomes, and ribosomes, and aggregate-prone or misfolded proteins (Lotz and Caramés, 2011). Autophagy is a constitutively active and seemingly protective process for the maintenance of articular cartilage homeostasis (Caramés et al., 2010), and it has been also found to be compromised at the early stage of OA in an animal model (Shi et al., 2016). Since autophagy is a druggable pathway, investigations have been started to confirm the effect of its activator in preventing the development of OA (Takayama et

al., 2014).

Appropriate hypertrophy-like changes are found in the chondrocytes in OA. There are several markers of hypertrophic chondrocytes, the most widely used are type X collagen and MMP13, the latter is also collagenase induced by inflammation stress (van der Kraan and van den Berg, 2012). These chondrocytes lead to the increase of the number of tidemarks in OA joint (Karpouzas and Terkeltaub, 1999). However, it is not clear whether chondrocyte hypertrophy per se or just the induction of selected hypertrophy-specific genes contributes to cartilage degeneration in OA.

3. OA and inflammatory factors

Overt inflammatory processes are present locally in OA joint. Among all the cytokines, IL-1 β , TNF α and IL-6 seem to be the main proinflammatory cytokines involved in the pathophysiology of OA, although others, including IL-15, IL-17, IL-18, IL-21, leukemia inhibitory factor (LIF) and chemokines have also been implicated. As the two major players, IL-1 β seems to be associated with cartilage destruction, and TNF α with driving the inflammatory cascade. In OA patient, both IL-1 β and TNF α levels are elevated in the synovial fluid, synovial membrane, subchondral bone and specially cartilage (Kapoor et al., 2011). Intra-articular injection of combinations of IL-1 β and TNF α into rabbit knee joint causes more leucocyte infiltration than either cytokine alone (Henderson and Pettipher, 1989).

The biological activation of cells by IL-1 β is mediated through its specific cell-surface receptor termed IL-1 receptor type I (IL-1RI). This receptor is more expressed on the human OA chondrocytes and synovial fibroblasts comparing with normal cells (Martel-Pelletier et al., 1992; Sadouk et al., 1995). IL-1 β also binds to a second specific cell-surface receptor, IL-1RII which is in fact a decoy receptor owing to its inability to transduce a signal (Colotta et al., 1994). IL-1 receptor antagonist (IL-Ra), another inhibitor of IL-1, is produced by several cell types including chondrocytes and functions as a competitive antagonist by binding to both IL-RI and IL-1RII without transducing signal (Palmer et al., 2002). The other major player TNF binds to two specific receptors

named TNF receptor I (TNFRI or p55) and TNF receptor II (TNFRII or p75). Between them, TNFRI appears to be the dominant receptor responsible for mediating TNF activity in the articular tissue cells and its expression is increased in OA chondrocytes (Alaeddine et al., 1997).

As described above, chondrocytes in OA cartilage, especially those in clonal clusters, express receptors which could respond to cytokines. Then the IL-1 β and TNF can inhibit anabolic activities of chondrocytes and downregulate the synthesis of major ECM components (Goldring et al., 1994; Saklatvala, 1986). In cell culture studies, treatment with IL-1 β reduces both type II collagen and aggrecan expression in chondrocytes isolated from several different species (Chadjichristos et al., 2003; Shakibaei et al., 2005; Stöve et al., 2000). In rat chondrocytes, the suppressive effect of IL-1 β on proteoglycan synthesis seems to be mediated through inhibition of β -1,3-glucuronyltransferase I, the key enzyme for the biosynthesis of GAGs linked to aggrecan core proteins (Gouze et al., 2001). Similarly, TNF has been shown to suppress the synthesis of proteoglycan, link protein and type II collagen in chondrocytes (Saklatvala, 1986; Séguin and Bernier, 2003).

Despite the production and secretion of active IL-1 β in OA is remains controversial (Bougault et al., 2012), this inflammatory factor synthesized by chondrocytes and synoviocytes is consider to be at concentrations that are capable of inducing the expression of catabolic genes. (Barakat et al., 2002; Goldring and Otero, 2011) MMP1, MMP3 and MMP13 are three key proteases of cartilage destruction, their expression in chondrocytes is significantly induced by IL-1 β and TNF (Lefebvre et al., 1990; Mengshol et al., 2000; Reboul et al., 1996). However, the regulation of ADAMTS-4 and ADAMTS-5 by inflammatory stimuli in cartilage seems to be species-specific. The mouse ADAMTS knock-out model shows that ADAMTS-5 play a more predominant role in OA pathogenesis (Glasson et al., 2004, 2005). In bovine, porcine and human samples, IL-1 β and TNF can only induce the expression of ADAMTS-4 but not that of ADAMTS-5. (Gabay et al., 2010; Palmer et al., 2002; Tortorella et al., 2001)

In addition to inducing the synthesis of proteinases, IL-1 β and TNF amplify and

perpetuate the OA process by promoting the production of proinflammatory cytokines such as IL-6 (Guerne et al., 1990) and chemokines such as IL-8 (Lotz et al., 1992), monocyte chemoattractant protein 1 (Villiger et al., 1992) and CC-chemokine ligand 5 (Alaeddine et al., 1997). IL-1 β and TNF also stimulate the synthesis of a number of other inflammatory mediators implicated in OA pathology. For instance, treatment of chondrocytes with these cytokines upregulates the expression of genes encoding inducible nitric oxide synthase (iNOS), soluble phospholipase A2, cyclooxygenase 2 (COX2) and microsomal prostaglandin E synthase 1, then stimulates the release of nitric oxide (NO) and prostaglandin E₂ (PGE₂) (Goldring and Otero, 2011). NO and PGE₂ play a role in articular inflammation and destruction by enhancing the production and activation of MMPs, inhibiting the anabolism of macromolecules such as collagen and proteoglycan, and the production of IL-1Ra, and promoting chondrocyte apoptosis (Kapoor et al., 2011). IL-1 β and TNF also induce the production of reactive oxygen species (ROS), mainly NO and the superoxide anion, that generate hydrogen peroxide, peroxynitrite and hydroxyl radicals (Afonso et al., 2007), which contribute to cartilage degradation. Moreover, IL-1 β and TNF inhibit the expression of the antioxidant enzymes that scavenge ROS, including superoxide dismutase, catalase and glutathione peroxidase (Mathy-Hartert et al., 2008; Scott et al., 2010), thus augmenting the damaging effects of ROS on cartilage.

4. OA and downstream signaling pathways in chondrocytes

An array of downstream signaling pathways are triggered in the chondrocytes by the injurious mechanical stress, inflammatory cytokines or other OA risk factors. Along with NF- κ B activation and translocation (Marcu et al., 2010; Nam et al., 2009), the activation of cell surface receptors also induces mitogen-activated protein kinase (MAPK) signaling (Fanning et al., 2003; Saklatvala, 2007), and Wnt-mediated mechanisms (Schett et al., 2008). Since these pathways not only promote the expressions of different catabolic enzymes, but also both induce and amplify the

expression of cytokine and chemokine genes, it therefore remains controversial whether inflammatory mediators are primary or secondary regulators of cartilage damage and defective repair mechanisms in OA (Goldring et al., 2011; Marcu et al., 2010).

4.1 NF- κ B pathway

The NF- κ B pathway (Figure 8) is a central regulator of the inflammatory cytokine-induced catabolic actions in chondrocytes, and its effectors are a family of ubiquitously expressed transcription factors involved in inflammatory diseases, stress responses, immunity, cell proliferation and cell death (Oeckinghaus et al., 2011).

Five members of the transcription factor NF- κ B family were found in mammals: RelA (p65), RelB, c-Rel, and the precursor proteins NF- κ B1 (p105) and NF- κ B2 (p100), which are processed into p50 and p52, respectively. All NF- κ B transcription factors share an N-terminal Rel-homology domain which is responsible for dimerization in homo- and hetero-dimeric active complexes, interaction with NF- κ B inhibitors (inhibitory NF- κ B proteins, I κ Bs), nuclear translocation and DNA binding to regulatory elements in the promoter/enhancer regions of Rel/ NF- κ B-target genes (Rigoglou and Papavassiliou, 2013).

Under unstimulated condition, the NF- κ B dimers are bound to inhibitory I κ B protein, which sequester NF- κ B complexes in the cytoplasm in an inactive form. When cells are stimulated by chemical or mechanical signals, the degradation of I κ B is initiated through phosphorylation by the I κ B kinase (IKK) complex, which consists of two catalytically active kinases, IKK α and IKK β , and the regulatory subunit IKK γ (NF- κ B essential modulator, NEMO). The I κ B proteins are phosphorylated by the IKKs, then targeted for ubiquitination and proteasomal degradation, which releases bound NF- κ B hetero-dimers to translocate into the nucleus then trigger the expression of a wide range of proteins. Transcriptional activity of nuclear NF- κ B is further regulated by post-translational modifications (Wan and Lenardo, 2009).

Overall, two distinct NF- κ B-activating pathways exist in cell. The classic pathway (canonical pathway) is activated by most physiological NF- κ B stimuli. For example, TNFR, IL-1R, Toll-like receptor (TL-R) or T-cell receptor (TC-R). This pathway

depend on the activation of IKK α /IKK β /IKK γ complex which leads to phosphorylation and subsequent degradation of I κ B α and nuclear translocation of mostly p65-containing heterodimers. Likewise, the alternative pathway (noncanonical pathway) is induced by the B-cell activating factor (BAFF), CD40, lymphotoxin β (LT β) or their receptors (BAFF-R, CD40-R and LT β -R, respectively). This signaling relies on NF- κ B-inducing kinase (NIK) which activates the IKK α via phosphorylation. Thereafter, IKK α mediates the phosphorylation-dependent proteolysis of p100 which is associated with RelB in cytoplasm, then leads to the release of p52 protein that is capable to switch on transcription of target genes (Rigoglou and Papavassiliou, 2013).

The NF- κ B pathway is a central regulator of the inflammatory cytokine-induced catabolic actions in chondrocytes. Chondrocytes express TLR 1-9, and the predominate one TLR-2 is more expressed in OA chondrocytes than normal cells, and it is up-regulated by IL- β and fibronectin (Kim et al., 2006; Su et al., 2005). The activation of NF- κ B transcription factors is responsible for the secretion of many degradative enzymes, including MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP13, ADAMTS-4 and 5. In addition, the NF- κ B mediates the action of a variety of catabolic cytokines and chemokines, such as TNF- α , IL-1 β , IL-6, receptor activator of NF- κ B ligand (RANKL), and IL-8, which perpetuate the cartilage destruction. Finally, the NF- κ B molecules enhance the articular damage through induction of NO, COX2, NOS and PGE₂, which promote the catabolic factors in OA cartilage (Marcu et al., 2010).

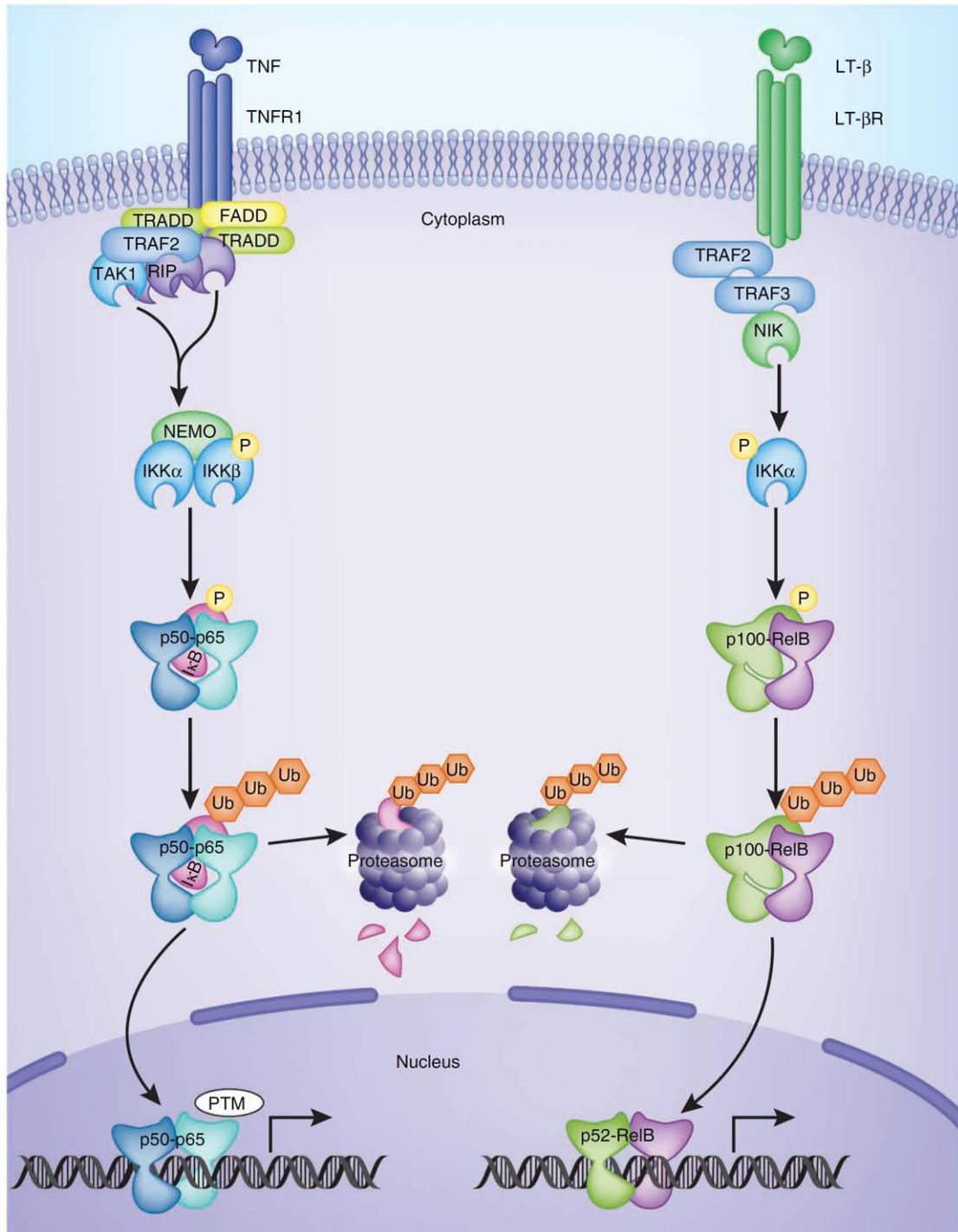


Figure 7. Canonical and noncanonical pathways of NF-κB activation

Stimulus-induced degradation of IκB proteins is initiated through phosphorylation by IKK complex, IKKα and IKKβ, and the regulatory subunit NEMO. Phosphorylated IκB proteins are targeted for ubiquitination and proteasomal degradation, which thus releases the bound NF-κB dimers so they can translocate to the nucleus. NF-κB signaling is often divided into two types of pathways. The canonical pathway (left) is induced by most physiological NF-κB stimuli and is represented here by TNFR1

signaling. In contrast, the noncanonical pathway (right), induced by certain TNF family cytokines, such as *LT-β*. Abbreviations: *IκB*, Inhibitor of κB ; *IKK*, *IκB* kinase; *NEMO*, *NF-kappa-B* essential modulator, *IKKγ*; *TNFR*, ; *TRADD*, Tumor necrosis factor receptor type 1-associated DEATH domain protein; *FADD*, Fas-Associated protein with Death Domain; *TRAF*, TNF receptor associated factors; *LT-β*, lymphotoxin- β ; *LT-βR*, receptor for lymphotoxin- β ; *NIK*, *NF-kappa-B*-inducing kinase. (Oeckinghaus et al., 2011).

4.2 MAPK pathways

In addition to *NF-κB*, mechanical and inflammatory stimuli activate the MAPK pathways through extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase or SAPK), and p38 kinase cascades in articular chondrocytes. The MAPKs are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation (Kim and Choi, 2010). MAPK signaling axis share the same model: a MAPK kinase kinase (MAP3K, aka MKK) phosphorylate and activate MAPK kinase (MAP2K, aka MEK), which in turn phosphorylate and activate MAPKs.

The JNK pathway consists of JNK, a MAP2K such as MAP2K4 or MAP2K7, and a MAP3K such as Apoptosis signal-regulating kinase 1 (ASK1), MAP3K1, mixed-lineage kinase (MLK), or transforming growth factor- β -activated kinase 1 (TAK1) (Davis, 2000). The p38 signaling pathway contains distinct MAP2Ks such as MAP2K3 and MAP2K6, the same MAP3Ks (such as ASK1 and TAK1) that function in the JNK pathway. In the ERK signaling pathway, ERK1 or ERK2 (ERK1/2) is activated by MEK1/2, which in turn is activated by a Raf isoform such as A-Raf, B-Raf, or C-Raf (Kim and Choi, 2010). RAF proteins are part of a conserved signaling module that passes signals from the cell surface to the nucleus. This module consists of a RAS family of small G proteins, which initiates RAF activation. This process leads to the activation of MEK1/2 and subsequently ERK1/2. It is proposed that B-Raf is the main isoform that couples RAS to MEK, then deliver signal from C-Raf and A-Raf to ERK

to fine-tune cellular responses (Wellbrock et al., 2004).

The activation of the MAPK cascades coordinates the activation of transcription factors, such as activator protein 1 (AP-1), E26 transformation-specific (ETS), and CCAAT-enhancer-binding proteins (C/EBP) families that regulate the expression of genes involved in catabolic and inflammatory events. JNK-driven AP-1 activation, MEK/ERK-induced phosphorylation of ETS factors, and p38-mediated activation of C/EBP β and RUNX2 participate in the induction of MMPs, including MMP-13 (Liu et al., 2010; Nishitani et al., 2010). The role of the ERK pathway has been studied in experimental OA. PD198306, the inhibitor of MKK/MEK1/2, was tested in a rabbit OA model, the highest dosage of this inhibitor demonstrated a significant protective effect by decreasing the cartilage macroscopic lesions and MMP1 level. In human chondrocytes, the activation of ERK1/2 is also involved in the increase of MMP13 expression stimulated by IL-1 (Santoro et al., 2015).

The activation of ERK seems to play an essential role in altering the chondrocyte phenotype. In culture, chondrocytes COL2A1 mRNA expression and collagen type II protein decrease with increasing passaging, accompanied with a remarkably increase of phospho-ERK2. In the presence of ERK inhibitor, COL2A1 expression was significantly increased at both P0 and P2 human chondrocytes (Ma et al., 2013). These results are in agreement with studies on primary bovine articular chondrocytes, in which ERK-pathway was found to be a negative regulator in staurosporine-induced re-differentiation (Rottmar et al., 2014).

4.3 TGF- β /BMP mediated signaling pathways

Transforming growth factor- β (TGF- β) proteins include two ligand subfamilies, defined by sequence similarity and the SMAD proteins they activate the the TGF- β /activin/nodal subfamily and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/Muellerian inhibiting substance (MIS) subfamily. In its classical pathways, the receptor complex propagates the signal through phosphorylation of receptor-regulated SMAD proteins (R-SMAD). The activated receptor complex associates with SAMD4 (Co-SMAD) in the cytoplasm when the R-SMADs are

phosphorylated. Pairs of activated R-SMADs and Co-SMAD form functional trimeric units that translocate into the nucleus, then bind other transcription factors to assemble distinct transcriptional complexes and thus activate distinct subsets of genes (Zhai et al., 2015).

TGF- β /BMP mediated signal is generally considered to be a protective factor for cartilage. In cultured human osteoarthritic cartilage, TGF- β 2 suppresses collagen resorption, glycosaminoglycan degradation and chondrocyte differentiation, supporting the protective role (Tchetina et al., 2006). However, recent study in human chondrocytes shows, TGF- β /SMAD3 decreases activity of miR-140 which downregulates the expression of genes such as MMP13 and ADAMTS5 (Tardif et al., 2013). So, the role of TGF- β /BMP signaling seems to be controversial in OA.

5. Syndecan4 signaling in chondrocytes

As mentioned above, syndecans exist on the membrane of the chondrocytes, it is a family of type I transmembrane heparin sulphate proteoglycans (HSPGs). The core protein of these single-pass transmembrane proteoglycans comprises two conserved membrane-proximal and carboxy-terminal regions (referred to as C1 and C2 respectively) that flank a small variable domain, a transmembrane region and an extracellular domain. The extracellular, N-terminal part of the core protein harbours a variable number of serine residues to which GAG chains of the heparan sulphate type are attached. One of the features of the C2 domains is a carboxy-terminal PDZ (Postsynaptic density 95, Disk large, Zona occludens-1)2-binding motif. Of all the four syndecans found in human tissues, syndecan4 is the only syndecan ubiquitously expressed, and it is distinct in having a positively charged lysine-rich motif. It has become evident that syndecan4 mediates numerous cellular processes through signaling pathways that affect cellular proliferation, migration, mechanotransduction and endocytosis (Elfenbein and Simons, 2013).

The extracellular region of syndecan4 binds with heparin-binding growth factors and cell adhesion molecules, that is indispensable for cells to interact with their

environments. At the cell membrane, the syndecan4 establishes an extracellular-intracellular connection through the recruitment of PKC α to sites where focal adhesions form and by mediating its subsequent activation (Lim et al., 2003). Diverse intracellular binding partners of syndecan4 facilitate its ability to initiate several parallel signaling pathways through the PDZ-binding domain. So via these three domains, syndecan4 independently control a myriad of extracellular and intracellular signaling processes (Elfenbein and Simons, 2013).

Although syndecan4 is not abundant in normal articular cartilage, its expression is significantly upregulated in both human and animal degenerated cartilage (Barre et al., 2000; Echtermeyer et al., 2009), it can be markedly induced by the proinflammatory cytokines (Wang et al., 2011). In the superficial zone of human osteoarthritic cartilage, the expression of syndecan4 reveal a correlation to the Mankin score and collagen type X expression. Study in knock-out mice indicated that, Syndecan4 regulates the activation of ADAMTS-5 through the phosphorylation of ERK1/2 and the expression of MMP-3 (Echtermeyer et al., 2009). However considering the indistinct role of ADAMT-5 in human cartilage, the function of syndecan4 in human chondrocytes is still misty.

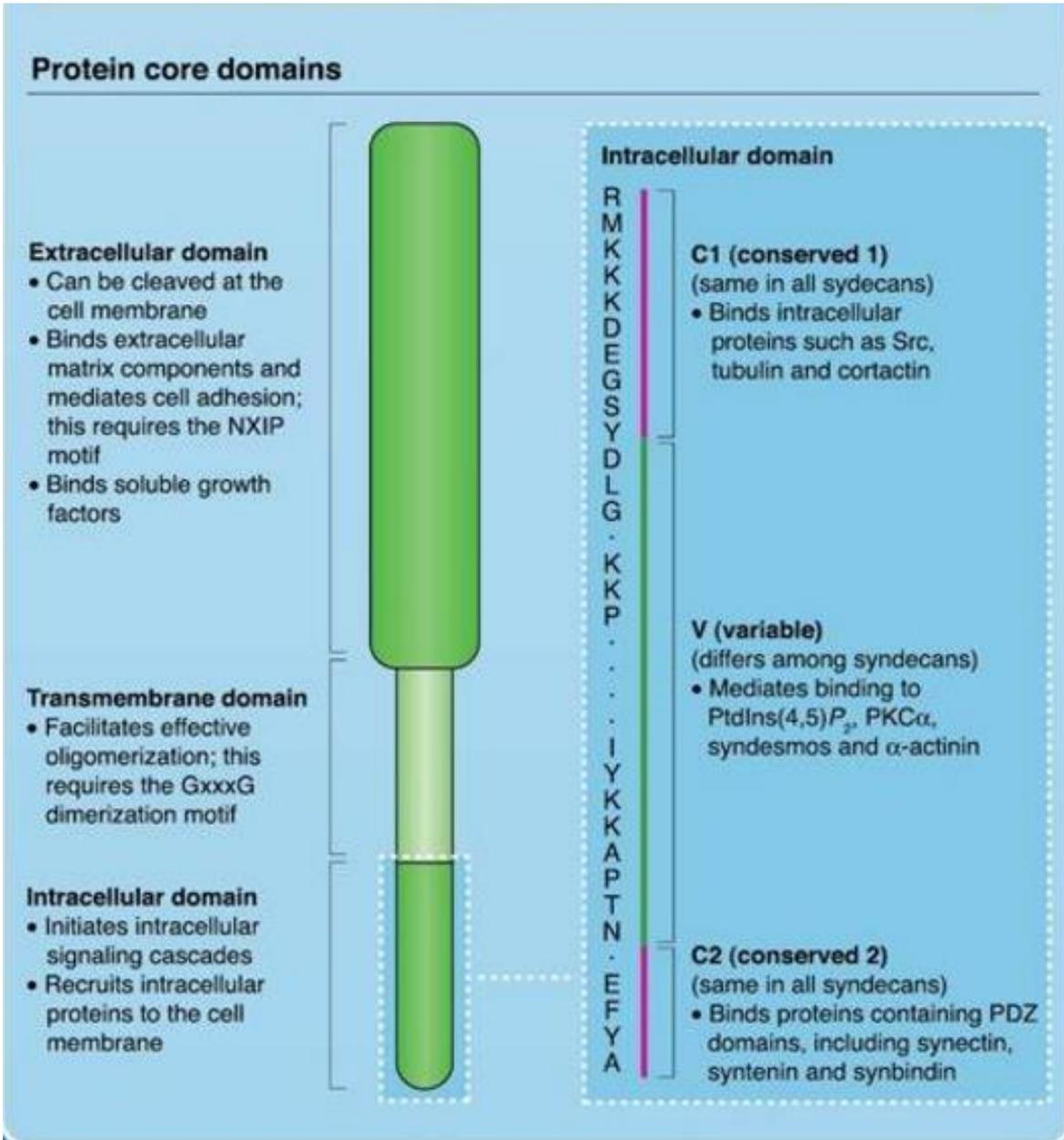


Figure 8. Core protein of syndecan4

The core protein of syndecan4 comprises three domains: extracellular domain, transmembrane domain and intracellular domain. The N-terminal part of the core protein (extracellular domain) harbours serine residues to which heparan sulphate GAG chains are attached. The intracellular domain is composed of one variable (V) region flanked by two conserved (C1 and C2) regions. (Elfenbein and Simons, 2013)

II. Wnt signaling pathway

The Wnt proteins are a family of 19 secreted cysteine-rich glycoproteins that are 350-400 amino acids in length. In 1976, the gene Wnt was first identified in *Drosophila*, and was named with its mutant phenotype – *Wingless* (Wg), absence of wing and haltere (Sharma and Chopra, 1976). In 1984, a mouse gene sequence named *int* was revealed to cause malignant transformation of mammary tissues (Nusse et al., 1984). Subsequently, the *Drosophila* and mouse genes were found to be homologs, so the gene was named as Wnt by combining these two names. Wnt proteins share features like a signal sequence for secretion, several highly charged amino-acid residues, and many potential glycosylation sites. This family also displays a characteristic distribution of 22 cysteine residues. The highly conserved spacing of these cysteines suggests that proper protein folding might require the formation of multiple intramolecular disulfide bonds (Mason et al., 1992).

A. Modification, secretion and transport of Wnt

Molecules that are secreted from cells are often posttranslationally modified. The primary amino-acid sequence of Wnts suggests that they should be quite soluble, the lipid modification by the attachment of a palmitate moiety on the first conserved cysteine residue makes them hydrophobic. That's also the reason why it's difficult to purify Wnt proteins. In fact, the lipid modification of Wnts potentially serves many purposes: targeting of Wnts to particular domains of the membrane, such as lipid rafts, detergent resistant fractions comprised of sphingolipids and cholesterol that have been implicated as platforms for signal transduction and cell activation (Simons and Toomre, 2000); serving to anchor the proteins to the endoplasmic reticulum membrane in close proximity of the oligosaccharyl transferase complex (Kadowaki et al., 1996; Tanaka et al., 2002; Willert et al., 2003; Zhai et al., 2004); even facilitating ligand reception on Wnt-responding cells (Cong et al., 2004). Wnt3a, for example, is modified with palmitoleic acid at Ser209, which is essential for its secretion (Kumar et al., 2014), and palmitate at Cys77, which is involved in its activities (Komekado et al., 2007).

Inhibitor of N-linked glycosylation by tunicamycin revealed that Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a and Wnt7b were glycosylated (Smolich et al., 1993). The mutations of the asparagine residues to glutamine showed that N-glycosylation plays an important role in the secretion but not signaling capabilities of Wnt3a and Wnt5a (Komekado et al., 2007; Kurayoshi et al., 2007). Wnts are cotranslationally imported into the Endoplasmatic Reticulum (ER) lumen where they are modified by Porcupine (Porcn), a conserved membrane bound O-acyltransferase (MBOAT) that can mediate the palmitoylation of Wnts (van den Heuvel et al., 1993; Tanaka et al., 2000; Zhai et al., 2004). Many studies have demonstrated that posttranslational fatty acyl modification was central to secretion of Wnts and their recognition by receptors (Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006).

Secretion of Wnt proteins depends on a dedicated multipass transmembrane protein Wntless (Wls)/Evenness interrupted (Evi)/Mom-3 (Evi/Wls), which shuttles all Wnts from the Golgi to the plasma-membrane (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), with the exception of WntD in *Drosophila* (Ching et al., 2008). At the meantime, a *retromer* complex which comprises a sorting nexin (snx) dimer that mediates membrane attachment and a cargo recognition trimer composed of Vps35, Vps29, and Vps26 is involved in retrograde transport from the endosomes to the trans-Golgi network and regulate the Wnt secretion (Bonifacino and Hurley, 2008; Coudreuse et al., 2006). Beside, this spreading of Wnts throughout the extracellular space is facilitated by HSPGs (You et al., 2011). Sulfatase 1 (Sulf1), a *Drosophila* homolog of vertebrate heparin sulfate 6-O endosulfatase, negatively modulates the Wg gradient by influencing both Wg stability and distribution (Kleinschmit et al., 2010). In accordance, the osteogenic activity of Wnt3a is cooperatively stimulated through physical interactions with exogenous heparin (Ling et al., 2010). Actually, the Wnt stability, diffusion and gradient formation outside cells is regulated in several ways: modification of Wnts structure, interaction with HSPGs and extracellular cleavage of Wnts. These context-specific cofactors can shape the Wnt/Wg trafficking, fine-tuning its range and robustness during development (Gross and Boutros, 2013).

B. Wnt signaling transduction

Wnt proteins can couple to more than 15 different receptors and co-receptors and thereby activate different downstream pathways. These receptors and co-receptors include members of the Frizzled proteins (Fz), low-density lipoprotein receptor-related protein 5/6 (LRP5/6), receptor Tyr kinase-like orphan receptor (ROR), protein Tyr kinase 7 (PTK7), receptor Tyr kinase (RYK), muscle skeletal receptor Tyr kinase (MUSK) and proteoglycan families including syndecan4.

On the basis of early studies, Wnt pathways have been classified as either canonical (β -catenin-dependent) or non-canonical (β -catenin-independent) signaling pathways. However, this classification can only serve as a rough guide, as within both Wnt signaling cascades especially non-canonical pathway various sub-branches are observed in different cellular contexts. Actually, the activity of Wnts depends on the cellular context and the receptors, thus Wnts cannot be rigorously subdivided according to the pathway they could induce. Although, it was a useful approximation that, for example, Wnt1, Wnt-3a and Wnt-8 are more commonly encountered in canonical signaling, and Wnt-5a and Wnt-11 are predominantly involved in non-canonical signaling (Niehrs, 2012), nowadays, Wnt-3a is proved to activate both canonical and no-canonical pathways (Nalesso et al., 2011), and Wnt-5a is confirmed to induce the no-canonical signaling and regulate the canonical signaling at the same time (Mikels and Nusse, 2006).

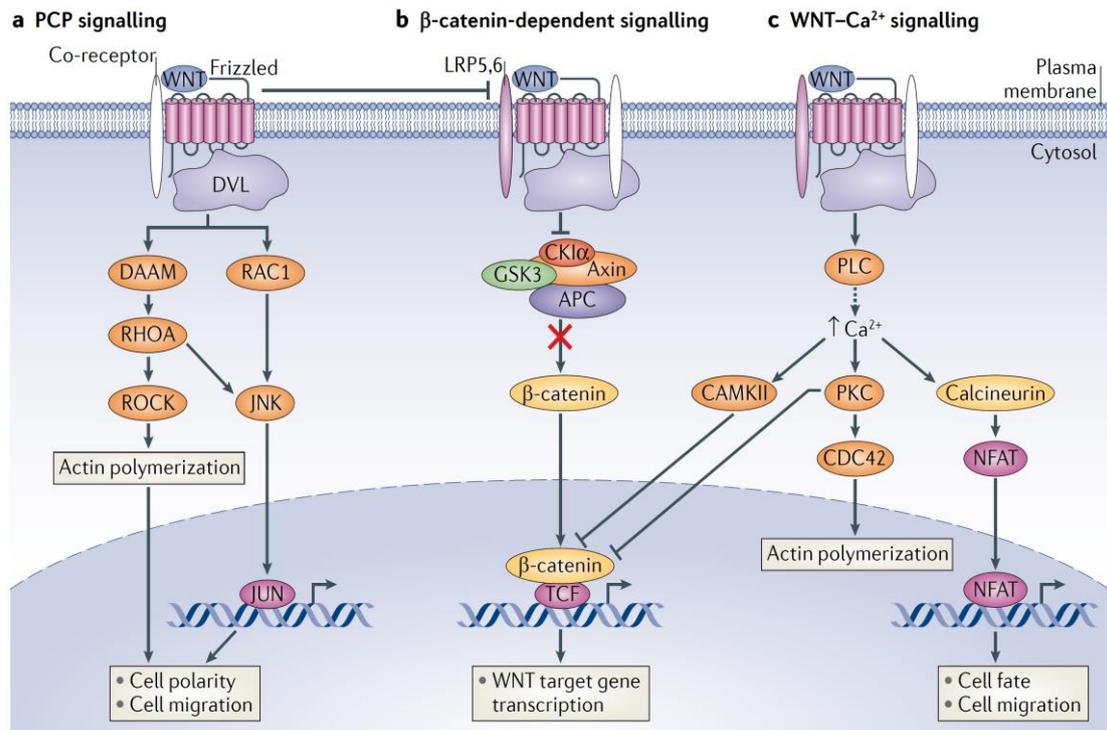


Figure 9. Simplified schemes showing the main Wnt pathways directed by specific Wnt, Frizzled and Wnt co-receptor interactions

a. PCP signalling induces the activation of RHOA and RAC1, which then activate ROCK and JNK, respectively, leading to actin polymerization and microtubule stabilization. PCP pathway regulates the cell polarity and migration. b. Under steady-state conditions, GSK3 triggers the degradation of β -catenin. But in the presence of Wnt, the destruction complex, composing GSK3, CK1 α , and APC, is inactivated. This allows β -catenin to accumulate and translocate into the nucleus, then to induce the transcription of target genes beneath the control of TCF. c. Wnt-Ca²⁺ pathway is mediated by CAMKII, PKC and calcineurin. This pathway regulates the cell fate and migration. Abbreviations: PCP, Planar cell polarity; DVL, Dishevelled; DAAM1, DVL-associated activator of morphogenesis 1; RAC1, Ras-related C3 botulinum toxin substrate 1; RHOA, Ras homolog gene family, member A; ROCK, RHO kinase; JNK, JUN-N-terminal kinase; GSK3, glycogen synthase kinase 3; CK1 α , casein kinase 1 α ; APC, adenomatous polyposis coli; TCF, T cell factor; PLC, phospholipase C; CAMKII, Ca²⁺ and calmodulin-dependent kinase II; PKC, protein kinase C; CDC42, Cell division control protein 42 homolog; NFAT, nuclear factor of activated T cells. (Niehrs, 2012)

1. Canonical pathway

The canonical pathway is the best-characterized pathway and is triggered by the interaction of Wnt with Frizzled and LRP5 or LRP6. The receptors transduce the signal

across the plasma membrane that results in the activation of the Dishevelled (DVL) protein. The activated DVL inhibits a “destruction complex” which composed of adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β) and AXIN, that targets β -catenin for ubiquitylation (Ub) and proteasomal degradation in unstimulated cells. Upon Wnt activation, cytoplasmic β -catenin is stabilized and enters the nucleus, where it associates with transcription factors, notably TCF (T cell factor) and LEF (lymphoid enhancer-binding factor), to regulate the transcription of target genes. This pathway have crucial roles in the regulation of diverse cell behaviors, including cell fate, proliferation, survival, differentiation, migration and polarity.

2. Noncanonical pathway

The noncanonical Wnt signal encompasses those pathways that do not use the β -catenin-TCF or β -catenin-LEF module but instead use other modes of downstream signaling to elicit a transcriptional response. These β -catenin-independent pathways imply different receptors, co-receptors and downstream effectors. For instant, there is no common rule to denominate the different no-canonical pathways, Wnt-CPC (planar cell polarity) signal pathway is named by function, while Wnt-Ca²⁺ pathway is named by downstream effector.

2.1 Wnt-PCP pathway

The best-characterized β -catenin-independent pathway is the PCP pathway, which was originally indentified in *Drosophila*. In most cases Frizzled and DVL are involved. PCP signal does not require LRP5 or LRP6 but instead uses ROR and PTK7 as co-receptors. In this pathway, three cascades are activated: the small GTPases RAC1 (Ras-related C3 botulinum toxin substrate 1), RHOA (Ras homolog gene family, member A) and JUN-N-terminal kinase (JNK). The PCP pathway is prominently involved in regulating cell polarity in morphogenetic processes. For example, in vertebrates PCP signalling regulates cell movement during gastrulation, neural tube closure and the orientation of stereocilia in the inner ear (Simons and Mlodzik, 2008).

2.2 Wnt-Ca²⁺ pathway

The other β -catenin-independent pathway that has been described is the Wnt-Ca²⁺ pathway, which was initially identified in zebrafish embryos. Ectopic expression of Xwnt-5a enhances the frequency of intracellular Ca²⁺ transients in the enveloping layer of the blastodisc (Slusarski et al., 1997a). In this case, Wnts trigger Frizzled-mediated activation of heterotrimeric G proteins. This activates phospholipase C (PLC), which in turn generate inositol-1,4,5-trisphosphate (IP3) production (Slusarski et al., 1997b). IP3 triggers Ca²⁺ release from intracellular stores leading to activation of effectors such as Ca²⁺- and calmodulin-dependent kinase II (CAMKII), calcineurin and protein kinase C (PKC), which activate the transcriptional regulator nuclear factor associated with T cells (NFAT). This pathway is involved in cancer, inflammation and neurodegeneration (De, 2011). Recently, this pathway was found also to be essential in chondrocyte differentiation (Nalesso et al., 2011).

C. Critical component of Wnt signaling

1. Frizzled proteins

Frizzled proteins, the first and best identified Wnt receptors, comprise 10 members in humans. As common receptors for both the β -catenin-dependent and β -catenin-independent pathways, these seven-pass transmembrane receptor Fzs are the principal Wnt receptors and contain a large extracellular Cys-rich domain (CRD), which mediates Wnt binding. The 3.25 Å structure of *Xenopus* Wnt-8 (XWnt-8) in complex with mouse Frizzled-8 CRD was reported by Janda and his colleagues (Janda et al., 2012). XWnt-8 grasp the Fz8-CRD at two opposing sites using extended thumb, comprising an N-terminal α -helical domain (NTD) and index fingers, comprising a C-terminal cysteine-rich region (CTD), projecting from a central “palm” domain, to contact “site 1” and “site 2,” respectively. The lipid of XWnt8 dominates the contact interface, burying approximately 580Å² of total surface area (330Å² from the lipid, 250Å² from the CRD), contacting 11 Fz-8 residues, and completely traversing the cleft on the Fz-8-CRD surface, which revealed partly the importance of lipidation of Wnt.

Furthermore, the site1/site2, Wnt/Fz interaction chemistry is incompatible with mono-specificity and therefore a binary, or highly-restricted ligand-receptor matching code probably do not exist.

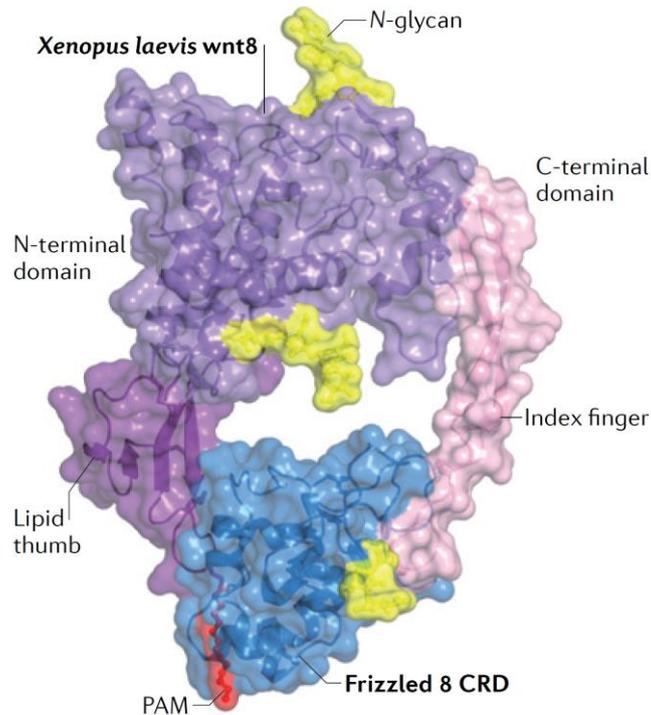


Figure 10. **Crystal structure of the Wnt-8-Frizzled-8 CRD complex**

Overall structure of *Xenopus laevis* Wnt-8 bound to the Frizzled 8 CRD shown in 'face on' representation. *X. laevis* Wnt-8 core is coloured in purple, the amino-terminal lipid thumb domain in deep purple, the carboxy-terminal index finger in light pink and PAM in red. The Frizzled 8 CRD is shown in blue and N-glycans in yellow. Abbreviations: CRD, Cys-rich domain; PAM, palmitoleic acid. (Janda et al., 2012)

2. DVLs

Like other important elements, Disheveled (Dsh), the hub of Wnt signaling was also originally identified in *Drosophila*. Three Dsh homologues, DVL1, 2, and 3 have been found in humans and mice, and the proteins consist of about 750 amino acids and display high sequence homology (Yang et al., 1996). DVL proteins possess three conserved domains, an amino-terminal DIX domain which also exist in Axin, a central

PDZ domain and a carboxyl-terminal DEP domain (Boutros and Mlodzik, 1999). In addition, another 2 conserved regions, the basic region and the proline-rich region, are also implicated in mediating protein-protein interaction and/or phosphorylation. These three homologues are widely expressed in embryonic and adult tissues, and they are involved in both Wnt canonical and non-canonical pathway. Despite that animal models revealed a high level of functional redundancy among DVLs, the phenotypes of single DVL mutants demonstrated unique functions of either of the DVL genes. DVL-1^{-/-} mice displayed social abnormalities and sensorimotor gating defects, DVL-2^{-/-} mice displayed skeletal defects, while DVL-3^{-/-} mice which all died at birth due to conotruncal heart defects was the only DVL mutant had cochlear defects (Wynshaw-Boris, 2012).

3. Wnt inhibitors: DKK, SFRPs and WIFs.

A variety of proteins, such as dickkopf (DKK), secreted frizzled-related proteins (SFRPs) and Wnt inhibitory factors (WIFs) also regulate the Wnt pathway.

DKKs is a family of evolutionarily conserved secreted glycoproteins, their first member DKK1 is initially discovered in 1988 as an embryonic head inducer and Wnt signaling antagonist in *Xenopus* (Glinka et al., 1998). Four main members of DKK (1-4) exist in vertebrates, they consist of 255-350 amino acids and share two conserved cysteine-rich domains (CRDs). DKKs specifically inhibit the canonical Wnt pathway. DKK1 bind to LRP 5/6 with high affinity then prevent the Wnt–LRP6 interaction and disrupt the activation of Wnt β -catenin pathway (Semënov et al., 2001).

The SFRPs resemble the ligand-binding CRD domain of the Frizzled receptors. This family comprise five members, SFRP1-5, with SFRP3 being the ortholog of Frzb (for Frizzled motif associated with bone development) which is first purified from bovine cartilage (Yasuhara et al., 2011). SFRPs bind to the Wnt proteins then sequester them away from active receptor complexes to suppress Wnt signaling (Lin et al., 1997; Wang et al., 1997). Thus, unlike DKKs, which specifically inhibit Wnt/ β -catenin signaling, SFRPs which bind many Wnt proteins can also inhibit noncanonical signaling (Li et al.,

2008; Matsuyama et al., 2009; Sugiyama et al., 2010).

WIF-1 is first described as an expressed sequence tag from the human retina and highly conserved orthologues exist in mouse, *Xenopus* and zebrafish (Hsieh et al., 1999). This protein consists of 379 amino acids with a unique and highly conserved WIF domain, five EGF (Epidermal growth factor)-like repeats, and a hydrophilic tail. Similar to SFRPs, WIF-1 prevents Wnt from binding to its receptors, then affecting both canonical and noncanonical pathways (Surmann-Schmitt et al., 2009).

D. Wnt signaling and cartilage development

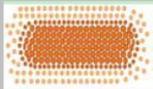
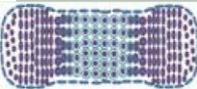
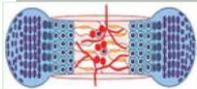
Many Wnt proteins and regulators are found to be expressed in the limb development. Wnt-3 and Wnt-6 are expressed in limb ectoderm, Wnt-4, Wnt-9a and Wnt-14 are expressed in cells of the joint region, Wnt-5a is expressed in the perichondrium, Wnt-5b is expressed in a subpopulation of prehypertrophic chondrocytes and in the outermost cell layer of the perichondrium, Wnt-7a shows in the early limb bud in dorsal ectoderm, while Wnt-7b is expressed in both dorsal and ventral ectoderm, and Wnt-11 is expressed in the perichondrium (Loganathan et al., 2005; Witte et al., 2009). Expression patterns of Wnt signaling antagonists such as Frzb and DKKs have been also found in the animal developing limb cartilage.

Due to the short range of their effect, Wnt signaling has distinct regulatory roles defined by the spatial differentiation of the cells. At the early phase of limb growth, Wnt signaling is active to keep the cells at the apical ectodermal ridge (AER) in a proliferative and undifferentiated state. Cells distant from the AER source of Wnt are subject to less Wnt signaling. As canonical Wnt signalling inhibits the expression of chondrogenic master regulator Sox9, these distal cells condense and commit to chondrogenic differentiation (Leucht et al., 2008).

In early skeletal progenitor cells, the Wnt pathway decides between osteogenic or chondrogenic differentiation. Overexpression of Wnt-1, Wnt-4, Wnt-7a or Wnt-8a inhibits the chondrogenesis of mesenchymal cells, in contrast overexpression of Wnt-5a, 5b, or Frzb stimulate it (Church et al., 2002; Enomoto-Iwamoto et al., 2002;

Hartmann and Tabin, 2000; Rudnicki and Brown, 1997). Wnt-14 (also known as Wnt-9a) is implicated as being a player in joint induction, it is crucial for shaping the joint surface by directing bi-potential chondro-synovioprogenitors to become synovial connective tissue, as opposed to chondrocytes, through actively suppressing their chondrogenic potential (Später et al., 2006). In general, the activation of canonical Wnt cascades inhibits the early stages of chondrogenesis, while the activation of noncanonical Wnt signaling promote early chondrogenesis. Overexpression of Frzb and Wnt-5a inhibit chondrocyte hypertrophy, matrix mineralization, and endochondral ossification (Enomoto-Iwamoto et al., 2002; Hartmann and Tabin, 2000). This indicated that the canonical and noncanonical pathways of Wnt have opposite effect on chondrocyte hypertrophy. Knock-out of β -catenin expression in mice strongly impairs long bone formation with inhibition of chondrocyte hypertrophy and osteoblast differentiation (Day et al., 2005; Hill et al., 2005). The conditional double knockout mice for LRP5 and LRP6 exhibited similar phenotype to that of β -catenin conditional mutant mice (Joeng et al., 2011), suggesting that skeletal precursor cells and chondrocytes redundantly utilize LRP5/6 as a co-receptor of Wnts mediating Wnt/ β -catenin signaling. As a major expressed Wnt protein in developing cartilage (Witte et al., 2009), Wnt-5a is required for longitudinal skeletal outgrowth, is crucial for cartilage and growth plate development, and both Wnt-5a and Wnt-5b regulate the transition between different chondrocyte zones (Yang et al., 2003). Wnt-5a regulates the PCP pathway which originally refers to the polarity of epithelial cells in a plane during development and organization (Devenport, 2014). Thus, limb elongation, longitudinal growth of long bones, and columnar structuring of growth plate chondrocytes may be the targets of the PCP pathway. Interaction of Wnt-5a with ROR2 and Vangl2 in limb development indicates that Wnt-5a regulate limb elongation and chondrocyte arrangement through this interaction and activation of PCP pathway (Gao et al., 2011). In brief, Wnts supports skeletal development via both canonical and non-canonical Wnt signaling pathways

Table 1 Role of Wnts and Wnt-related molecules in cartilage development

Developmental events	Role in cartilage development	
	Stimulation or requirement	Inhibition
 <p>Chondrogenesis</p>	Wnt-5a, Wnt-5b, and Frzb	Wnt-1, Wnt-4, Wnt-8a, Wnt9a, Wnt-11, and Wnt/β-catenin
 <p>Growth plate assembly columnar formation</p>	Wls, Wnt-5a, and ROR2	Exces Wnt/β-catenin
 <p>Hypertrophy mineralization</p>	Wnt-9a, Wnt-4, Wnt/β-catenin, and LRP5/6	Frzb, Wnt-5a, and ICAT
 <p>Perichondrial bone formation</p>	Wnt/β-catenin	

A number of Wnts and Wnt-related molecules have been implicated in the regulation of cartilage development. The regulation of Wnt signaling is not only dependent on the presence of ligands but also on the action of endogenous antagonists of Wnt signaling. (Usami et al., 2016)

E. Wnt signaling and Osteoarthritis

Genetic risk variants are considered to be one of the risk factors of OA. An association between loci 2q31.1 and hip OA was firstly reported by Loughlin et al (Loughlin et al., 2000), Substitutions of two highly conserved arginine residues (Arg200Trp and Arg324Gly) in *FRZB* was found to be a strong risk factor for primary hip OA (Loughlin et al., 2004). *FRZB* encodes sFRP3 which is a soluble antagonist of Wnt signaling, and the Arg324Gly substitution diminishes sFRP3's ability to antagonize Wnt signaling in vitro. Many studies confirmed this result in different populations (Lane et al., 2006; Min et al., 2005; Valdes et al., 2007), however a large-scale meta-analysis of individual-level data fails to support the notion that these 2 polymorphisms in *FRZB*, rs7775 (Arg324Gly) or rs288326 (Arg200Trp), has any sizable genetic effect on hand, knee or

hip OA phenotypes (Evangelou et al., 2009). More studies would be required to get the final conclusion since this analysis cannot disregard the potential association of rs288326 with hip OA in women. The other gene DOT1-like histone H3 methyltransferase (*DOT1L*) is found to be involved in OA in a genome-wide association studies, Castaño Betancourt et al (Castaño Betancourt et al., 2012) reveal a correlation between radiographic joint-space widths and the *DOT1L* rs12982744 polymorphism. Study in the Chinese Han population also shows the same correlation (Zhou et al., 2014). *DOT1L* gene is expressed in the articular chondrocytes (Castaño Betancourt et al., 2012), and is an essential activator of Wnt/ β -catenin pathway in animal model (Mahmoudi et al., 2010). The role of *DOT1L* in cartilage hemostasis still needs to be clarified in human cells.

The Wnt family gene expression analyses performed in articular cartilage and synovial tissues in knee joints taken from rheumatoid arthritis, OA, and normal patients showed that, the expression of Wnt-3a, Wnt-5b, Wnt-9b, Wnt-10b, Wnt-11 decrease in OA samples, while Wnt-7b is significantly up-regulated (Nakamura et al., 2005). A systematic analysis of the overexpression of Wnt signaling members revealed a down-regulation of *FRZB*, up-regulation of Wnt-16 and Wnt target genes (such as *c-MYC*, *CYCLIN D1*, and *AXIN2*), and nuclear localization of β -catenin in injured cartilage (Dell'Accio et al., 2008). Likewise, in human OA cartilage, higher expressed Wnt-1-inducible-signaling pathway protein 1 (WISP-1) (Blom et al., 2009) and more translocation of β -catenin into nuclear of the chondrocytes (Zhu et al., 2009) are detected. Alteration of Wnt family members exists also in the serum and synovium samples. Higher serum levels of DKK-1 are found to be associated with diminished risk of radiographic hip OA progression, while higher serum sFRP-3 levels at baseline are less likely to develop hip OA (Lane et al., 2007). Both plasma and synovial fluid DKK-1 levels are proved to be inversely correlated with radiographic severity in knee OA patients (Honsawek et al., 2010). Additionally, in human OA synovium, FRP1, FRP2, and FRP4 are frequently detected (Imai et al., 2006). These studies demonstrated that the Wnt signaling is alerted in the OA joint.

In animal models, Wnt/ β -catenin signalling activation induces the cartilage degeneration. The β -catenin level have been reported to be higher in chondrocytes within areas of degenerative cartilage (Hwang et al., 2005a; Kim et al., 2002). Through the activation of canonical pathway, Wnt-3a can induce the expression of catabolic genes (such as MMP-3, MMP-13, ADAMTS4 and ADAMTS5) in chondrocytes , leading to matrix degeneration (Yuasa et al., 2008). The Wnt related molecules also promote the cartilage degeneration. Overexpression of WISP1 is associated with increased aggrecanase and MMPs activities and expressions, resulting in increased cartilage damage in murine knee joints (Blom et al., 2009). In rat model, activation of Wnt/ β -catenin pathway by inter-articular injection of an inhibitor of GSK3 β induces the appearance of OA-like features. Moreover, when Frzb gene expression is genetically abrogated in mice, the instability, enzymatic injury, or inflammation can cause more cartilage loss (Lories et al., 2007). However, confusing results also exist. The loss-of function of β -catenin increases apoptosis and contributes significantly to the articular cartilage destruction in transgenic mice (Zhu et al., 2008). Knock out of DKK1 in a rat model and overexpression of DKK1 in a mouse model both protect the cartilage from OA destruction. These studies suggest that Wnt/ β -catenin signalling not only activates cartilage matrix catabolism causing cartilage destruction under pathological conditions, but also participates in cartilage homeostasis by supporting the chondrocytes survive. In addition, caution need to be applied when animal and human are compared, as effects of Wnt proteins in human cells can be different from those seen in animal model. Ma et al demonstrate that activation of β -catenin by Wnt-3a or inhibition of GSK3 β inhibits the MMPs expression in human cells or cartilage explants, whereas opposite effect occur in similarly stimulated mouse or bovine chondrocytes (Ma et al., 2012).

As describe in the last section, Wnt signaling has a role in the terminal differentiation of chondrocytes, in fact, this is a deleterious outcome in the context of OA. In rabbit chondrocytes, Wnt-3a induces the expression of c-Jun and its phosphorylation by c-Jun N-terminal kinase (JNK), then causes the activation of AP-1 which can suppress the

expression of Sox-9, a major transcription factor regulating type II collagen expression (Hwang et al., 2005b). Furthermore, Nalesso et al. (2011) showed that the differentiation of human chondrocytes stimulated by Wnt-3a is mediated by the noncanonical pathway, in particular by CaMKII and calcium signaling, this signaling cascade and β -catenin pathway have reciprocal inhibitory effects (Nalesso et al., 2011). So both canonical and noncanonical Wnt pathway play a part in the OA development by affecting chondrocytes.

F. Wnt pathway as therapeutic target

Since Wnt was discovered by its oncogenesis, the interference of this complex signaling pathway was firstly performed in the treatment of cancer. Five classes of molecularly targeted agents were reported: β -catenin-TCF antagonists; drugs that bind the PDZ domain of DVL; other mechanism-based inhibitors that principally target enzymes (for example, Porcupine, tankyrase and kinase inhibitors); biologics; and drugs that target Wnt co-activators (Kahn, 2014). Despite of their good performance in the cell and animal studies, these agents targeting the Wnt signaling pathway in the clinic using are in their infancy. In a few years, after the termination of several clinical Phase I trials, we will know whether any of these therapeutic agents that specifically target the Wnt pathway will be efficient with an acceptable safety profile or not. Before that, the knowledge of the complexity of Wnt pathway and its role in both normal physiology and pathophysiology is essential to expand application of the agents, explain their side effects and even find new targeting point. Considering the participation of Wnt signaling in skeletal development and OA, the joint diseases will be another promising treating target.

AIM AND OBJECTIVE

AIM AND OBJECTIVE

OA disease is characterized by inflammation and cartilage degradation. This disease affects more than half of the world population over 65 years-old and causes huge financial burden. Due to the lack of self-healing capacity of articular cartilage, no curative therapy which can halt disease progress or reverse the cartilage damage is currently available. In recent years, numbers of studies are performed to comprehend the molecular mechanism of the OA initiation and development. The high expression of proteinase and aggrecanase, and dysfunction of chondrocytes are confirmed to be crucial in the cartilage destruction. Many cytokines initiate and promote this process and various signaling pathways are activated, such as NF κ B signaling pathway, MAPK signaling pathway, TGF/TMP signaling pathway and Wnt. Wnt signaling pathway is divided into canonical and noncanonical cascades. Firstly described as an oncogene, Wnt was demonstrated to be essential in the development, tissue homeostasis, hematopoiesis, cell migration, genetic stability and apoptosis. Apart from its role in limb development, Wnt signaling is revealed to be involved in the cartilage homeostasis and OA initiation. However, how Wnt signaling regulate the cartilage degeneration and chondrocytes differentiation is not well elucidated yet. So in this thesis we use Wnt-3a which can activate both β -catenin dependent and nondependent pathway to study Wnt's effect on human chondrocytes.

The aim of the first part is to investigate the effect of Wnt canonical pathway on cartilage degeneration. The objective is to determine whether Wnt induces the expression of cartilage degrading enzymes in the human cartilage and chondrocytes, explore which pathway is involved, and identify which components (Frizzled receptors and DVLs) mediate this signaling and how the signaling regulate the expression of these enzymes. On the other hand, the purpose is to determine whether Wnt synergizes or counteracts the effect of IL-1 β on cartilage degeneration.

The aim of the second part is to investigate effect of Wnt on chondrocyte phenotype.

The main objective is to determine which components (Frizzled receptors and DVLS) mediate the noncanonical pathway, and how this pathway inhibit the expression of *COL2A1*, and induces loss of chondrocyte phenotype.

MATERIAL AND METHOD

MATERIAL AND METHOD

I. Cell and cartilage explants isolation, culture and treatment

A. Chondrocytes isolation and culture

Human primary chondrocytes were isolated from cartilage obtained from patients who underwent joint replacement for knee OA. Cartilage tissue was dissected from preserved areas of the femoral condyles and patellar groove avoiding subchondral bone and fibrocartilage. Cartilage was washed twice with DMEM F12 complete medium (2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 10% (v/v) fetal bovine serum) and aliquot was then digested with protease (1 mg/ml) for 90 min at 37 °C . Following two times washes with the medium, an overnight digestion in collagenase B (1 mg/ml) in DMEM F12 complete medium on the low speed shaker at 37 °C was performed. The second day, cells were precipitated by a 5 min 300 × g spin. Then chondrocytes recovered from the digestion were seeded at a density of 25,000 cells/cm² in DMEM F12 complete medium at 37°C in a humidified atmosphere supplemented with 5% CO₂ (Figure 11). All experiments were performed using confluent P0 cells.

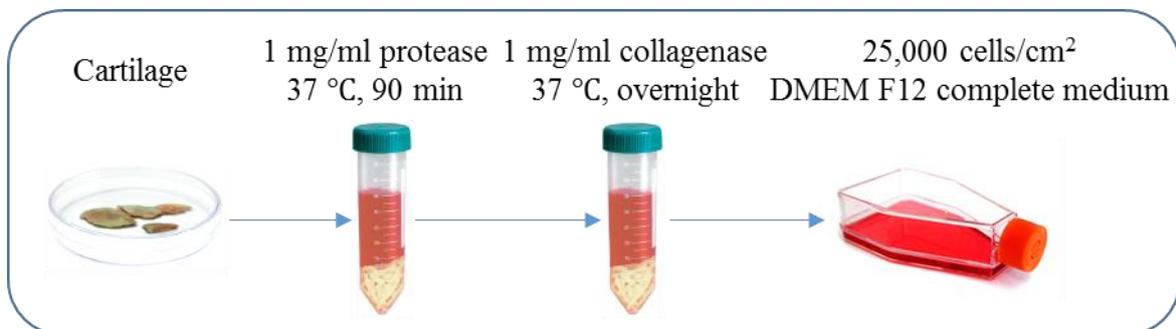


Figure 11. **Schematic diagram of chondrocytes isolation**

Cartilage isolated from preserved areas of the femoral condyles and patellar groove was digested by the protease and collagenase successively. Chondrocytes were spin

down and the pellet was suspended with DMEM F12 complete medium and seed at a density of 25,000 cells/cm² in 75 cm² flasks.

B. Marrow-derived mesenchymal stem cells isolation and culture

Bone marrow-derived mesenchymal stem cells (MSCs) were obtained from femur samples of patient undergoing total hip replacement surgery. After aspiration in femoral neck, and lysis of the red blood cells, human bone MSCs were cultured in a 75 cm² flask with proliferation medium (DMEM low glucose (1 g/L) supplemented with 10% FBS, 1% glutamine and 1% penicillin/ streptomycin) in a dense of 50, 000/ cm² until the third passage for the experiments.

C. Cartilage explant isolation and culture

For cartilage explant isolation, cartilage specimens were dissected within 1 h of operation, and full-thickness cartilage was removed from preserved areas of the femoral condyles using a 6 mm biopsy punch. Extreme care was taken to avoid subchondral bone, osteophytic cartilage and fibrocartilage. Explants were washed with DMEM F12 complete medium and cultured in 24-well plates in the same medium and condition as for chondrocytes. They were cultured for 48 h before use.

Aliquot of cartilage was used for histological scoring for features of OA as described previously (Mankin et al., 1971). Only cells and explants obtained from samples with a Mankin score < 4 were used for subsequent experiments. This study was approved by our local research institution (Commission de la Recherche Clinique; registration no. UF 9757, CPRC 2004, Cellules souches et chondrogénèse). The protocol conforms to the ethical guidelines of the Declaration of Helsinki, and written informed consent has been obtained from each patient.

D. Cell and cartilage explant treatments

Human primary chondrocytes or bone MSCs were seeded onto six-well plates at 5×10^5 cells/well and allowed to attach overnight in standard culture conditions. The cells were then treated for 12 h with 100 ng/ml of recombinant Wnt-3a or 10 ng/ml IL-1 β

(R&D Systems, Minneapolis, MN USA) or vehicle (0.1% BSA in PBS) in culture medium with 2% (v/v) fetal bovine serum prior to gene or protein expression analyses. When co-treatments with inhibitor was carried out, cells were pre-treated for 30 min with the inhibitor *ie.* U0126 (10 μ M; Cell signaling, Danvers), DKK1 (100 ng/ml; R&D Systems, Minneapolis, MN USA) KN93 and KN92 (10 μ M; R&D Systems, Minneapolis, MN USA) prior to treatment with 100 ng/ml of recombinant Wnt-3a or vehicle. When co-treatments of Wnt-3a and IL-1 β is performed, chondrocytes were pre-treated with 100 ng/ml of Wnt-3a for 12 h before addition of 10 ng/ml IL-1 β for another 12 h.

II. Gene expression analysis

A. Extraction of total RNA

Total RNA from cartilage explants was extracted using TRIzol (Lifetech, Carlsbad, CA) and purified with RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. After the treatment, cartilage explants were cut to small pieces, then 1 ml TRIzol was added for each sample, and homogenization was performed with a homogenizer (ULtra Turrax Disperseur DI 25). Homogenized cartilage samples were incubated for 15 min at room temperature, then centrifuged for 3 min, at 4 $^{\circ}$ C , at 12000 \times g, the supernatant was transferred into RNase-free tubes. 0.2 mL of chloroform per sample was added, then the tubes were shaken vigorously by hand for 15 sec and an incubation of 3 min at room temperature was performed. Following centrifugation for 15 min, 4 $^{\circ}$ C , 12000 \times g, and the aqueous phase was transferred to a fresh tube. This aqueous phase was incubated with 0.5ml of 100% isopropanol for 10 min, then a centrifugation (10 min, 4 $^{\circ}$ C , 12000 \times g) was performed and the supernatant was removed. The RNA pellet was then washed once by 75% ethanol and resuspended by RNase-free water. Finally the samples were purified with RNeasy kit.

Total RNA from cells was extracted by RNeasy kit directly. The RLT buffer was used to lyse the cells. The samples were then applied to the RNeasy Mini spin columns. After

washing with 700 ul RW1 buffer and 500 ul RPE buffer twice, the total RNA was eluted with the 30 ul RNase-free water. RNA concentration was measured by Nanodrop 1000 (Thermo Scientific, Waltham, MA). Ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA (A ratio of ~2.0 is generally accepted as pure for RNA).

B. The reverse transcription

The reverse transcription was performed using 500 ng (primary cells) or 200 ng (cartilage explants) of total RNA from each sample with SuperScript® VILO™ Master Mix (Lifetech, Carlsbad, CA). Indicated quantities of RNA and 4 µl of Master Mix were supplemented by DEPC-treated water until a final volume 20 µl. The mixed solution was gently mixed and incubate at 25 °C for 10 min, 42 °C for 60 min finally 85 °C at 5 min. The cDNA was stored at -20° C.

C. Relative quantitative real time PCR

Relative quantitative PCR was performed with iTaq™ Universal SYBER Green Supermix kit (BIO-RAD, Hercules, CA) using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Cycling parameters were 20 s at 95 °C; 40 cycles of 1 s at 95 °C, and 20 s at 60 °C . The melt curve was used to examine the specificity of PCR amplifications. Gene expression was determined in triplicate in three separate experiments and normalized with the housekeeping gene ribosomal protein S29. Analyses and fold differences were determined using the comparative CT method. Fold change was calculated from the $\Delta\Delta CT$ values with the formula $2^{-\Delta\Delta CT}$, and data are relative to control values. Primer sequences are listed in Table 2.

Table 2. **Primer sequences used in the relative quantitative real time PCR**

Gene name	Primer sequence	Gene name	Primer sequence
RP29 F	5'-TTCAAACCGGCACGGTCTGA-3'	Fz-1 F	5'-GGCTTTGTGTCGCTCT-3'
RP29 R	5'-TGCCGTACTGACGGAAACAC-3'	Fz-1 R	5'-GGGATAGCGTAGCTCTT-3'
COL2A1 F	5'-CTGCTCGTCGCCGCTGCCTT-3'	Fz-2 F	5'-GAGCGTGATTGTGCTG-3'
COL2A1 R	5'-AAGGGTCCCAGGTTCTCCATC-3'	Fz-2 R	5'-GCTCTGGGTAGCGGAA-3'

Aggrecan F	5'-GTTGTCATCAGCACACCAGCATC-3'	Fz-3 F	5'-GCTTACAGTGAGTGTTCG-3'
Aggrecan R	5'-ACCACACAGTCCTCTCCAGC-3'	Fz-3 R	5'-CACGCACATGCAGAAA-3'
Sox9 F	5'-GAACGCACATCAAGACGGAG-3'	Fz-4 F	5'-GCTACAACGTGACCAAG-3'
Sox9 R	5'-TCTCGTTGATTCGCTGCTC-3'	Fz-4 R	5'-TCAGGACGGGYCACA-3'
RUNX2 F	5'-GCTGTTATGAAAAACCAAGT-3'	Fz-5 F	5'-CTGTCGCTAAACTTTCCG-3'
RUNX2 R	5'-GGGAGGATTGTGAAGAC-3'	Fz-5 R	5'-CGTCCAAAGATAAACTGCT-3'
SYND4 F	5'-CGAGAGACTGAGGTCATCGAC-3'	Fz-6 F	5'-TCCCTAATCTGATGGGTC-3'
SYND4 R	5'-GCGGTAGAACTCATTGGTGG-3'	Fz-6 R	5'-TTCAAGCTCCTCAGGC-3'
Axin2 F	5'-AGTGTGAGGTCCACGGAAAC-3'	Fz-7 F	5'-GACGTGCAAGAGCTATG-3'
Axin2 R	5'-CTGGTGCAAAGACATAGCCA-3'	Fz-7 R	5'-TGCTGCTGTGGCTAAGT-3'
DVL-1 F	5'-TTATCTACCACATGGACGAGGA-3'	Fz-8 F	5'-ACCCGAGATTTGGAGC-3'
DVL-1 R	5'-CAAAGATCTCCTCCTCACCAC-3'	Fz-8 R	5'-GGCATTTCCTGGTCA-3'
DVL-2 F	5'-CGTCACAGATTCCACAATGTCT-3'	Fz-9 F	5'-AAGGTCAGGCAAGTGAG-3'
DVL-2 R	5'-TCGTTGCTCATGTTCTCAAAGT-3'	Fz-9 R	5'-CTCCAGAGGAACGCAA-3'
DVL-3 F	5'-CATGTCACTCAACATCATCACG-3'	Fz-10 F	5'-GAACGCCTCAACATGGA-3'
DVL-3 R	5'-ACAACATATCTCTGGCTCGAT-3'	Fz-10 R	5'-CTTTAACCTACGGCTGC-3'
ADAMTS4 F	5'-TCCTGCAAACTGAGGACTG-3'	ADAMTS5 F	5'-TATGACAAGTGGGAGTATG-3'
ADAMTS4 R	5'-CGTGGCTCCAGCACATAGTA-3'	ADAMTS5 R	5'-TGACGATAAAGCTGGACTT-3'
IL-1 α F	5'-AGTAGCAACCAACGGGAAGG-3'	IL-6 F	5'-GGCACTGGCAGAAAACAACC-3'
IL-1 α R	5'-TGGTTGGTCTTCATCTTGGG-3'	IL-6 R	5'-GCAAGTCTCCTCATTGAATCC-3'
RELA	5'-TCTGCCGAGTGAACCGAAAC-3'		
	5'-AGCCTGGTCCCGTGAATAC-3'		

F: Forward, R: Reverse

III. Plasmids

A. Source of the plasmids

B-raf vector was a gift from Dustin Maly (University of Washington), Flag-DVL-2 and Flag-DVL-2(∂ 361-736) vectors were a gift from Jeff Wrana (University of Toronto, Canada) and TOP-FLASH-firefly reporter plasmid was a gift from Randall Moon (University of Washington). They were obtained from Addgen.

B. Site-directed mutagenesis

B-raf V600E and B-raf D594V were generated by site directed mutagenesis using B-raf as template. Construction of amino acid substituted mutants (V600E, D594V) of B-raf were performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the recommendations of the manufacturer. The PCR was performed in the presence of 125 ng of sense and antisense oligonucleotides carrying indicated mutation, 200 μ M dNTPs, 5 μ l 10 \times concentrated buffer, 100 ng plasmid and 2.5 U *Pfu*Turbo® DNA polymerase, DNase-free water was added to complement the solution to a final volume of 50 μ l. Cycling parameters were 95 $^{\circ}$ C for 30 s; 16 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 1min; then 68 $^{\circ}$ C for 8 min. Non-mutated parental strands were removed by the digestion with *Dpn*I restriction enzyme.

C. Cloning of HA-SYND4 plasmid, SYND4 promoter and

NF κ B reporter

HA-SYND4 was generated by PCR and cloned into EcoRI and BamHI sites of pCMV empty vector (Stratagene, Valencia, CA). SYND4 human gene promoter sequence encompassing nucleotide -1500 to +50 was generated by PCR and cloned into BamHI and HindIII sites of pGL3-Basic (Promega, Madison, WI) to generate SYND4prom-firefly reporter vector. All the vectors were verified by sequencing.

D. Transformation

One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) was incubated with the plasmid on ice for 30 min, then for 30 sec at 42 $^{\circ}$ C in a water bath. Vials were placed back on ice for several seconds then cultured in pre-warmed S.O.C medium for 45 min at 225 rpm in a shaking incubator. Cells were spread on Luria-Bertani (LB)-agar (Difco, France) plates containing of 100 μ g/ml ampicillin and incubated at 37 $^{\circ}$ C overnight. The second day, plasmids were isolated and analysed.

E. Purification of plasmid

The plasmids were purified using QIAGEN Kits (Qiagen, Hilden, Germany) following the manufacturer's recommendation. Bacterial cells were harvested by centrifugation for 15 min, at 4 °C, at 6000 × g, then were resuspended in the Buffer P1. The Buffer P2 was added and the sealed tube was vigorously inverted 4-6 times. After 5 min, Buffer P3 was added and mixed immediately. Following an incubation on ice for 5 min, the solution was centrifuged at 13000 × g, at 4°C for 10 min, and the supernatant was applied to the QIAGEN-tip 20 which had been equilibrated by the Buffer. The DNA in the tip was washed twice with the Buffer QC then eluted by the Buffer QF. Isopropanol (0.7 volumes) was added to precipitated the DNA by centrifugation for 30min, at 4°C, at 13000 rpm, the supernatant was carefully decanted. Afterwards, the DNA pellet was washed by 70% ethanol then air-dried for 5-10 min, and dissolved in DNase-free water.

IV. siRNA

Small interfering RNA (siRNA) targeting SYND4, MAPK1, MAPK3, PKC α , DVL-1, DVL-2, DVL-3, CTNNB1, Fz-6, p65 and control siRNA were purchased from QIAGEN and are listed in Table 2.

For siRNA transfection, cells were transfected with 25 nM siRNAs using DharmaFECT transfection reagent (Thermo Scientific, Waltham, MA). A mix of siRNA and DharmaFECT transfection reagent was prepared according to manufacturer's instructions, cells were incubated with the mix in the DMEM F12 medium with 2 mM glutamine and 10% (v/v) fetal bovine serum. 24 h after transfection, the medium was replaced to DMEM F12 medium with 2 mM glutamine and 10% (v/v) fetal bovine serum and at 48 h post-transfection, cells were stimulated with 100 ng/ml of Wnt-3a, 10 ng/ml IL-1 β or vehicle for 12 h at 37°C and 5% CO₂.

Table 3. Sequences of siRNA used

Gene	Si RNA sequence
SYND4	5'-UACGGGAAUCUCUGUGGUAUA-3'
CTNNB1	5'-CUCGGGAUGUUCACAACCGAA-3'
MAPK1	5'-AACACUUGUCAAGAAGCGUUA-3'
MAPK3	5'-CUCCCUGACCCGUCUAAUAUA-3'
DVL-1	5'-CAGCGUUUAGAUGGUUAACAU-3'
DVL-2	5'-GGUUCCUCCUCCAUGAGCACCAU-3'UA
DVL-3	5'-CUCCUGGUACUUCAUCACAA-3'
Fz-6	5'-GGUUCCACCUUGUCGUAAA-3'
P65	5'-CCUGAGCACCAUCAACUAU-3

V. Transfection and luciferase reporter assays

Subconfluent human primary chondrocytes in 6-well plates were transfected in triplicate with 2 μ g of plasmid using lipofectamine 2000 transfection reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA), the transfection mixture was prepared in the Opti-MEM[®]I Reduced Serum Medium (Invitrogen) according to manufacturer's instructions. Cells were incubated with the transfection mixture for 6 h cultured in DMEM F12 medium with 2 mM glutamine and 10% (v/v) fetal bovine serum for 24 h.

Chondrocytes used in the luciferase reporter assay were seeded in 24-well plates and transfected in triplicate with TOP-FLASH-firefly reporter vector, SYND4prom-firefly reporter vector, NF κ B-firefly reporter vector or pGL3-Basic and pRL-TK used as internal control (in a ratio 1:10). Twenty-four hours after transfection, the medium was replaced and the cells were stimulated for 12 h with 100 ng/ml recombinant Wnt-3a (R&D systems) or vehicle at 37°C and 5% CO₂. For cells of each well Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Assay System (Promega, Madison, WI) using a Berthold luminometer (Bad Wildbad, Germany).

Chondrocytes in each well were lysed with 100 μ l Passive Lysis Buffer, the plate was gently rocked for 15 min at room temperature. 20 μ l supernatant of cell lysate was mixed with 100 μ l LAR II in the luminometer tube, then the Firefly fluorescent activity was measured immediately. Afterwards, 100 μ l of Stop & Glo[®] Reagent was added and the *Renilla* luciferase activity was recorded. Luciferase activities were normalized to pRL-TK vector activity and were expressed relative to the basal activity of empty pGL3-Basic vector.

VI. ADAMTS-4 activity assay

ADAMTS-4 activity was measured using SensoLyte[®]520 Aggrecanase-1 Assay Kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Briefly, human chondrocytes in the 12-well-plates were treated or not with 100ng/ml Wnt-3a and/or 10 ng/ml IL-1 β in serum-free medium for 48 h, then this conditioned medium was collected and centrifuged for 15 min at 1000 g at 4°C to discard the cells. 50 μ l of the supernatant was incubated with the aggrecanase substrate (5-FAM/TAMRA FRET) solution for 1 h avoiding the light. After adding 50 μ l of stop solution, the fluorescence intensity was measured at Ex/Em=490 nm/520 nm with the Varioskan[™] Flash Multimode Reader (Thermo Scientific, Waltham, MA).

The fluorescence of 5-FAM is quenched by TAMRA. However, upon the cleavage of the FRET peptide by ADAMTS-4, the fluorescence of 5-FAM is recovered and can be monitored at excitation/emission = 490 nm/520 nm. The concentration of 5-FAM was determined using a standard curve and the increase in FAM concentration is correlated to ADAMTS-4 activity as showed in the Figure 12.

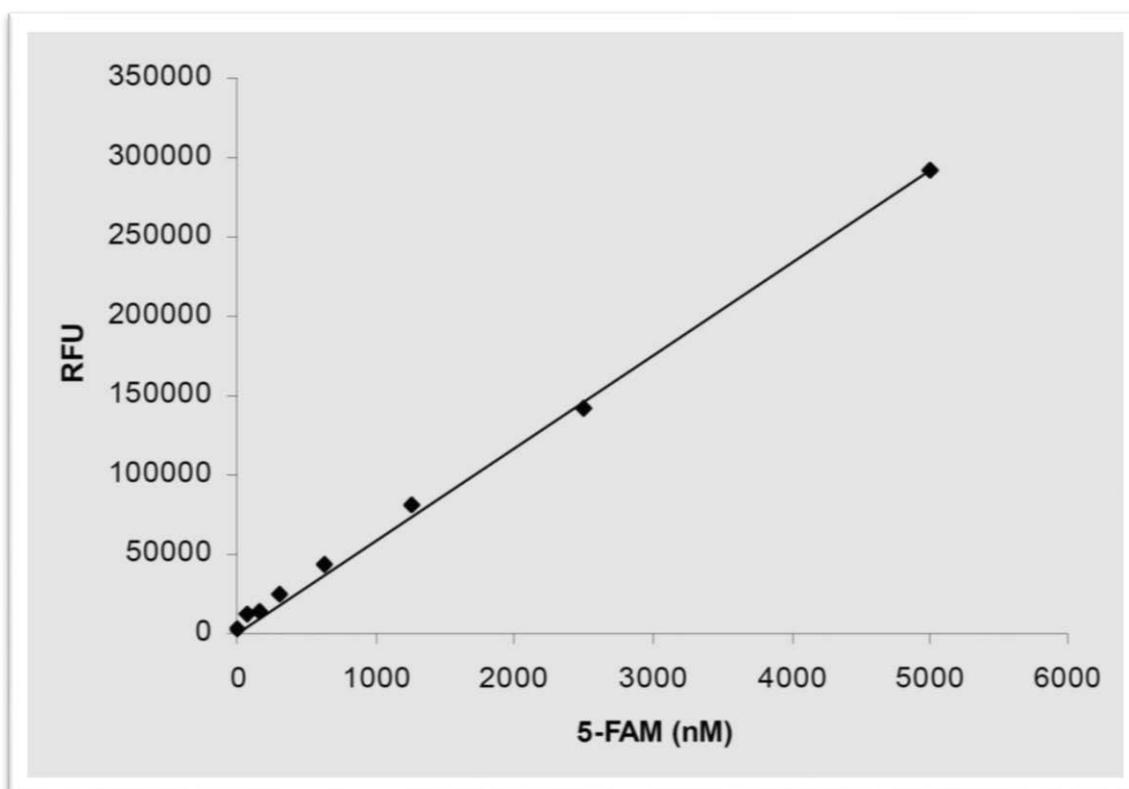


Figure 12. **5-FAM reference standard**

The final concentrations of 5-FAM in reference standards are 5000, 2500, 625, 312.5, 156.78, and 0 nM. The fluorescence is recorded at Ex/Em=490 nm/520 nm.

VII. Western Blotting

Total protein from chondrocytes was extracted using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Nuclear proteins extraction was performed using Nuclear Extract Kit (Carlsbad, Active Motif, CA). Cell lysates were sonicated on ice and protein

concentration of the samples was determined by the Bradford method. Proteins (50 µg/lane) were separated on 10% SDS-PAGE gels, transferred to a PVDF membrane (Millipore, Eschborn, Germany), and subsequently blocked in PBS-Tween 20 containing 5% nonfat milk or 5% BSA. Membranes were then incubated overnight with primary antibodies directed against NFκB P65, IκBα, p-SAPK/JNK, JNK-2, Acetyl-NFκB/p65, p44/42 MAPK, phospho-p44/42, β-catenin, DVL-2, DVL-3, CaMKIIα, Lamin B, β-actin (diluted 1:1000; Cell signaling, Danvers, USA), DVL-1, phospho-CaMKIIα (diluted 1:1000 Santa Cruz, Texas), Flag (diluted 1:1000, Sigma, Saint Louis MO) or HA (diluted 1:1000, Biologend, San Diego, CA) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (diluted 1:2000, Cell signaling). Antibodies were diluted in 5% nonfat milk/0.01% tween 20 in PBS. In the case of NFκB P65, IκBα, p-SAPK/JNK, JNK-2, Acetyl-NFκB/p65, p44/42 MAPK, phospho-p44/42, DVL-2, DVL-3 antibodies were diluted in 5% BSA/0.01% tween 20 in PBS. The blots were then developed using Clarity Western ECL substrate (BIO-RAD, Hercules, CA) according to the instructions of the manufacturer. β-actin was used as loading control for the cell lysate and Lamin B was used as control for the nuclear proteins.

VIII. Immunoprecipitation

For immunoprecipitation, subconfluent primary chondrocytes in 10-cm plates were treated with Wnt-3a (100 ng/ml) or vehicle for 1 h then lysed on ice with TNE buffer (50 mM Tris pH 8, 1% IGEPAL®CA-630, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche Diagnostics). The lysates were sonicated and centrifuged, and the supernatants were stored at -20°C until use. Protein concentration of the samples was determined by the Bradford method and ddH₂O was added to make the samples at the same protein concentration. 1 µg of protein for each sample was incubated with protein A beads (Millipore, Eschborn, Germany) for 30 min at 4°C. Beads were pulled down and the resultant supernatants were used to recover B-raf, Flag-tagged DVL-2 or HA-tagged SYND4 by incubating with anti-B-raf (diluted

1:40, Santa Cruz), anti-Flag (diluted 1:100, Sigma) or anti-HA (diluted 1:100, Sigma) antibodies overnight at 4°C, respectively followed by incubation with protein A beads for 2 h at room temperature. After washing with lysis buffer for 4 times, bound proteins were eluted with loading buffer at 95°C for 5 min, immunoprecipitated proteins were analysed by immunoblotting. Immunoblotting was performed as described previously with antibodies against Flag, HA, B-raf, DVL-2 or phospho-CaMKII α .

IX. Immunofluorescence

For immunofluorescence, primary chondrocytes were seeded on coverslips in 24-well plates. After the treatment, cells were rinsed 3 times with PBS. Cells were then fixed with 4% (w/v) para-formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 4 min and blocked with 0.2% fish skin gelatine in PBS for 20 min. Primary antibodies against β -catenin (diluted 1:100 in PBS/0.2% gelatine, Cell signaling) or HA (diluted 1:200 in PBS/0.2% gelatine, Sigma) were incubated with the fixed cells for 20 min. Coverslips were then rinsed 3 times with PBS and incubated with secondary antibodies anti-rabbit-Alexa Fluor 555 (diluted 1:200 in PBS/0.2% gelatine, Invitrogen) or anti-mouse-Alexa Fluor 488 (diluted 1:200 in PBS/0.2% gelatine, Invitrogen) for 20 min. The nuclei were stained with DAPI and the cells were finally mounted with Moviol (National Diagnostics, Hull, U.K) containing 1% propylgallate (Sigma, Saint Louis MO). Digital images were captured with an inverted microscope, Leica DMI3000 B (Leica Microsystems, Germany).

X. Proteoglycan synthesis and release

A. Proteoglycan release essay

Cartilage explants were incubated with 10 μ Ci/ml of ³⁵S-sulfate (PerkinElmer Life Sciences, MA USA) for 16 h to pre-label proteoglycans. After three times wash with PBS, cartilage explants were treated with Wnt-3a, IL-1 β or both in the DMEM-F12 complete medium for 48 h. Media were collected, ³⁵S-labeled proteoglycans released into the media were digested with papain (1 mg/ml) overnight at 60°C. The samples

were boiled for 10 min to deactivate the papain, then centrifuged for 15 min at the highest speed. The proteoglycans in the supernatant were precipitated by gradient concentrations of cetylpyridinium chloride (CPC), 2% CPC in 0.3M NaCl for 2 h, 1% CPC for 2 h and 0.5% CPC for 2 h, then dissolved in scintillation fluid (Ultima Gold, PerkinElmer Life Science, MA USA). The radioactivity associated with GAGs was measured by liquid scintillation counting (Packard, Rungis, France). Wet weight of each cartilage explants was recorded and used for normalisation.

B. Proteoglycan synthesis assay

Chondrocytes were cultured in six-well plates in DMEM F-12 until 80% confluence then radiolabeled with 10 μ Ci/ml 35 S-sulfate for 6 h. Culture medium was collected and digested overnight with papain (1 mg/ml) at 60 $^{\circ}$ C, then boiled for 10 min to inactivate the enzyme. Aliquots of 35 S-labeled GAGs were precipitated by gradient concentrations of CPC and dissolved in scintillation fluid as described above. The radioactivity associated with GAGs was measured by liquid scintillation counting. The cells in the wells were kept and rinsed with PBS, then the total DNA was quantified by Qubit[®] dsDNA HS Assay Kit (Thermo Scientific, Waltham, MA), and used for normalisation.

XI. Data Analysis and Statistical Procedures

Each experiment was repeated at least three times independently. Quantitative data were expressed as mean \pm S.D. Statistical analysis was performed with an unpaired two-tailed Student's t-test, and effects were considered statistically significant at $*P < 0.05$. One representative immunoblot of three independent experiments was shown in results.

RESULTS

RESULTS

I. Part I Wnt/ β -catenin signaling counteracts interleukine-1-induced ADAMTS-4 expression and activity in human articular chondrocytes

A. Wnt-3a down-regulates the expression of ADAMTS-4 through the canonical Wnt/ β -catenin pathway

Wnt/ β -catenin signaling has been recognized as key regulator of cartilage development and chondrocyte function (Tamamura et al., 2005; Wu et al., 2010). Activation of β -catenin in mice articular chondrocytes resulted in OA-like phenotypes with progressive cartilage degradation (Zhu et al., 2009). To gain insight into the mechanism involved in Wnt-3a-induced cartilage degradation, we investigated whether Wnt-3a regulates the expression of the aggrecan-degrading aggrecanases ADAMTS-4 and ADAMTS-5 in human primary chondrocytes and cartilage explants. Treatment of primary chondrocytes with Wnt-3a resulted in β -catenin accumulation (Figure 13A), activation of TCF/LEF-dependent transcriptional reporter TOPflash, which records the transcriptional activation of canonical Wnt/ β -catenin pathway (Figure 13B).

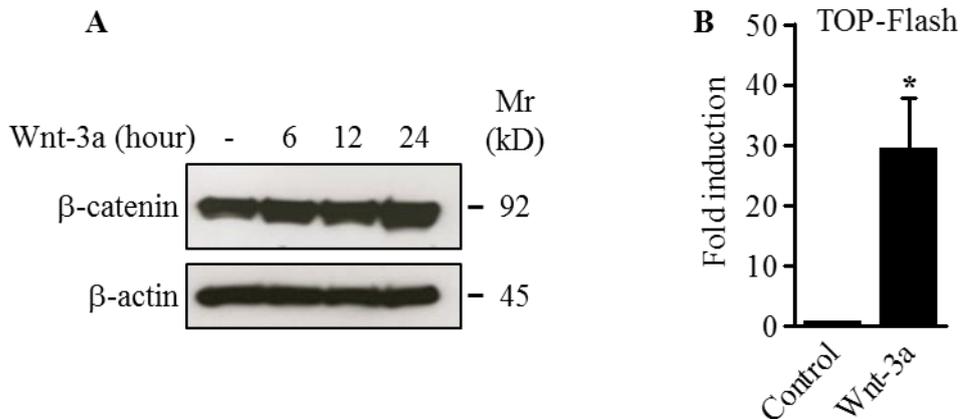


Figure 13. **Wnt-3a induces the activation of Wnt canonical pathways**

(A) *Western blotting for β -catenin indicated time-dependent accumulation of β -catenin.*

Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle (control) for 6 h, 12 h and 24 h and the protein level of β -catenin was determined by Western blotting, β -actin was used as loading control (n=3). (B) Wnt-3a activates the TCF/LEF1 reporter assay TOP-Flash in human primary chondrocytes after 24 h of stimulation (n=3). Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$).

Unexpectedly, activation of Wnt signaling by Wnt-3a strongly decreased (50%) the expression of ADAMTS-4 in primary chondrocytes, whereas no significant changes in the expression of ADAMTS-5 were observed (Figure 14A). Similar results were obtained in human cartilage explants (Figure 14B). ADAMTS-4 was also significantly down regulated (30%) by Wnt-3a in bone marrow-derive mesenchymal stem cells (Figure 14C), indicating that the inhibitory response of Wnt signaling on ADAMTS-4 expression is conserved during mesenchymal stem cells differentiation and cartilage development.

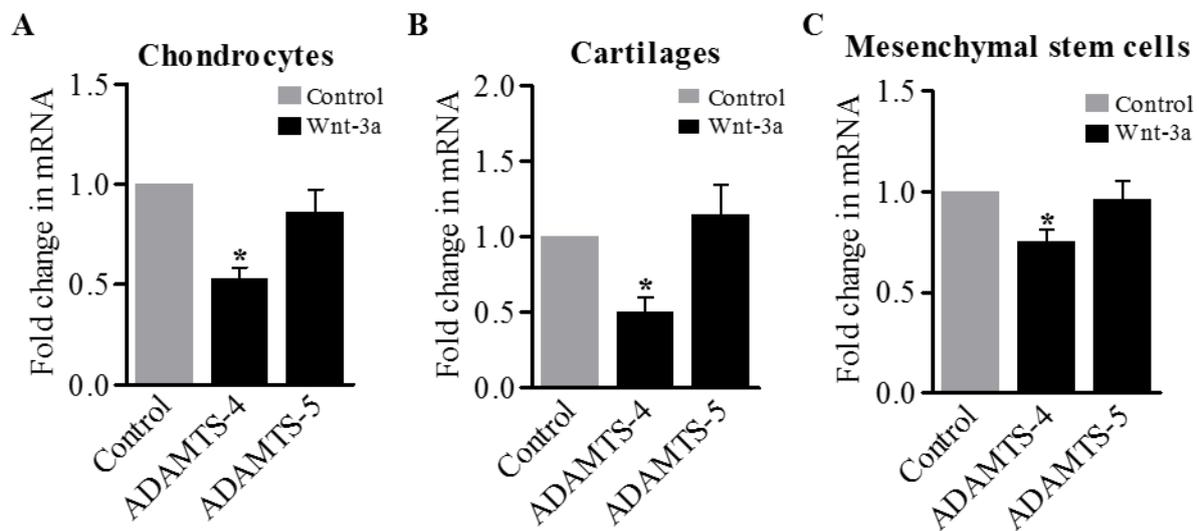


Figure 14. Wnt-3a inhibits the expression of ADAMTS-4

(A-C) Chondrocytes (A, n=5), cartilage explants (B, n=5) and Bone marrow-derive mesenchymal stem cells (C, n=5) were treated with vehicle or 100ng/ml Wnt-3a for 12 h, mRNA levels determined by real time PCR showed that ADAMTS-4 were inhibited by Wnt-3a. Values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. Data are expressed as

mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$).

To determine whether Wnt-3a mediates down-regulation of ADAMTS-4 through canonical Wnt pathway, primary chondrocytes were treated with Wnt-3a alone or in combination with DKK1, a specific inhibitor of canonical Wnt signaling pathway. As expected, Wnt-3a increased the level of β -catenin, whereas co-treatment with DKK1 prevented it (Figure 15A). Interestingly, Wnt-3a failed to down regulate the expression of ADAMTs-4 in presence of DKK1 (Figure 15B), indicating that Wnt-3a inhibits the expression of ADAMTS-4 through canonical Wnt signaling.

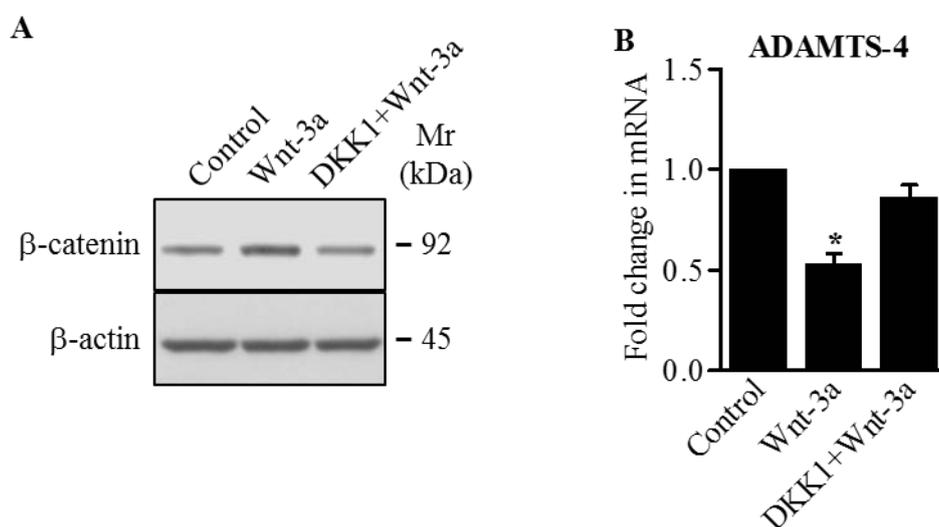


Figure 15. DKK1 prevents the inhibition of ADAMTS-4 induced by Wnt-3a
(A and B) Human chondrocytes were pretreated with vehicle or DKK1 for 30 min and exposed to 100ng/ml Wnt-3a for 12 h, β -catenin was determined by Western blotting when β -actin was used as loading control (A, $n=3$), and the expression of ADAMTS-4 was determined by RT-PCR (B, $n=3$). Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$).

To further confirm this result, siRNA knockdown of β -catenin was performed. Silencing of β -catenin efficiently decreased (45%) its expression (Figure 16A) and unexpectedly induced (2-fold) ADAMTS-4 expression (Figure 16B), suggesting that β -catenin exerts

an inhibitory effect on the basal expression of ADAMTS-4 in human primary chondrocytes.

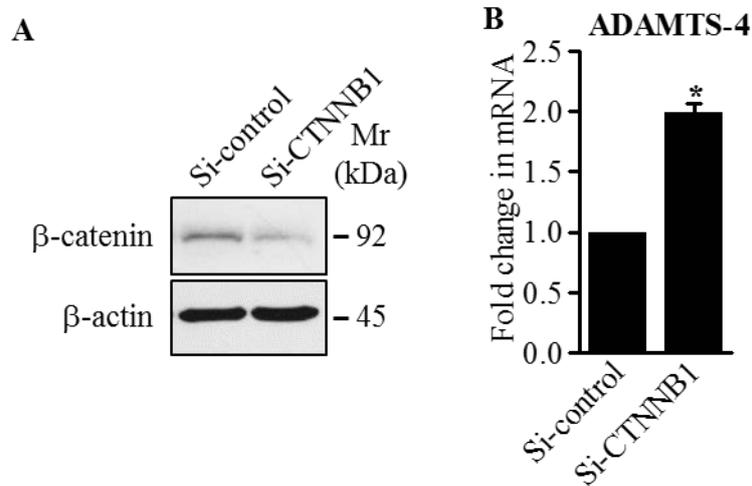


Figure 16. **Block of Wnt canonical pathway induces the expression of ADAMTS-4** (A and B) Silencing of β -catenin was performed by the transfection of si-CTNNB1 in human chondrocytes, β -catenin level was determined by Western blot (A, $n=3$), and the expression of ADAMTS-4 was evaluated by RT-PCR (B). Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

In addition, knockdown of β -catenin efficiently prevented its accumulation following Wnt-3a stimulation (Figure 17A) and not only rescued Wnt-3a-induced decrease of ADAMTS-4 expression but promoted it (Figure 17B), indicating that Wnt-3a regulation of ADAMTS-4 expression in human primary chondrocytes is β -catenin-dependant.

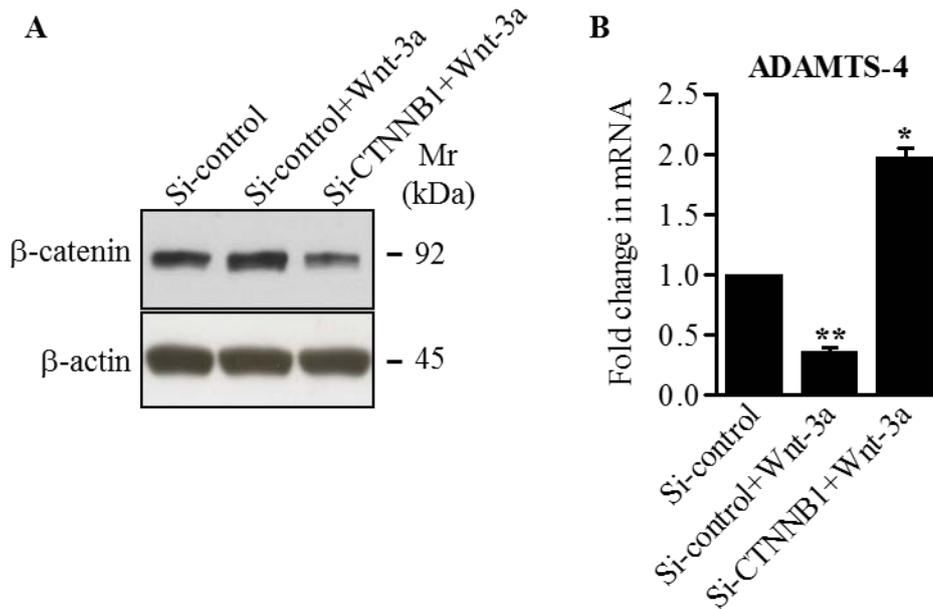


Figure 17. Knockout of β -catenin abolishes the effect of Wnt-3a on ADAMTS-4
*(A and B) Chondrocytes were transfected with siRNA to β -catenin (Si-CTNNB1) or siRNA control (Si-control) for 48 h then stimulated with Wnt-3a (100 ng/ml) or vehicle for 12 h (n=3). Western blotting for β -catenin (A, n=3), and the RT-PCR for ADAMTS-4 (B, n=3) shows the effect of blocking Wnt canonical pathway. Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).*

B. DVL-1, DVL-3 and Frizzled-8 mediate canonical Wnt signaling and down-regulation of ADAMTS-4 in human primary chondrocytes

Considering of DVL's role of hub in Wnt signaling and in order to determine which of the DVLs mediates Wnt-3a-induced down-regulation of ADAMTS-4, we knocked down each of DVLs individually and evaluated the effect on Wnt-3a-induced down-regulation of ADAMTS-4. The knockdown of each DVL efficiently decreased (about 70%) the expression of the corresponding mRNA DVLs (Figure 18A-C) without

affecting other DVLS (Figure 18D-E).

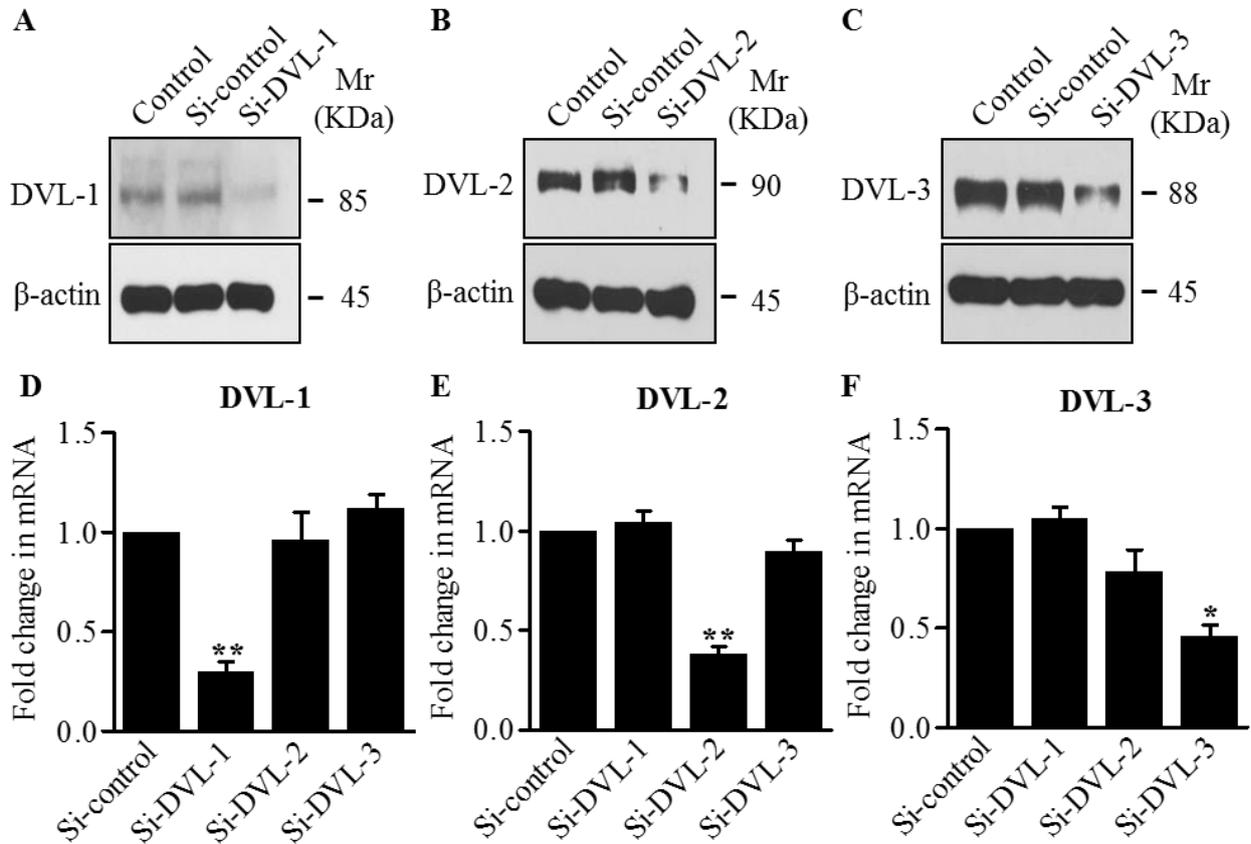


Figure 18. siRNA efficiency on the expression of DVLS in human primary chondrocytes

(A-C) Knockdown of DVL-1, DVL-2 and DVL-3 efficiently decreased the corresponding protein level. Primary chondrocytes were transfected with siRNA specific to (A) DVL-1 (Si-DVL-1), (B) DVL-2 (Si-DVL-2), (C) DVL-3 (Si-DVL-3), or siRNA control (Si-control) for 48 h and the level of DVL-1, DVL-2 and DVL-3 was determined by Western blotting, β -actin was used as loading control ($n=3$). (D-F) Knockdown of DVL-1, DVL-2 and DVL-3 did not affect other DVLS respectively ($n=5$). Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

The knockdown of DVL-1 and DVL-3 reduced partly the accumulation of β -catenin induced by Wnt-3a (Figure 19A), suggesting that canonical Wnt pathway signal mainly through DVL-1 and DVL-3 in human primary chondrocytes. This was further confirmed by the analysis of the expression of the Wnt/ β -catenin-dependent targeted

gene AXIN2. Indeed, the knockdown of DVL-1 and DVL-3 reduced by 50% the induction of the expression of AXIN2 by Wnt-3a (Figure 19B). Importantly, knockdown of DVL-1 and DVL-3 prevented Wnt-3a-induced down-regulation of ADAMTS-4 and even, in the case of DVL-1, promoted its expression (Figure 19C), indicating that Wnt-3a-induced down-regulation of ADAMTS-4 is mediated by DVL-1 and DVL-3. These results also clearly indicated that DVL-1 exerted a repressive effect on basal expression of ADAMTS-4 in human primary chondrocytes.

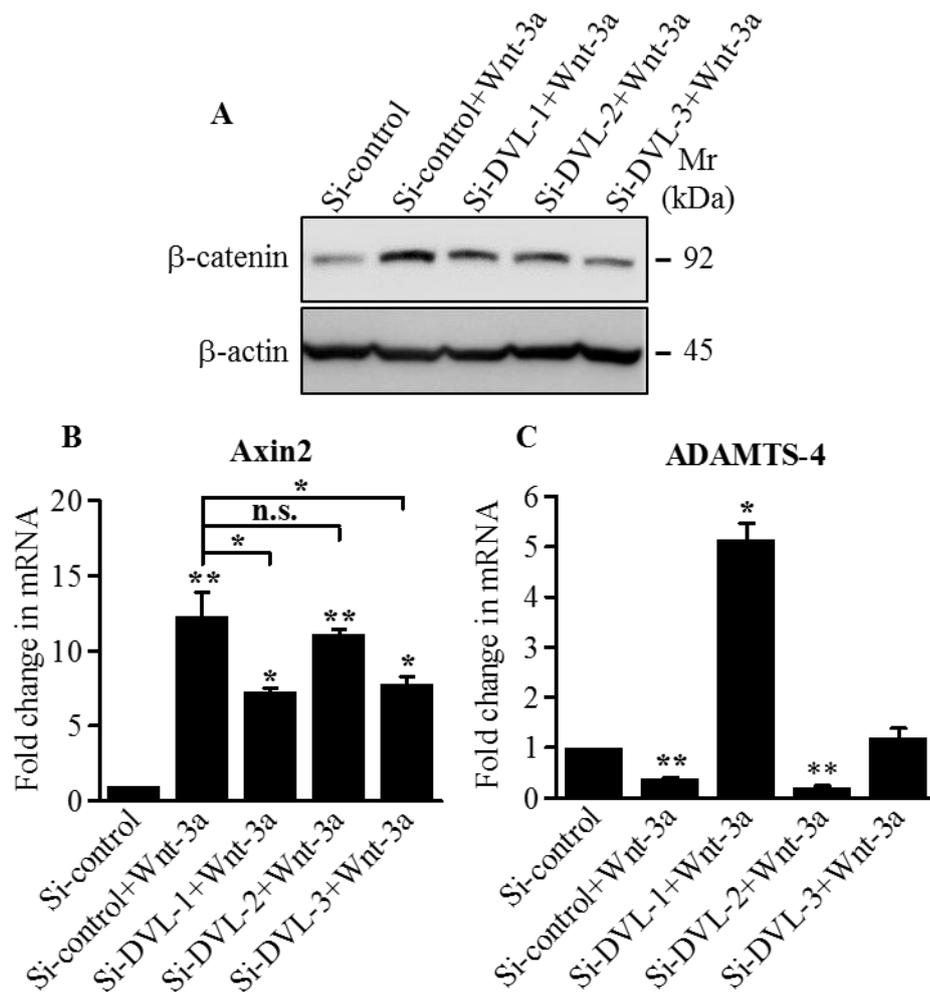


Figure 19. DVL-1, DVL-3 mediate canonical Wnt signaling and down-regulation of ADAMTS-4

(A) Knock down of DVL-1 and DVL-3 inhibited Wnt-3a-induced accumulation of β -catenin. Primary chondrocytes were transfected with siRNA specific to DVL-1, DVL-2, DVL-3 or siRNA control for 48 h then treated with Wnt-3a (100 ng/ml) or vehicle for 12h and the protein level of β -catenin was determined by Western blotting, β -actin was

*used as loading control (n=3). (B and C) After the 48 h transfection with si-control, siDVL-1, siDVL-2 or siDVL-3, chondrocytes were treated with 100ng/ml Wnt-3a for 12 h. Expression of Axin2 (B) and ADAMTS-4 (C) was determined by RT-PCR. PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (n.s., statistically not significant; *, $p < 0.05$; **, $p < 0.01$)*

Frizzled (Fz) is a family of G protein-coupled receptor proteins that serves as receptors in the Wnt signaling pathway. To address which of the 10 known Fz genes are involved in Wnt-3a-induced activation of the Wnt canonical pathway, we first performed qRT-PCR to monitor the expression pattern of all genes encoding Wnt receptors in human primary chondrocytes. The result obtained showed that Fz-6 and Fz-8 were predominant in chondrocytes (Figure 20A). To explore their function in Wnt signaling and in Wnt-3a-induced down-regulation of ADAMTS-4 in human primary chondrocytes, siRNA knockdown of Fz-6 and Fz-8 was performed. QPCR showed that siRNAs reduced by 90% and 50% the mRNA levels of Fz-6 and Fz-8, respectively (Figure 20B, C). Interestingly, the knockdown of Fz-8 but not that of Fz-6 prevented Wnt-3a-induced accumulation of β -catenin (Figure 21A), and the augmentation of Axin2 (Figure 21B), indicating that Fz-8 is essential for Wnt-3a activation of canonical Wnt signaling. Importantly, knockdown of Fz-8 but not that of Fz-6 prevented Wnt3a-induced down-regulation of ADAMTS-4 (Figure 21C). Altogether, these results indicate that Wnt-3a-induced activation of canonical Wnt signaling in human primary chondrocytes is mediated by Fz-8 receptor and by DVL-1 and DVL-3 then led to down-regulation of the expression of ADAMTS-4.

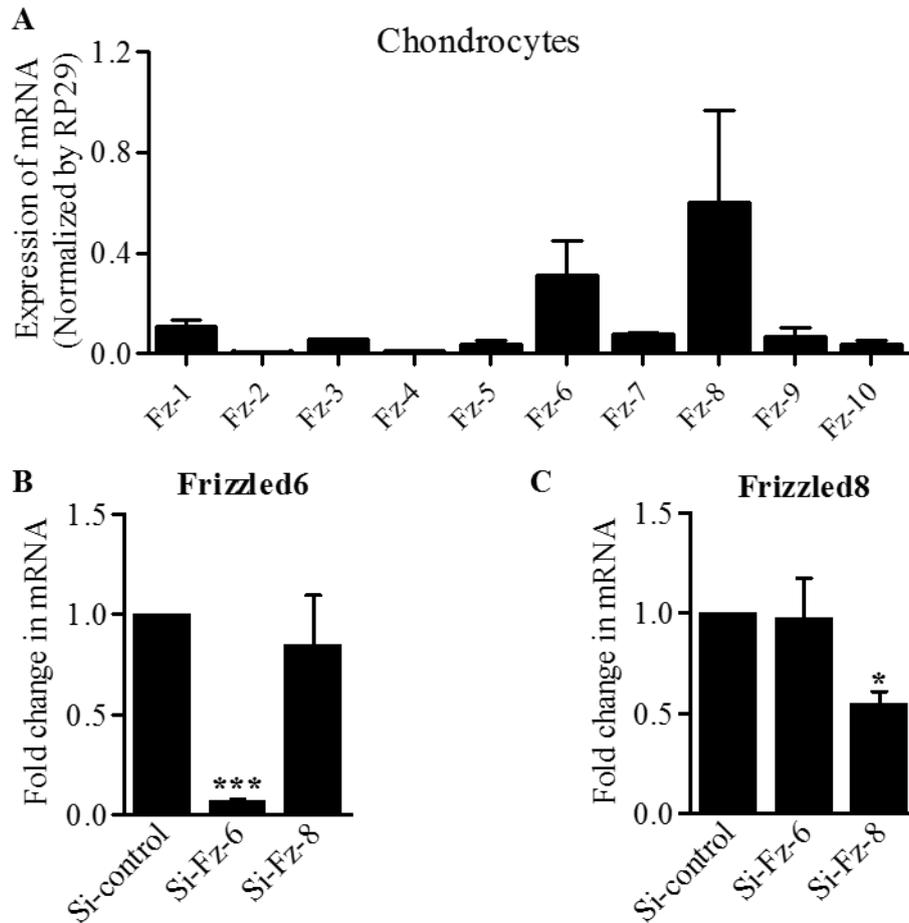


Figure 20. The relative expression of Fz receptors in human primary chondrocytes and the effects of si-Fz-6 and Fz-8

(A) *Fz-6 and Fz-8 receptors are predominantly expressed in human primary chondrocytes (n=3). Expression of different Fz receptors was evaluated by absolute Q-PCR. Specific DNA segment for each of the ten Fz receptors (standards) was amplified by RT-PCR, purified on agarose gel, quantified and used as template for Q-PCR. Absolute quantification was determined compared to standards that are run in parallel.*

(B and C) *Human chondrocytes were transfected with si-control, si-Fz-6 or si-Fz-8 for 48 h, knockdown efficiency of si-Fz-6 (B, n=6) and si-Fz-8 (C, n=5) was determined by RT-PCR. Data are expressed as mean \pm S.D. of 3 separate experiments. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; ***, $p < 0.001$).*

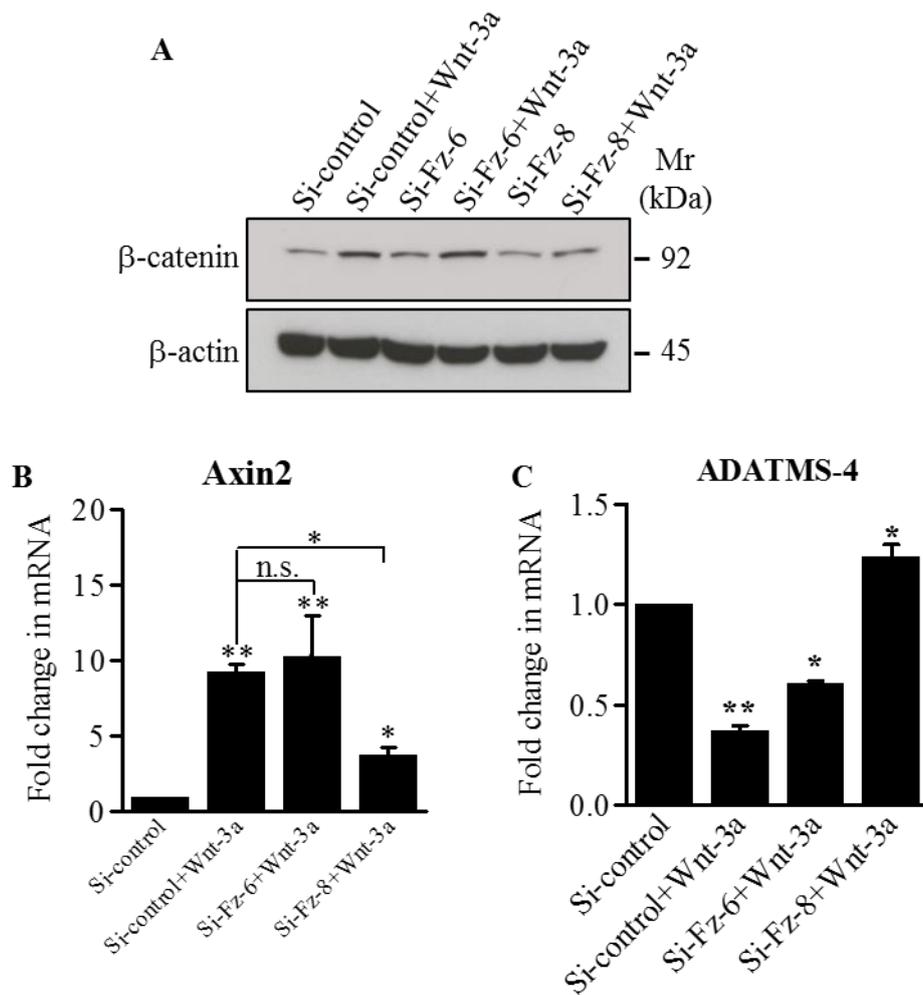


Figure 21. Fz-8 mediate the canonical Wnt signaling and the down-regulation of ADAMTS-4

(A-C) *Wnt-3a*-induced activation of canonical pathway was impaired by *Fz-8* knockdown. Western blotting for β -catenin in chondrocytes transfected with *si-Fz-6*, *si-Fz-8* or *Si-control* for 48 h and treated with *Wnt-3a* (100 ng/ml) or vehicle for a further 12 h, β -actin was used as loading control (A, $n=3$), expression of *Axin2* (B, $n=3$) and *ADAMTS-4* (C, $n=3$) was determined by RT-PCR. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (n.s., statistically not significant; *, $p < 0.05$; **, $p < 0.01$).

Surprisingly, the PCR results showed that *Wnt-3a* inhibited the mRNA expression of *Fz-8* (Figure 22A), and this inhibition is β -catenin dependent (Figure 22B). These results suggest that β -catenin might exert a negative feed-back loop control on the

canonical Wnt signaling mediated by Fz-8 receptor.

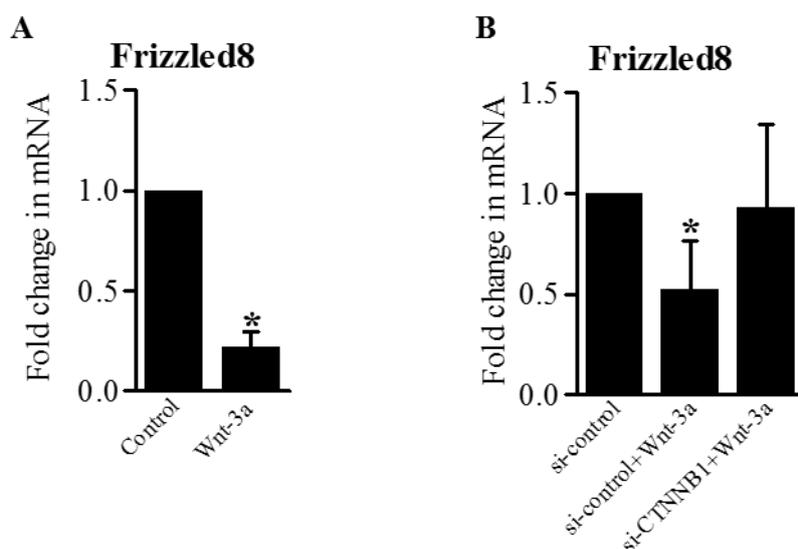


Figure 22. **Wnt-3a inhibits expression of Fz-8 through canonical pathway**

(A) Chondrocytes were treated with vehicle or 100ng/ml Wnt-3a for 12 h, mRNA levels of Fz-8 was determined by real time PCR. (B) Chondrocytes were transfected with siRNA to β -catenin (Si-CTNNB1) or siRNA control (Si-control) for 48 h then stimulated with Wnt-3a (100 ng/ml) or vehicle for 12 h ($n=3$). Fz-8 expression was measured by real time PCR. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$).

C. Wnt-3a down-regulates the expression of ADAMTS-4 by inhibiting NF- κ B signaling

ADAMTS-4 expression has been shown to be regulated through NF- κ B-dependent pathway (Bondeson et al., 2007; Séguin et al., 2006). To further examine whether ADAMTDS-4 expression is regulated by NF- κ B in human primary chondrocytes, NF- κ B/p65/RELA was knocked down and the expression of ADAMTS-4 was evaluated. The Knockdown of NF- κ B/p65/RELA efficiently reduced (90%) the expression of NF- κ B/p65/RELA (Figure 23A, B). Interestingly, silencing of NF- κ B/p65/RELA strongly reduced (67%) the expression of ADAMTS-4 (Figure 23C), indicating that the NF- κ B

signaling play an essential role in maintaining basal expression of ADAMTS-4 and suggested that Wnt-3a may regulates NF- κ B pathway.

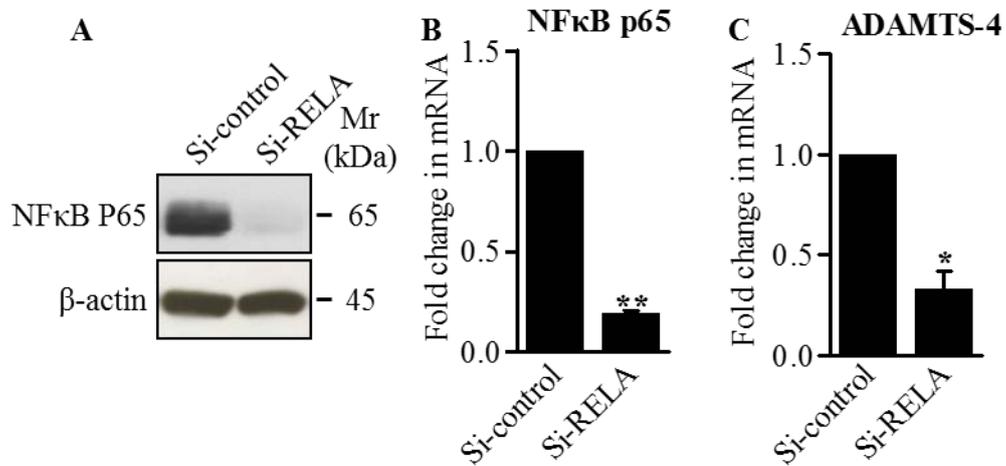


Figure 23. Basal expression of ADAMTS-4 is NF- κ B pathways dependent

(A-C) Knockdown efficiency of RELA in human primary chondrocytes, as evaluated by Western blotting (A, $n=3$) and Q-PCR (B, $n=3$), its effect on the ADAMTS-4 mRNA levels was also measured by RT-PCR (C, $n=3$). Data are expressed as the fold change relative to control. Q-PCR results were normalized as described previously. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t -test (*, $p < 0.05$; **, $p < 0.01$).

Of note, Wnt-3a down regulated the expression of IL6 and IL-1 α , two established target genes of NF- κ B pathway in human chondrocytes (Figure 24A). To further confirm the regulatory effect of Wnt-3a on NF- κ B signaling, the effect of Wnt-3a on NF- κ B activity was examined using NF- κ B responsive promoter construct. As shown in figure 24B, treatment of chondrocytes with Wnt-3a significantly reduced NF- κ B reporter activity, clearly indicating that Wnt-3a inhibits NF- κ B signalling.

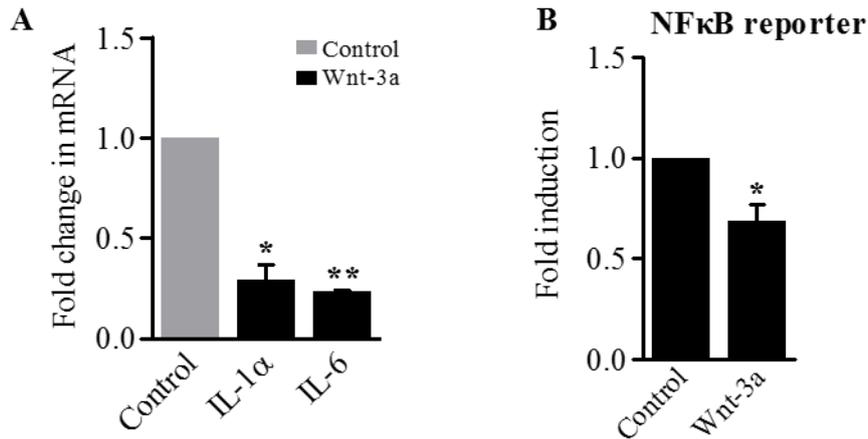


Figure 24. Wnt-3a inhibited the activation of NF-κB-dependent pathways

(A) Shown by RT-PCR, *IL-1α* and *IL6* mRNA levels were inhibited by 12 h treatment of 100ng/ml *Wnt-3a* ($n=3$). (B) Human chondrocytes were transduced with plasmid expressing an NF-κB reporter and *Renilla* for 24 h then luciferase reporter activity was measured. Data are expressed as the fold change relative to control. Q-PCR results were normalized as described previously. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

D. Wnt-3a inhibits NFκB signaling by reducing nuclear translocation of NFκB/p65

Because IκB degradation is a major signaling step leading to NF-κB activation, we examined IκB levels in chondrocytes that were treated or not with Wnt-3a. As shown in figure 25A, Wnt-3a did not produce any significant alteration of IκB levels, indicating that Wnt-3a reduced NF-κB activation through a mechanism that is independent of IκB degradation. Activation of the NF-κB transcription factor is associated with nuclear translocation of the p65 component of the complex. We then examined the translocation of NF-κB/p65 into the nucleus in chondrocytes treated or not with Wnt-3a. As indicated by Western blot NF-κB/p65 was detected in nuclear extract of control cells, indicating the presence of basal NF-κB activity in human primary chondrocytes (Figure 25B and C). Interestingly, treatment with Wnt-3a reduced the translocation of NF-κB/p65 into the nucleus (Figure 25B and C).

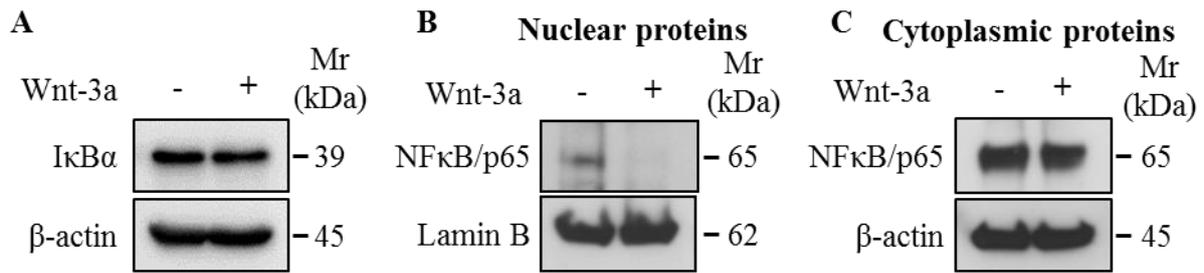


Figure 25. Wnt-3a inhibited the translocation of p65 in to nuclear

(A) Chondrocytes were treated with vehicle control or 100ng/ml Wnt-3a for 12 h, the IκBα was detected by western blot (n=3). (B and C) After 12 h treatment of 100ng/ml Wnt-3a, chondrocytes nuclear proteins (B, n=3) and cytoplasmic proteins (C, n=3) were separated to detect the translocation of NFκB P65. One representative blot of three independent experiments is shown.

Epifluorescence microscopy was employed to further explore the translocation of NF-κB/p65 into the nucleus. Significant staining of NF-κB/p65 was observed in the nucleus of unstimulated cells, while in Wnt-3a stimulated cells it was not (Figure 26A). These results strongly suggested that Wnt-3a reduces the activation of NF-κB pathway by blocking the translocation of NF-κB/p65 into the nucleus. Given that loss of β-catenin abolished the effect of Wnt-3a on ADAMTS-4, these data support the notion that inhibition of NF-κB pathway by Wnt-3a is β-catenin-dependent. In line with this, silencing of β-catenin not only prevented the inhibition of the NF-κB regulated gene IL-1α by Wnt-3a but even promoted its expression (Figure 26B). Altogether, these results demonstrated that Wnt-3a down regulates the expression of ADAMTS-4 by attenuating NF-κB signaling through β-catenin-dependent pathway.

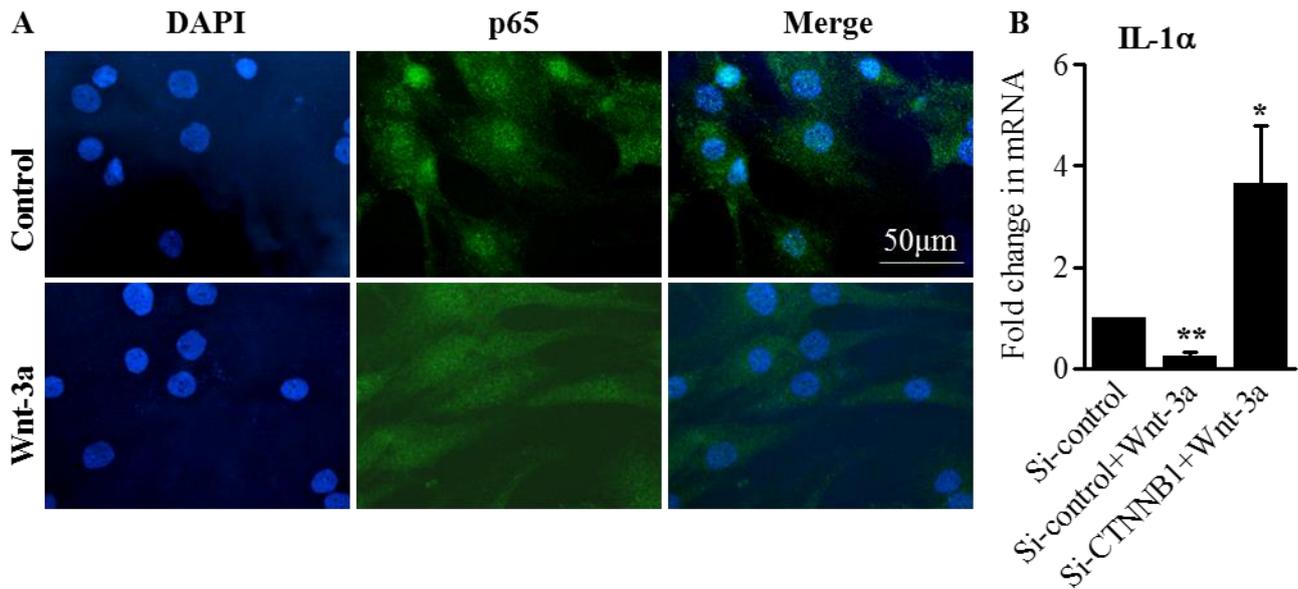


Figure 26. **Wnt-3a inhibited the activation of NF- κ B via canonical pathways**
 (A) Chondrocytes were treated with vehicle control or 100ng/ml Wnt-3a for 12 h, then NF κ B P65 was stained with immunofluorescence (n=3). The basic translocation of p65 to the nuclear was eliminated by the treatment of Wnt-3a. (B) The down regulation of IL-1 α is canonical Wnt pathway-dependent. The knock-out of β -catenin well prevent the inhibition of IL-1 α induced by Wnt-3a (n=3). Q-PCR results were normalized as described previously. One immunofluorescence of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

E. Wnt-3a counteracts IL-1 β -induced upregulation of ADAMTS-4

Depletion of aggrecan from articular cartilage is an early and critical pathophysiological event in OA (Caterson et al., 2000; Nagase and Kashiwagi, 2003). In human chondrocytes, the aggrecanase ADAMTS-4 but not ADAMTS-5 is induced by IL-1 β , the main cytokine incriminated in OA pathogenesis, through activation of NF κ B signaling pathway (Bondeson et al., 2008; Chockalingam et al., 2007; Matsukawa et al., 2013; Song et al., 2007), therefore suggesting that induction of ADAMTS-4 is involved in the pathogenesis of OA. In line with these studies, treatment of human primary chondrocytes with IL-1 β strongly increased (about 700-fold) the expression of ADAMTS-4 and did not significantly affect that of ADAMTS-5 (Figure 27A). In

addition, the knockdown of NF- κ B/p65/RELA reduced by 80% the expression of ADAMTS-4 induced by IL-1 β in primary chondrocytes (Figure 27B). Given that Wnt-3a reduced the activation of NF κ B signaling pathway, the ability of Wnt-3a to counteract IL-1 β -induced up-regulation of ADAMTS-4 was tested. Primary chondrocytes were treated with IL-1 β alone or in combination with Wnt-3a and the expression of ADAMTS-4 was evaluated. As expected, treatment with IL-1 β strongly increased (750-folds) the expression of ADAMTS-4 (Figure 27C). However, co-treatment with Wnt-3a attenuated by about 60% the induction of ADAMTS-4 by the cytokine (Figure 27C), indicating that Wnt-3a is able to antagonize the effect of IL-1 β on ADAMTS-4 expression. Similarly, analysis of ADAMTS-4 activity indicated an increase of about 250-folds in chondrocytes treated with IL-1 β , while co-treatment with Wnt-3a reduced by 30% the IL-1 β -induced activity of ADAMTS-4 (Figure 27D). Altogether, these results clearly demonstrate that Wnt-3a display an ability to counteract IL-1 β -induced stimulation of the expression and activity of ADAMTS-4 in human primary chondrocytes.

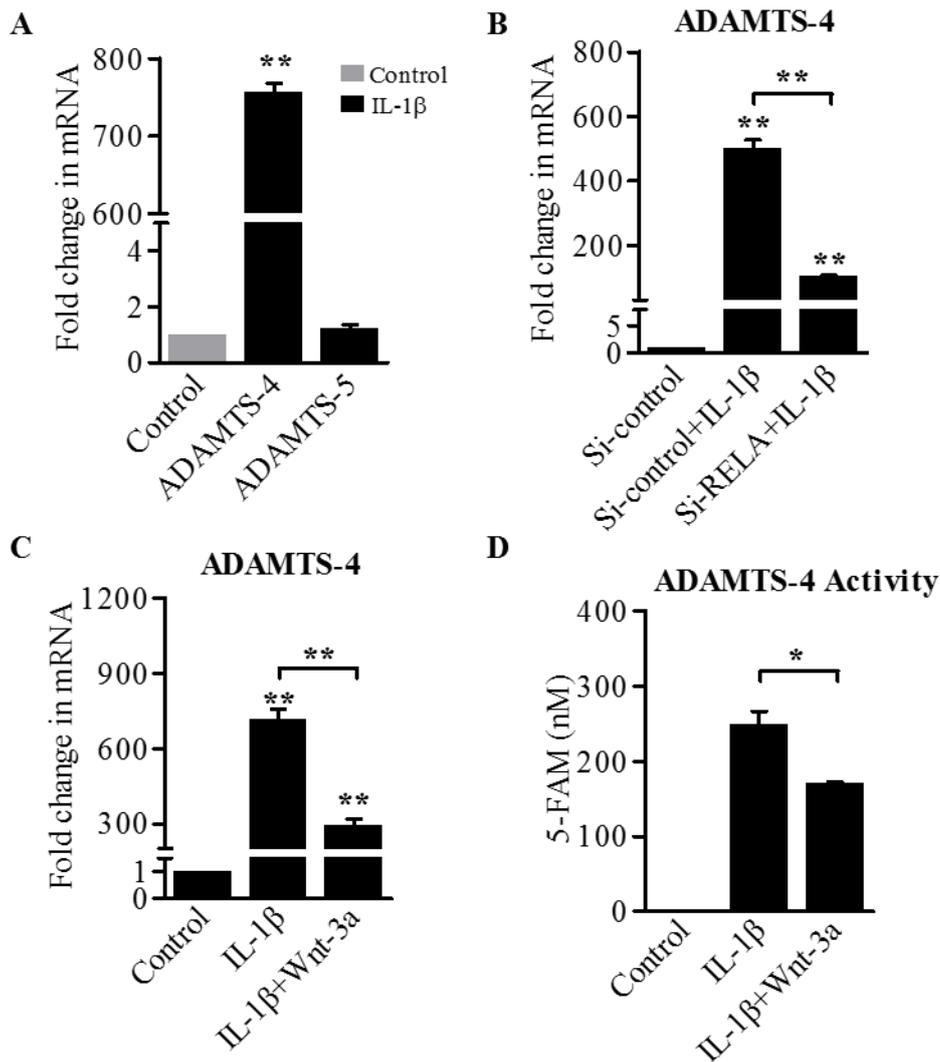


Figure 27. Wnt-3a counteracts IL-1 β -induced up-regulation of ADAMTS-4 and cartilage degradation

(A) Human primary chondrocytes were treated with 10ng/ml IL-1 β for 12 h and mRNA levels of ADAMTS-4 and -5 were measured by RT-PCR (n=3). (B) Human chondrocytes were transfected with control siRNA or si-RELA for 48 h then treated with vehicle or 10ng/ml IL-1 β for another 12 h, then the expression of ADAMTS-4 was measured by RT-PCR (n=3). (C) Treatment with 10ng/ml IL-1 β or combined with 100ng/ml Wnt-3a was performed for 12 h in primary chondrocytes, then ADAMTS-4 mRNA level was determined by RT-PCR (n=3). (D) Chondrocytes were treated with 10ng/ml IL-1 β or combined with 100ng/ml Wnt-3a for 48 h, the activity of ADAMTS-4 in medium was detected by its ability to cleave the 5-FAM/TAMRA FRET substrate to 5-FAM fragment (n=3). Q-PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

F. Wnt-3a attenuates IL-1 β -induced activation of NF κ B signalling

by reducing nuclear translocation of NF κ B/p65

SAPK/JNK and NF κ B signaling pathways have been suggested to play a role in the regulation of the expression of ADAMTSs by IL-1 β . To gain insight into the mechanism by which Wnt-3a reduced IL-1 β -induced expression of ADAMTS-4, we tested whether IL-1 β -induced activation of SAPK/JNK and NF κ B signaling pathways was affected by Wnt-3a. Analysis of the phosphorylation status of SAPK/JNK kinases indicated similar activation by IL-1 β either in absence or in presence of Wnt-3a (Figure 28A). However, analysis of the activation of NF- κ B signaling showed that stimulation of chondrocytes with IL-1 β strongly increased the level of NF- κ B/p65 in the nucleus, compared to unstimulated cells (Figure 28B), whereas co-treatment with Wnt-3a reduced the nuclear translocation of NF- κ B/p65 induced by the cytokine (Figure 28B).

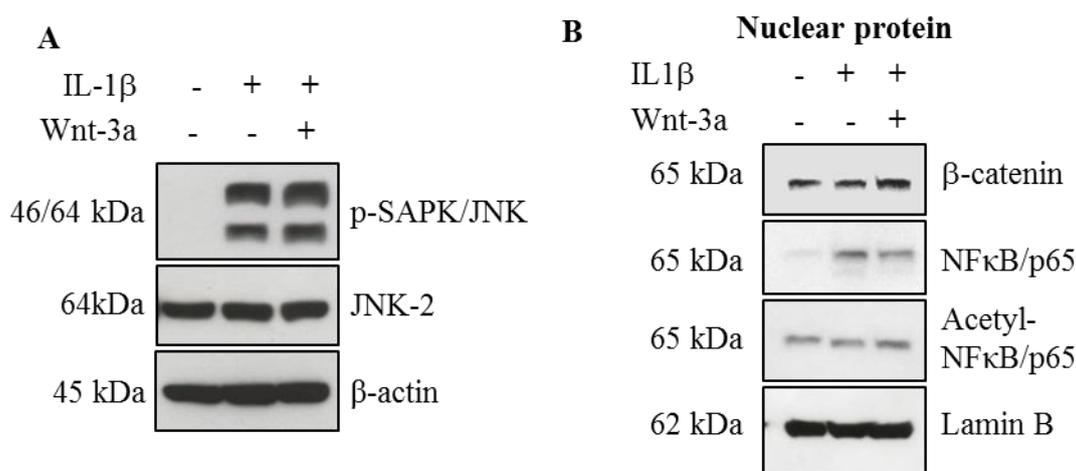


Figure 28. **Wnt-3a counteracts IL-1 β -induced activation of NF- κ B**

(A and B) Human chondrocytes were treated with 100ng/ml Wnt-3a for 12 h then treated with vehicle or 10ng/ml IL-1 β for 20 min, p-SAPK/JNK and JNK-2 in whole-cell extract (A, n=3) and β -catenin, NF κ B/P65, acetyl- NF κ B/P65 in nuclear extract (B, n=3) were detected by western blot. One representative blot of three independent experiments is shown.

To further explore the translocation of NF- κ B/p65 into the nucleus epifluorescence

microscopy was used. Stimulation of chondrocytes with IL-1 β resulted in a strong staining of the nucleus, compared to control (Figure 29), indicating a high level of NF- κ B/p65 translocation into the nucleus in IL-1 β -stimulated cells. Interestingly, co-treatment with Wnt-3a reduced the level of NF- κ B/p65 staining in the nucleus compared to the treatment with IL-1 β alone (Figure 29), suggesting that Wnt-3 counteracts IL-1 β -induced translocation of NF- κ B/p65 into the nucleus. These results suggested that Wnt-3a counteracts IL-1 β -induced NF- κ B activity by reducing the nuclear translocation of NF- κ B/p65.

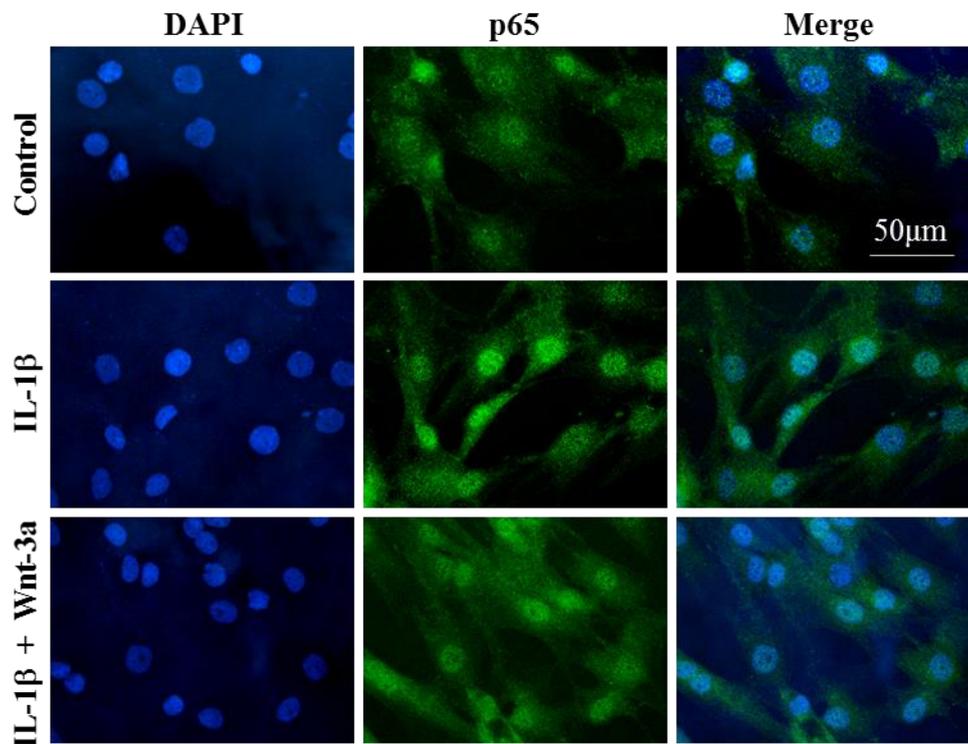


Figure 29. Wnt-3a counteracts IL-1 β -induced translocation of p65

Chondrocytes were treated with 100ng/ml Wnt-3a or vehicle control for 12 h then simulated with 10ng/ml IL-1 β , the NF κ B P65 was stained with immunofluorescence. One representative immunofluorescence of three independent experiments is shown.

In line with this, Wnt-3a reduced by about 5-fold the IL-1 β -induced up-regulation of the NF- κ B responsive gene, IL1 α (Figure 30A). However, change of the translocation of p65 by Wnt-3a is not dependent of I κ B α (Figure 30B). After all, we conclude that

Wnt-3a negatively modulates NF- κ B activity and therefore reduced IL-1 β -induced stimulation of the expression of ADAMTS-4 in human primary chondrocytes.

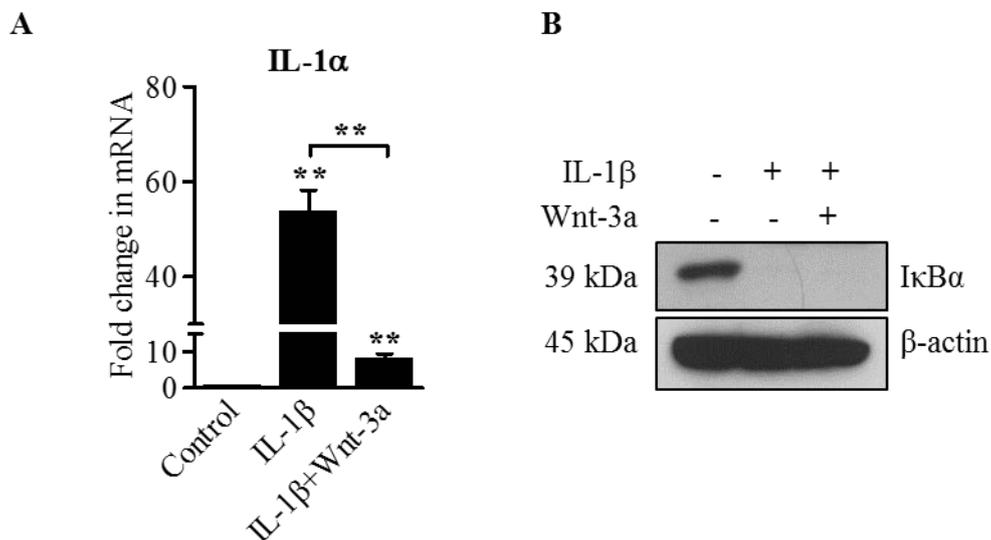


Figure 30. Counteractions of IL-1 β -induced activation of NF- κ B by Wnt3a is independent of I κ B α

(A) Chondrocytes were treated with 10ng/ml IL-1 β or combined with 100ng/ml Wnt-3a for 12 h, the expression of IL-1 α was determined by RT-PCR (n=3). (B) Human chondrocytes were treated with 100ng/ml Wnt-3a for 12 h then treated with vehicle or 10ng/ml IL-1 β for 20 min, I κ B α were detected by western blot (n=3). Q-PCR results were normalized as described previously. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (**, $p < 0.01$).

G. Wnt-3a counteracts IL-1 β -induced inhibition of proteoglycan synthesis

Proteoglycan loss is a major event in the process of OA that leads to cartilage alteration and loss of joint function. Proteoglycan loss occurs early in OA and can be induced by IL-1 β . To examine whether Wnt-3a could counteract loss of proteoglycans induced by IL-1 β , proteoglycans in the medium of human chondrocytes treated with either IL-1 β or in combination with Wnt-3a was evaluated by 35 S-sulfate incorporation. As shown in figure 31A, treatment with IL-1 β reduced by 75% the level of proteoglycans

compared to control, whereas co-treatment with Wnt-3a produced a reduction of only 50%, indicating that Wnt-3a attenuated IL-1 β -induced loss of proteoglycans. To verify these results, human cartilage explants were pre-labelled with ^{35}S , then treated with vehicle, Wnt-3a, IL-1 β , or both of the cytokines, the measure of released ^{35}S -proteoglycan demonstrated Wnt-3a reduced the release of proteoglycan induced by IL-1 β (Figure 31B). Altogether, these results revealed that Wnt-3a might have a protective effect against the development of human OA.

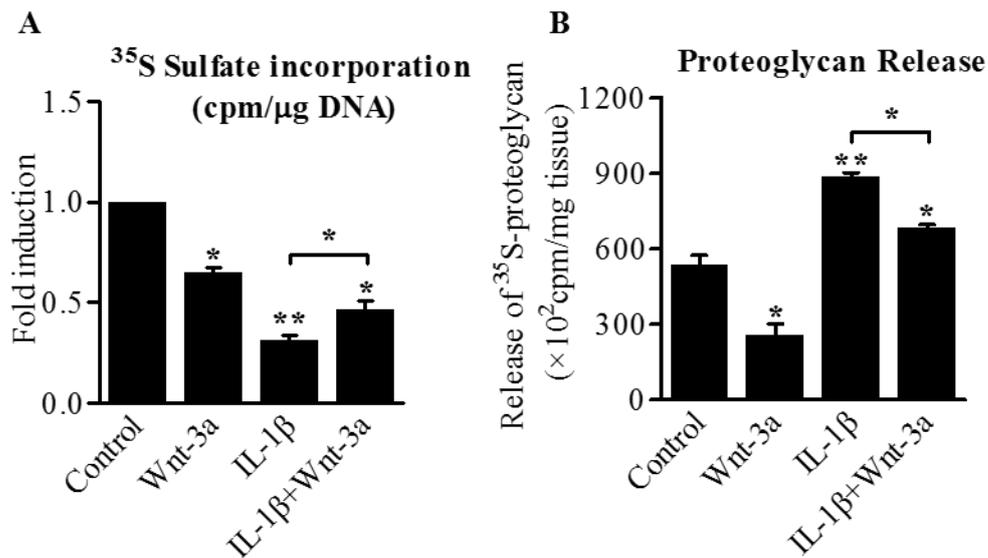


Figure 31. Wnt-3a counteracts IL-1 β -induced cartilage degradation

(A) PG synthesis studied by [^{35}S] sulfate incorporation in primary human chondrocytes treated with vehicle, Wnt-3a, IL-1 β and both Wnt-3a and IL-1 β for 24 h was demonstrated after normalized by cell total DNA ($n=3$). (B) The release of ^{35}S -Proteoglycan was measured in the medium of the cartilage treated with vehicle, Wnt-3a, IL-1 β and both Wnt-3a and IL-1 β for 48h, results were normalized by the wet weight of cartilage ($n=3$). Q-PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

II. Part II Non-canonical Wnt-3a signaling regulates chondrocyte phenotype via syndecan4

A. Non-canonical Wnt mediates loss of chondrocyte phenotype

To gain insight into the mechanism by which Wnt-3a mediates loss of chondrocyte phenotype, we first investigated the expression of chondrocyte-lineage markers *COL2A1*, *SOX9* and *aggrecan* in human primary chondrocytes and articular cartilage explants treated with either Wnt-3a or vehicle (control). In agreement with other studies (Nalesso et al., 2011), the expression of chondrocyte-lineage markers *COL2A1*, *SOX9* and *aggrecan* was decreased by Wnt-3a in primary chondrocytes. We also found that the expression of the hypertrophy marker *RUNX2* was increased (Figure 32A). Similar results were obtained with human cartilage explants (Figure 32B), indicating that Wnt-3a activates de-differentiation of articular chondrocytes.

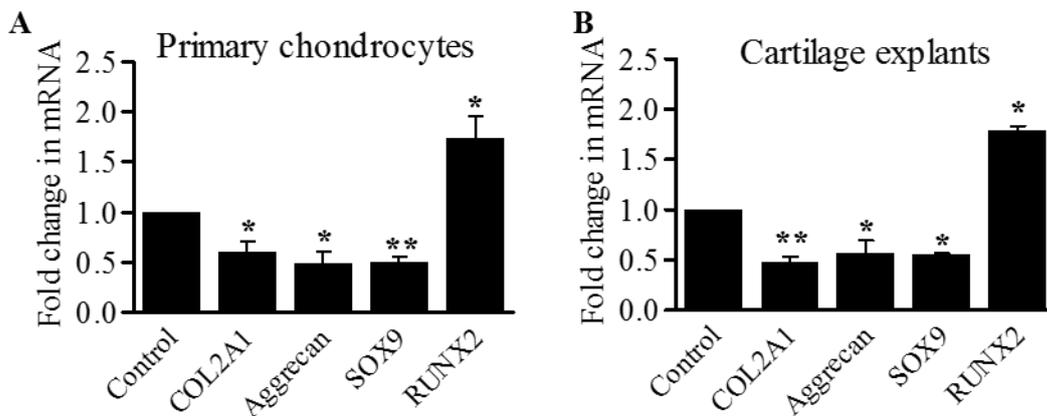


Figure 32. **Wnt-3a induces loss of chondrocyte phenotype**

(A and B) *Wnt-3a* inhibited the expression of chondrocyte phenotype markers. *Wnt-3a* (100 ng/ml) down-regulated the expression of *COL2A1*, *Aggrecan* and *SOX9* and up-regulated that of *RUNX2* in human chondrocytes (A, n=5) and cartilage explants (B, n=5) as evaluated by Q-PCR. Values were normalized for the housekeeping gene ribosomal protein *S29* and are expressed as relative expression compared with control. Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

Wnt-3a induced both canonical and non-canonical Wnt pathways. To determine whether Wnt-3a mediates loss of chondrocyte phenotype through canonical Wnt pathway, we investigated the effect of β -catenin knockdown on Wnt-3a-induced down-regulation of the chondrocyte phenotypic marker *COL2A1*. Treatment of primary chondrocytes with Wnt-3a inhibited the *COL2A1*, however, knockdown of β -catenin failed to rescue the Wnt-3a down regulation of *COL2A1* (Figure 33A), indicating that Wnt-3a regulates *COL2A1* through β -catenin-independent pathway. This was further confirmed using DKK1. Co-treatment with DKK1 could not prevent Wnt-3a-induced down-regulation of *COL2A1* (Figure 33B), therefore suggesting that down-regulation of *COL2A1* expression by Wnt-3a is mediated through non-canonical Wnt signaling pathway in human primary chondrocytes.

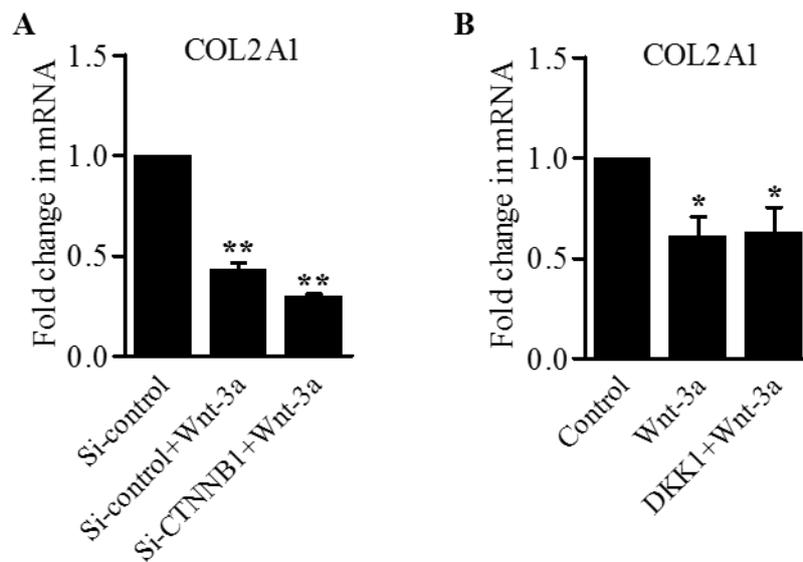


Figure 33. Wnt-3a induces loss of chondrocyte phenotype through β -catenin-independent pathway

(A and B) Inhibition of β -catenin-dependent pathway could not rescue Wnt-3a-induced down-regulation of the chondrocyte phenotypic gene marker *COL2A1*. (A) Silencing of β -catenin or (B) blockage of the Wnt canonical pathway by DKK1 failed to rescue the Wnt-3a-induced down regulation of *COL2A1* ($n=3$). Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

B. Syndecan 4 regulates non-canonical Wnt pathway

SYND4 has been shown to regulate the non-canonical Wnt signaling in *Xenopus* embryos by interacting with the receptor Frizzled7 (Fz-7) and its signal transducer DVL (Muñoz et al., 2006). To determine the role of SYND4, if any, in Wnt-3a-induced loss of chondrocyte phenotype, we performed loss-of-function experiments in human primary chondrocytes using siRNA strategy. The knockdown of SYND4 efficiently decreased SYND4 expression (Figure 34A), but did not prevent Wnt-3a-induced β -catenin stabilization (Figure 34B), indicating that SYND4 is not mandatory for the activation of Wnt/ β -catenin pathway in primary chondrocytes. This was confirmed by the up-regulation by Wnt-3a of the established Wnt/ β -catenin target gene *AXIN2* (Figure 34C).

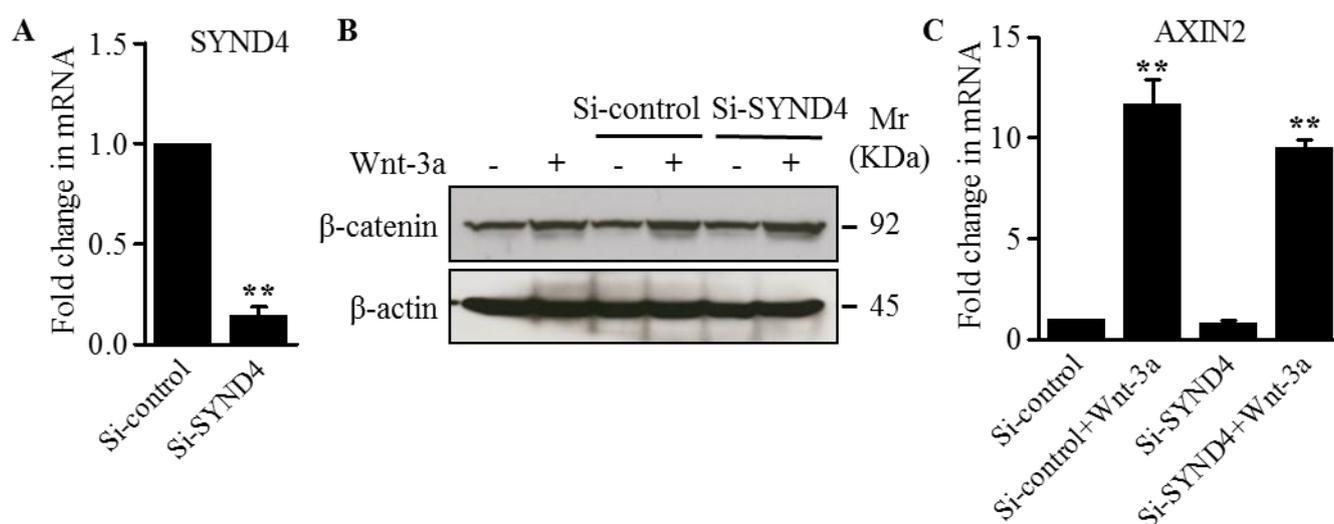


Figure 34. Syndecan4 is not essential for Wnt canonical pathway

(A-C) Knockdown of SYND4 did not impair the activation of canonical Wnt/ β -catenin-dependent pathway. (A) Transfection of chondrocytes with Si-SYND4 strongly reduced its expression as evaluated by Q-PCR (n=4). (B) Wnt-3a-induced accumulation of β -catenin was not impaired by SYND4 knockdown. Western blotting for β -catenin in chondrocytes transfected with Si-SYND4 or Si-control for 48 h and treated with Wnt-3a (100 ng/ml) or vehicle for a further 12 h, β -actin was used as loading control (n=3). (C) Knockdown of SYND4 did not block Wnt-3a-induced up-regulation of Wnt canonical target gene *AXIN2* in primary chondrocytes (n=3), as evaluated by Q-PCR. Values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. Data are expressed as mean

± S.D. Statistical analysis was performed with an unpaired Student's *t*-test (NS, statistically not significant; *, $p < 0.05$; **, $p < 0.01$).

Analysis of the effect of the knockdown of SYND4 on Wnt-3a-induced down-regulation of *COL2A1* revealed that silencing of SYND4 induced by its own a decrease in the expression of *COL2A1* (Figure 35A), suggesting that SYND4 is necessary for basal expression of *COL2A1* in chondrocytes. Interestingly, treatment with Wnt-3a did not produce any further decrease of *COL2A1* expression in SYND4-deficient chondrocytes (Figure 35A), indicating that SYND4 is essential for Wnt-3a-mediated down-regulation of *COL2A1*. To confirm the role of SYND4 in Wnt-3a-induced down-regulation of *COL2A1*, we performed gain-of-function experiment with an engineered HA tagged SYND4 expression vector (Figure 35B). Chondrocytes transfected with HA-SYND4 vector efficiently expressed this HSPG at the cell surface and stimulation with Wnt-3a led to internalization and accumulation of SYND4 in punctate structures in chondrocytes, as shown by immunofluorescent labelling (Figure 35C). Noteworthy, expression of HA-SYND4 did not produce any significant change on basal expression of *COL2A1*, however it abrogated Wnt3a-induced down-regulation of *COL2A1* (Figure 32D), indicating that forced expression of SYND4 counteracts Wnt-3a-induced decrease of *COL2A1* expression.

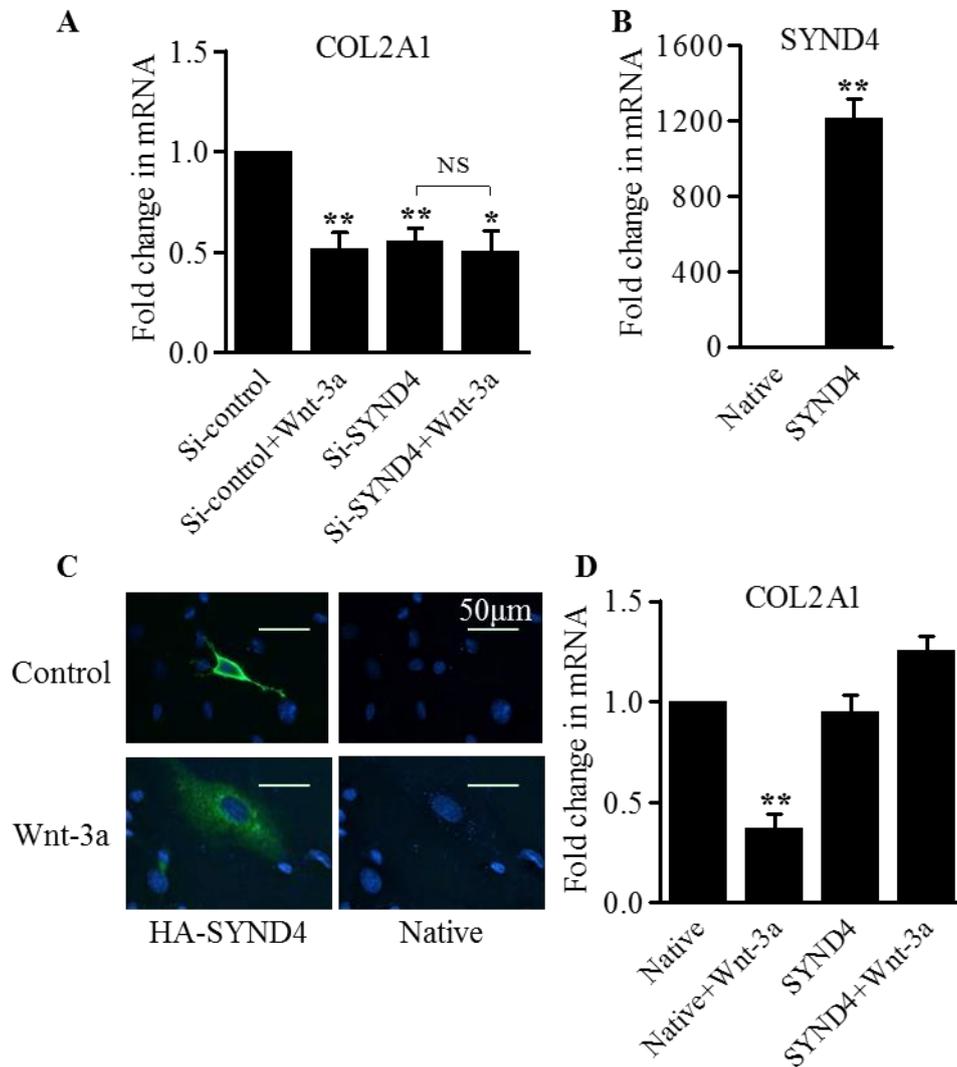


Figure 35. Syndecan4 is essential for Wnt-3a-induced loss of chondrocyte phenotype

(A) Knockdown of *SYND4* decreased the expression of *COL2A1*, and prevented a further decrease induced by *Wnt-3a* ($n=4$). (B-C) Gain-of-function of *SYND4* counteracted *Wnt-3a*-induced loss of chondrocyte phenotype. (B) Transfection of human primary chondrocytes with HA-*SYND4* expression vector for 24 h produced strong expression of *SYND4* mRNA compared to cells transfected with empty vector (Native) ($n=3$), as evaluated by Q-PCR. (C) Immunofluorescence analysis of HA-*SYND4* in chondrocytes treated with *Wnt-3a* or vehicle for 30 min at 37°C ($n=3$). Chondrocytes transfected with HA-*SYND4* vector efficiently expressed this HSPG at the cell surface and stimulation with *Wnt-3a* (100 ng/ml) led to internalization and accumulation of *SYND4* in punctate structures in chondrocytes ($n=3$) (scale bar, 50µm). (D) Overexpression of *SYND4* did not produce any significant change on basal expression of *COL2A1*, but it completely abrogated the down-regulation induced by

Wnt-3a (n=3), as evaluated by Q-PCR. Values were normalized for the housekeeping gene ribosomal protein *S29* and are expressed as relative expression compared with control. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (NS, statistically not significant; *, $p < 0.05$; **, $p < 0.01$).

C. Syndecan 4 expression is down-regulated by non-canonical

Wnt

The ability of SYND4 to prevent down-regulation of *COL2A1* by Wnt-3a raised the possibility that the molecular mechanism by which Wnt-3a-induced loss of this chondrocyte phenotypic marker is by modulating the expression of SYND4. We therefore monitored the expression of this HSPG in primary chondrocytes upon treatment with Wnt-3a. The results showed that Wnt-3a significantly decreased the expression of SYND4 (Figure 36A). Similarly, direct treatment of human cartilage explants with Wnt-3a strongly reduced SYND4 expression (Figure 36B).

To gain further insight into the mechanism by which Wnt-3a down-regulates the expression of SYND4, we cloned the proximal promoter sequence of human *SYND4* gene and monitored the effect of Wnt-3a on its transcriptional activity using a luciferase reporter assay. SYND4 promoter showed significant basal activity in primary chondrocytes. Importantly, treatment with Wnt-3a significantly reduced the activity of the promoter, indicating that Wnt-3a down-regulates the expression of SYND4 by inhibiting the transcriptional activity of the gene promoter (Figure 36C).

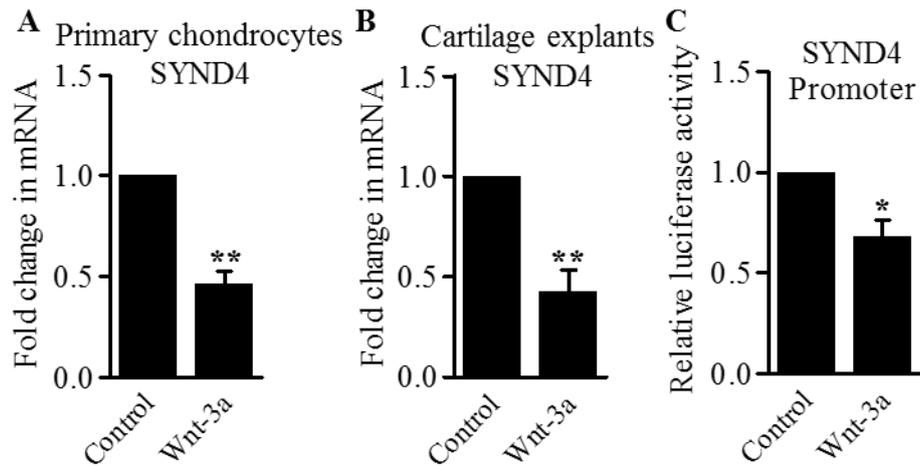


Figure 36. Syndecan4 expression is reduced by Wnt-3a

(A-C) *Wnt-3a* inhibited the expression of *SYND4*. *Wnt-3a* (100 ng/ml) down-regulated the expression of *SYND4* in human chondrocytes (A, $n=5$) and cartilage explants (B, $n=5$), as evaluated by *Q*-PCR. (C) *SYND4* promoter activity was significantly inhibited by *Wnt-3a* in human primary chondrocytes ($n=4$). Primary chondrocytes were transfected with *SYND4*prom-firefly reporter vector or *pGL3-Basic* (empty vector, control) and *pRL-TK* vector (used as internal control) and treated with *Wnt-3a* (100 ng/ml) or vehicle for 24 h. Relative luciferase activity was calculated as fold of activation over that of *pGL3-Basic* after normalization. *Q*-PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

To determine whether down-regulation of *SYND4* by *Wnt-3a* is mediated through canonical *Wnt*/ β -catenin pathway, chondrocytes were treated with *Wnt-3a* alone or in combination with *DKK1*. Co-treatment with *DKK1* failed to rescue *Wnt-3a*-induced down-regulation of *SYND4* (Figure 37A). In addition, β -catenin knockdown did not prevent *Wnt-3a*-induced decrease of *SYND4* expression (Figure 37B), indicating that modulation of *SYND4* expression by *Wnt-3a* is not dependent on the *Wnt*/ β -catenin signaling pathway. Therefore, we hypothesized that *Wnt-3a* regulates *SYND4* expression through non-canonical *Wnt* pathway.

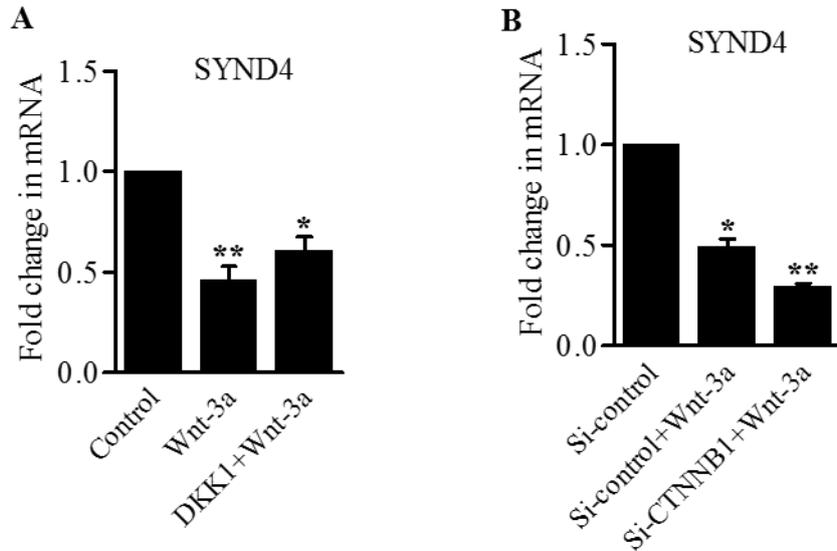


Figure 37. **Downregulation of Syndecan4 expression by is β -catenin independent** (A) Blockage of Wnt canonical pathway by DKK1 or (B) knockdown of β -catenin failed to rescue the down-regulation of SYND4 by Wnt-3a ($n=4$). Q-PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

It has been shown that Wnt-3a also signal through β -catenin-independent mechanisms to mediate distinct physiologically relevant responses. Indeed, Wnt-3a induces proliferation of NIH3T3 fibroblasts and regulates inflammation in primary microglia cells through activation of ERK1/2 MAPK (Halleskog and Schulte, 2013; Yun et al., 2005). To determine whether non-canonical Wnt/ERK1/2 pathway mediates Wnt-3a-induced down-regulation of SYND4, we first analyzed whether Wnt-3a activates this pathway in primary chondrocytes. The activation status of ERK1/2 kinases was determined in primary chondrocytes following Wnt-3a treatment. The p-ERK level was increased in a time-dependent manner starting within 5 min of the treatment and last over 2 h (Figure 38), indicating that Wnt-3a activates ERK1/2 pathway in primary chondrocytes.

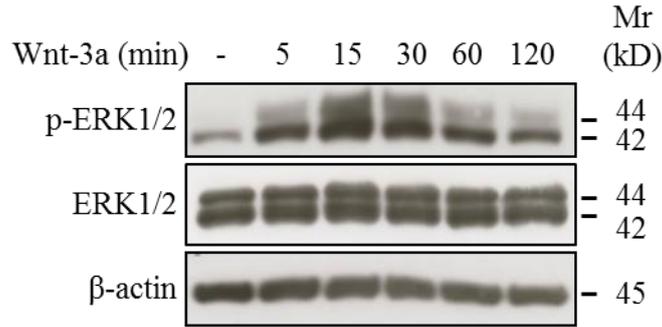


Figure 38. Wnt-3a activates ERK1/2 in a time dependent manner

Wnt-3a rapidly activates ERK1/2 signaling in primary chondrocytes. Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for different time points and the protein level of ERK1/2 and p-ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). One representative blot of three independent experiments is shown.

Co-treatment with the MEK inhibitor, U0126 abrogated the activation of ERK1/2 pathway by Wnt-3a (Figure 39A). We next analyzed the effect of inhibition of Wnt-3a-induced activation of ERK1/2 on SYND4 expression. Inhibition of ERK1/2 pathway rescued Wnt-3a-induced down-regulation of SYND4 (Figure 39B), thus indicating that Wnt-3a regulates the expression of SYND4 through activation of ERK1/2 pathway. Consistent with this finding, knockdown of ERK1 and ERK2 (Figure 39C) reverted the effect of Wnt-3a on the expression of SYND4 (Figure 39D), confirming the key role of ERK1/2 in Wnt-3a-induced down-regulation of SYND4. We showed above that inhibition of *COL2A1* expression by Wnt-3a is mediated through down-regulation of SYND4, therefore we hypothesized that inhibition of *COL2A1* expression may be mediated by ERK1/2 pathway.

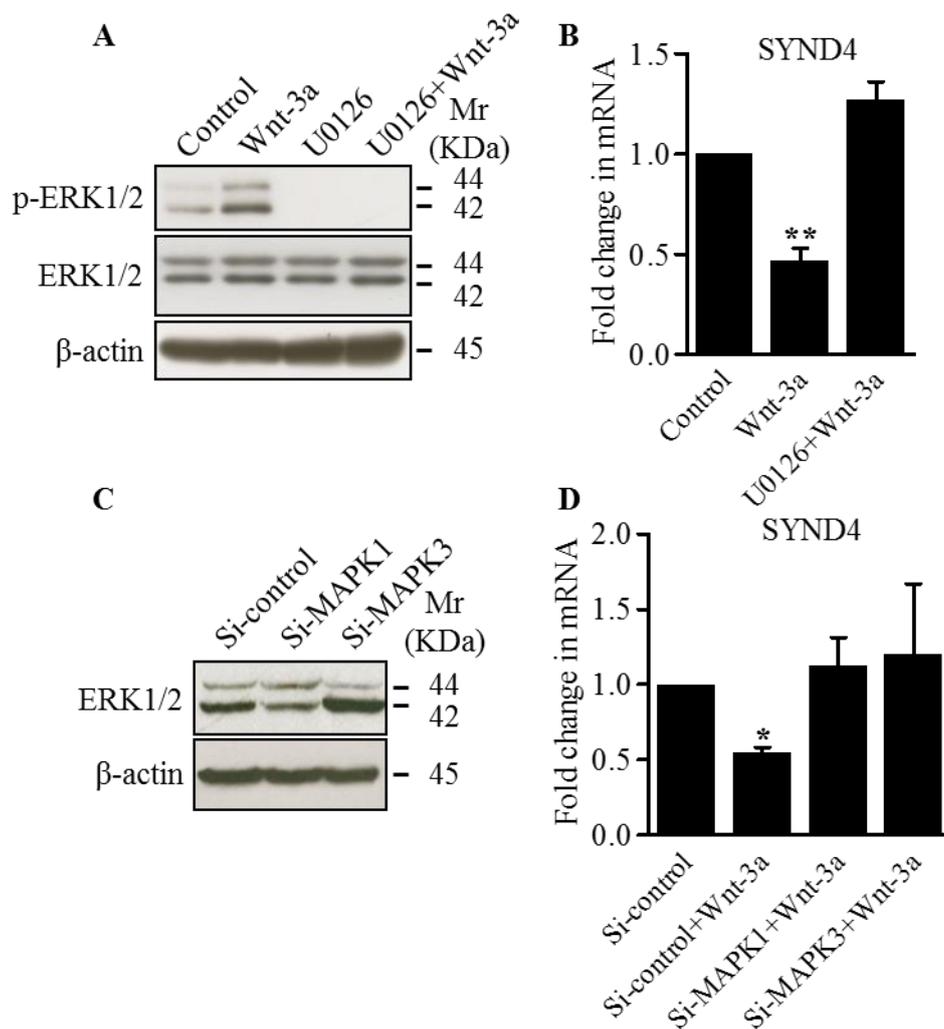


Figure 39. Syndecan4 expression is negatively regulated by non-canonical Wnt/ERK1/2 pathway

(A) Pharmacological inhibition of MEK1/2 prevented activation of ERK1/2 by Wnt-3a. Primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for 12 h, in presence and absence of MEK1/2 inhibitor U0126 and the protein level of p-ERK1/2 and ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). (B) Pharmacological inhibition of ERK1/2 activation prevented Wnt-3a-induced down-regulation of SYND4. Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) in combination or not with the MEK1/2 inhibitor U0126 (1 μ M) for 12 h and the expression of SYND4 was evaluated by Q-PCR (n=4). (C) Knockdown of MAPK1 and MAPK3 efficiently decreased their expression. Primary chondrocytes were transfected with siRNA specific to MAPK1 (Si-MAPK1), MAPK3 (Si-MAPK3) or siRNA control (Si-control) for 48 h and the level of ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). (D) Inhibition of ERK1/2 signaling

by the knockdown of *ERK1* or *ERK2* prevented down-regulation of *SYND4* by *Wnt-3a*. Primary chondrocytes were transfected with *Si-MAPK1*, *Si-MAPK2* or *Si-control* for 48 h and treated with *Wnt-3a* (100 ng/ml) or vehicle for a further 12 h ($n=4$) and expression of *SYND4* was analysed by Q-PCR. Q-PCR results were normalized as described previously. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

Hence, we tested whether *COL2A1* expression was affected by inhibition of *Wnt-3a*-induced activation of *ERK1/2*. Co-treatment with the MEK inhibitor U0126 prevented down-regulation of *COL2A1* by *Wnt-3a* (Figure 40A). In addition, this effect of *Wnt-3a* on *COL2A1* expression was rescued by knockdown of *ERK2* (Figure 40B). Altogether, these results indicated that *Wnt-3a*-induced activation of *ERK1/2* signaling pathway negatively regulates the expression of *SYND4* and subsequently that of *COL2A1*.

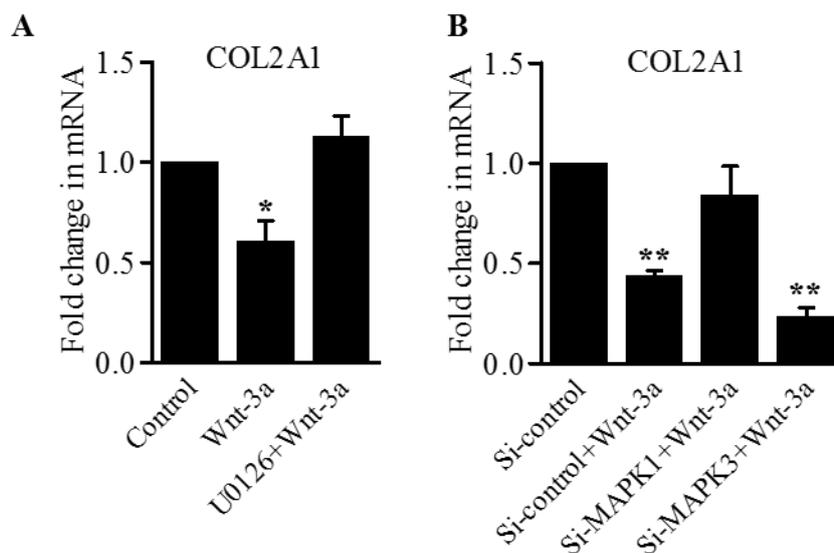


Figure 40. *COL2A1* is reduced by non-canonical *Wnt/ERK1/2* pathway

(A) Pharmacological or (B) siRNA inhibition of *ERK1/2* signaling prevented the down-regulation of *COL2A1* expression by *Wnt-3a* ($n=4$). Q-PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

D. DVL-2 mediates non-canonical Wnt down-regulation of

COL2A1

DVL is key component of both canonical and non-canonical Wnt signaling pathways and, depending on the cellular context, specifically activates each Wnt signaling branch (Logan and Nusse, 2004). Mammalian DVLs include three isoforms, DVL-1, DVL-2 and DVL-3 which display differential expression and are not truly “redundant“ with respect to function (Etheridge et al., 2008; Hamblet et al., 2002; Lee et al., 2008). Analysis of the expression DVL-1, DVL-2 and DVL-3 by Western blotting showed that DVL-3 is the most abundant DVL expressed in primary chondrocytes, whereas DVL-1 is the less abundant (Figure 41A). Considering of DVL’s role of hub in Wnt signaling and in order to determine which of the DVLs mediates Wnt-3a-induced down-regulation of *COL2A1*, we knocked down each of DVLs individually and evaluated the effect on Wnt-3a-induced decrease in the expression of *COL2A1*. DVL-2 knockdown not only prevented down-regulation of *COL2A1* by Wnt-3a but, promoted the expression of this chondrocyte phenotype marker (Figure 41B). The knockdown of DVL-1 and DVL-3 did not prevent Wnt-3a-induced down-regulation of *COL2A1* instead, silencing of DVL-1 potentate it (Figure 41B). These results indicate that DVL-2 mediates Wnt-3a down-regulation of *COL2A1* and suggests that, in chondrocytes, non-canonical Wnt pathway signal through DVL-2. These results also revealed that DVL-2 exerts a repressive effect on the expression of *COL2A1* in chondrocytes.

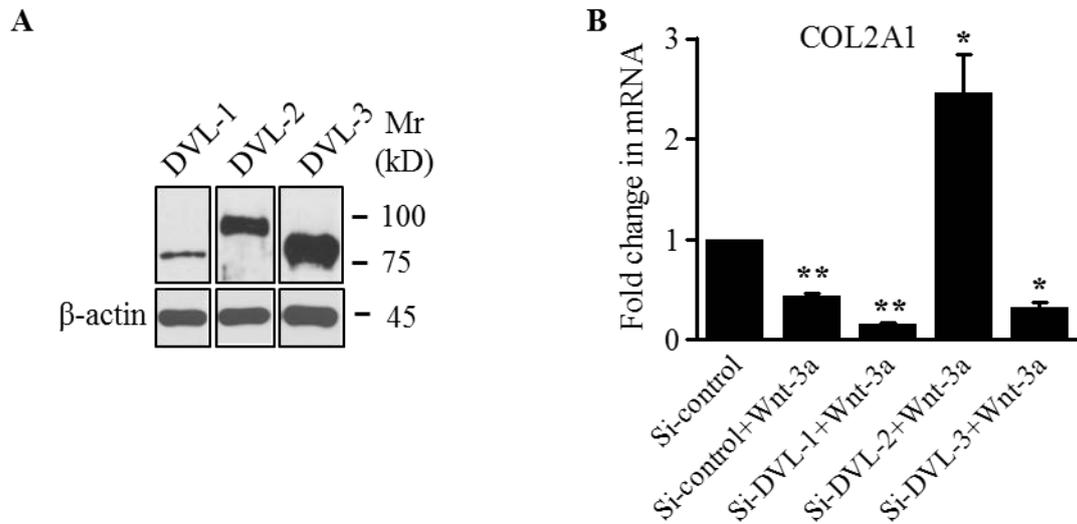


Figure 41 DVL-2 mediates the down-regulation of COL2A1 induced by non-canonical Wnt signaling

(A) Expression levels of different subtypes of DVL are significantly different in primary chondrocytes. Western blotting for DVLs indicated that DVL-3 is the most expressed DVL protein in human chondrocytes, while DVL-1 is the least expressed ($n=3$). (B) DVL-2 is essential for Wnt-3a-induced down-regulation of COL2A1. Knockdown of DVL-2 reversed the inhibition by Wnt-3a of COL2A1 as evaluated by PCR ($n=3$). Western blotting, β -actin was used as loading control ($n=3$). Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$)

As DVL-2 mediates Wnt-3a-induced down-regulation of COL2A1, we hypothesized that down-regulation of SYND4 by Wnt-3a may be mediated by DVL-2. To test this hypothesis, we analyzed the effect of DVLs knockdown on Wnt-3a-induced down-regulation of SYND4. The knockdown of DVL-1 and DVL-3 did not prevent Wnt-3a-induced down-regulation of SYND4, instead it was prevented by knockdown of DVL-2 (Figure 42A), indicating that down-regulation of SYND4 by Wnt-3a is mediated by DVL-2.

We showed above that Wnt-3a-induced down-regulation of SYND4 is mediated through activation of ERK1/2 pathway. Since knockdown of DVL-2 suppresses Wnt-3a-induced down-regulation of SYND4, we asked whether DVL-2 silencing affects activation of ERK1/2 in response to Wnt-3a. Although Wnt-3a provoked the activation of ERK1/2 signaling, it failed to stimulate this pathway in chondrocytes made deficient in DVL-2 (Figure 42B). The knockdown of DVL-2 provoked a decline, rather than increase in ERK1/2 activation in response to Wnt-3a, indicating that DVL-2 play a critical role in Wnt-3a-induced activation of ERK1/2 pathway that is linked to non-canonical Wnt signaling.

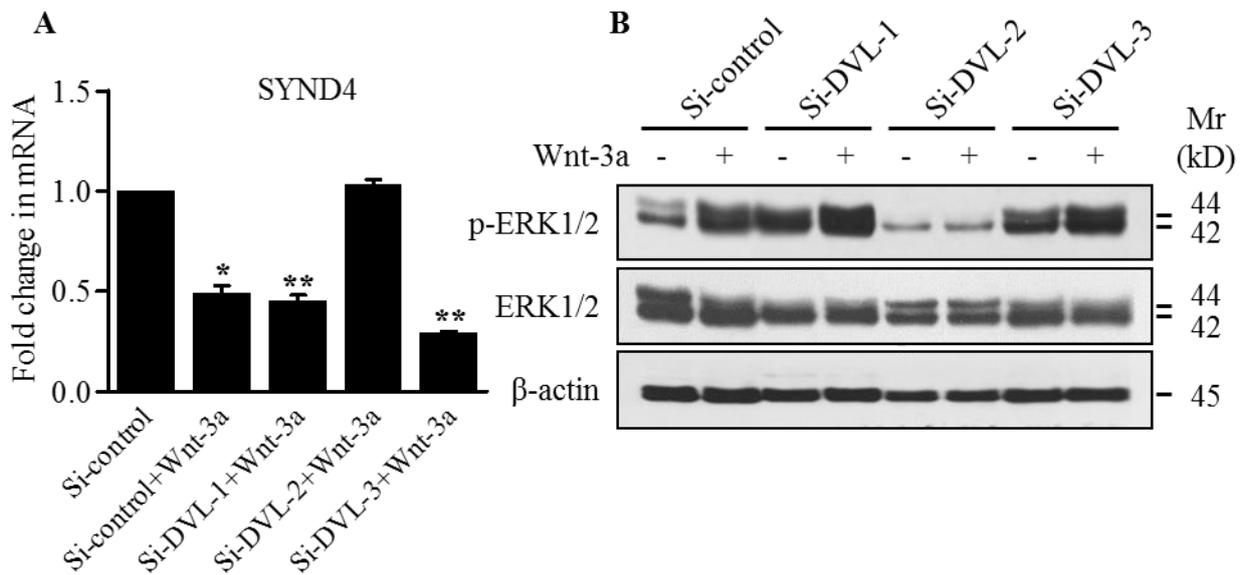


Figure 42. DVL-2 mediates the down-regulation of SYND4 and activation of ERK1/2 induced by Wnt-3a

(A) Knockdown of DVL-2 reversed the inhibition of SYND4 by Wnt-3a, as evaluated by PCR ($n=3$). (B) Knock down of DVL-2 but not of DVL-1 or DVL-3 inhibited Wnt-3a-induced activation of ERK1/2 signaling. Primary chondrocytes were transfected with siRNA specific to DVL-1, DVL-2, DVL-3 or siRNA control for 48 h then treated with Wnt-3a (100 ng/ml) or vehicle for 30 min and the protein level of ERK1/2 and pERK1/2 was determined by Western blotting, β -actin was used as loading control ($n=3$). Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of

three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (, $p < 0.05$; **, $p < 0.01$)*

E. DVL-2 docks B-raf to activate ERK1/2 signaling

DVL-2 is the scaffold protein that relays Wnt signaling by bridging receptor and adaptor proteins in both canonical and non-canonical Wnt pathways (Boutros et al., 1998; Wallingford and Habas, 2005). As DVL-2 was required for the activation of ERK1/2 by Wnt-3a, we hypothesized that B-raf which transduces signals to MEK and ERK1/2 MAPK may relay Wnt signaling through interaction with DVL-2. To test whether DVL-2 interacts with B-raf, co-immunoprecipitation assays were performed. Human primary chondrocytes were treated or not with Wnt-3a and cells extracts were immunoprecipitated with anti-B-raf specific antibodies. Co-immunoprecipitation showed that DVL-2 associates with B-raf and this occurs only in the presence of Wnt-3a (Figure 43A), therefore revealing that B-raf interacts with DVL-2 in Wnt-dependent manner. To determine whether recruitment of DVL-2 to the membrane is required for DVL-2 interaction with B-raf, we used a DVL-2 mutant lacking the DEP domain (Flag-DVL-2 Δ 361-736) that is important for membrane recruitment (Simons et al., 2009). We stimulated cells containing the Flag-tagged wild-type or Δ 361-736 mutant of DVL-2 with Wnt-3a and then immunoprecipitated B-raf with specific antibodies. As shown in figure 43B, the Wnt-3a-dependent binding between DVL-2 and B-raf was observed only in the presence of Wnt-3a in cell expressing the wild-type DVL-2, but not the DVL-2 (Δ 361-736) mutant. These results indicate that DVL-2 interaction with B-raf requires the presence of the DEP domain and suggest that recruitment of DVL-2 to the membrane is critical for DVL-2 docking of B-raf. Taken together, these data strongly suggest that activation of ERK1/2 pathway by Wnt-3a is regulated through recruitment of DVL-2 to the plasma membrane and docking of B-raf.

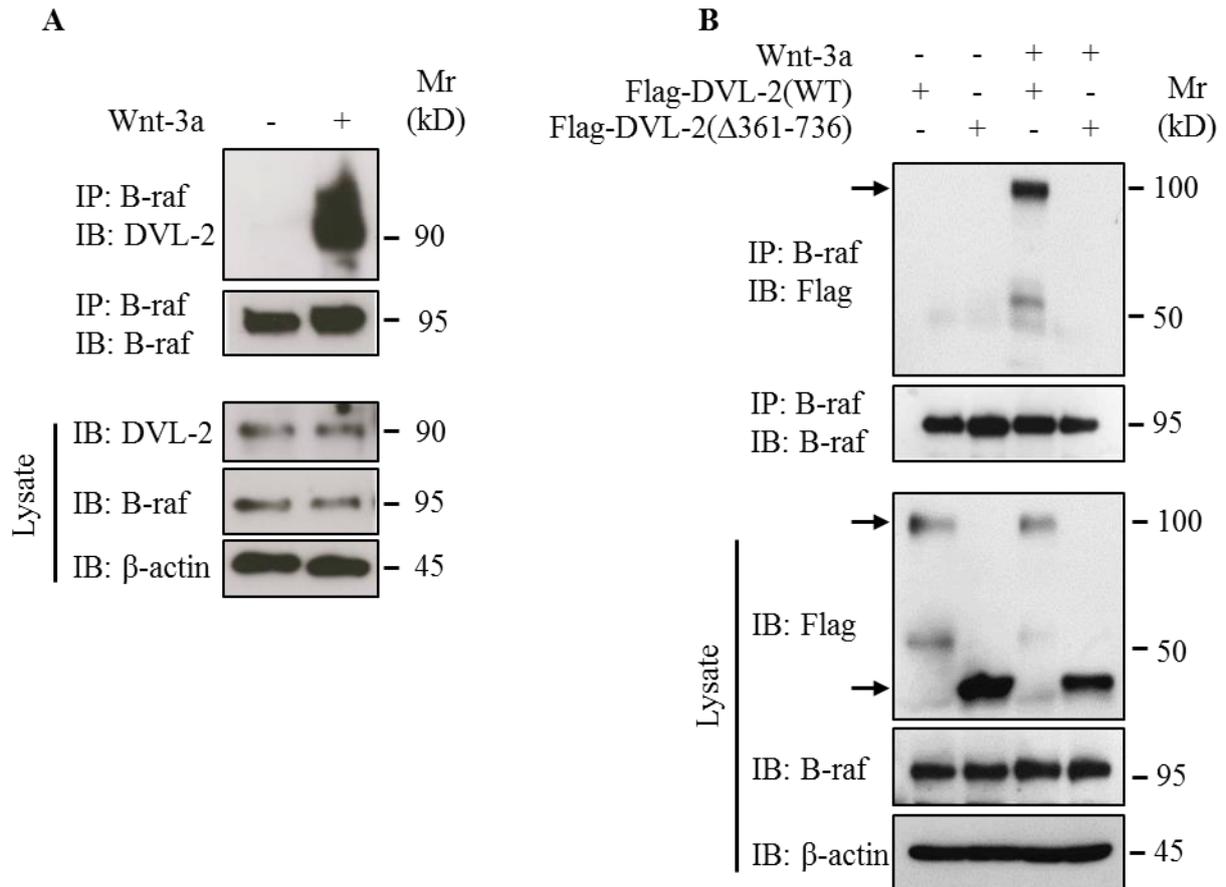


Figure 43. DVL-2 docks B-raf in Wnt-dependent manner

DVL-2 interacts with B-raf through the C-terminal domain. (A) Primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for 1 h and cell lysates were assessed after immunoprecipitation with anti-B-raf by immunoblot analysis with anti-DVL-2 or anti-B-raf antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-DVL-2 and anti-B-raf antibodies. β -actin was used as loading control. (B) Primary chondrocytes were transfected with expression vector for Flag-tagged wild-type DVL-2 or Flag-DVL-2(Δ 361-736) for 24 h, then treated with Wnt-3a (100 ng/ml) or vehicle for 1 h, assessed after immunoprecipitation with anti-B-raf by immunoblot analysis with anti-Flag or anti-B-raf antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-Flag and anti-B-raf antibodies. β -actin was used as loading control. One representative blot of three independent experiments is shown.

To further investigate the role of B-raf in Wnt-3a-induced activation of ERK1/2 signaling pathway, we stimulated chondrocytes containing the wild-type or D594V kinase-dead mutant of B-raf with Wnt-3a then analyzed the phosphorylation of ERK1/2. As shown in figure 44A, forced expression of wild-type B-raf increased

phosphorylation of ERK1/2 which was increased further in response to Wnt-3a. In contrast, expression of D594V mutant significantly reduced basal activation of ERK1/2 and abrogated the activation of ERK1/2 by Wnt-3a (Figure 44B), indicating that B-raf mediates Wnt-3a-induced activation of ERK1/2 signaling pathway.

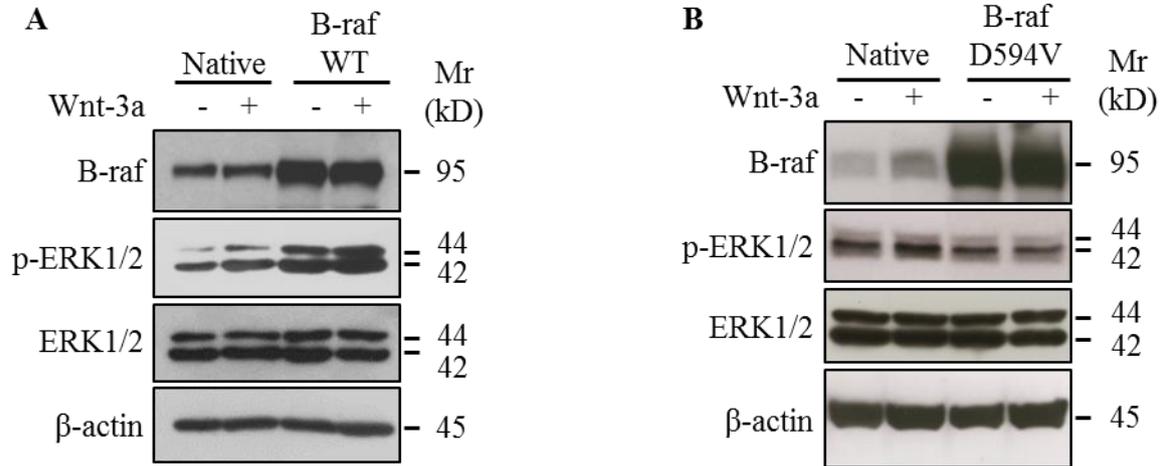


Figure 44. **B-Raf is essential for Wnt-3a activation of ERK1/2 signaling**

(A) With empty vector (Native) or expression vector for wild-type B-raf, or (B) with empty vector (Native) or expression vector for dead mutant of B-raf (D594V), and treated with Wnt-3a (100 ng/ml) or vehicle for 12 h, probed with anti-B-raf, anti-p-ERK1/2 or anti-ERK1/2. β -actin was used as loading control ($n=3$). One representative blot of three independent experiments is shown.

F. CaMKII mediates Wnt-3a-induced activation of ERK1/2

It has been reported that Wnt-3a activates the non-canonical Ca^{2+} /CaMKII-dependent pathway in chondrocytes (Nalesso et al., 2011). In agreement with this study, we found that treatment of chondrocytes with Wnt-3a induced the phosphorylation of CaMKII α in a time-dependent manner (Figure 45A). Noteworthy, CaMKII has been shown to mediate the Ca^{2+} -induced activation of ERK1/2 in rat aortic VSM cells (Abraham et al., 1997). We then hypothesized that CaMKII α may mediate Wnt-3a-induced activation of ERK1/2 in chondrocytes. To test this hypothesis, primary chondrocytes were pre-treated or not with the specific CaMKII inhibitor KN-93 or with KN92, an analogue of KN-93 which does not inhibit CaMKII, then stimulated with Wnt-3a. Analysis of cell extracts showed that not only KN-93 prevented Wnt-3a-induced activation of CaMKII α

but almost completely abrogated the activation of ERK1/2 signaling by Wnt-3a (Figure 45B). In contrast, KN92 did not prevent the activation by Wnt-3a of either CaMKII α or ERK1/2 (Figure 45B). These results indicate that the activation of non-canonical Wnt/ERK1/2 signaling pathway by Wnt-3a requires the activation of CaMKII α . In line with this, inhibition of CaMKII α prevented down-regulation of the expression of SYND4 (Figure 45C) and COL2A1 (Figure 45D) by Wnt-3a.

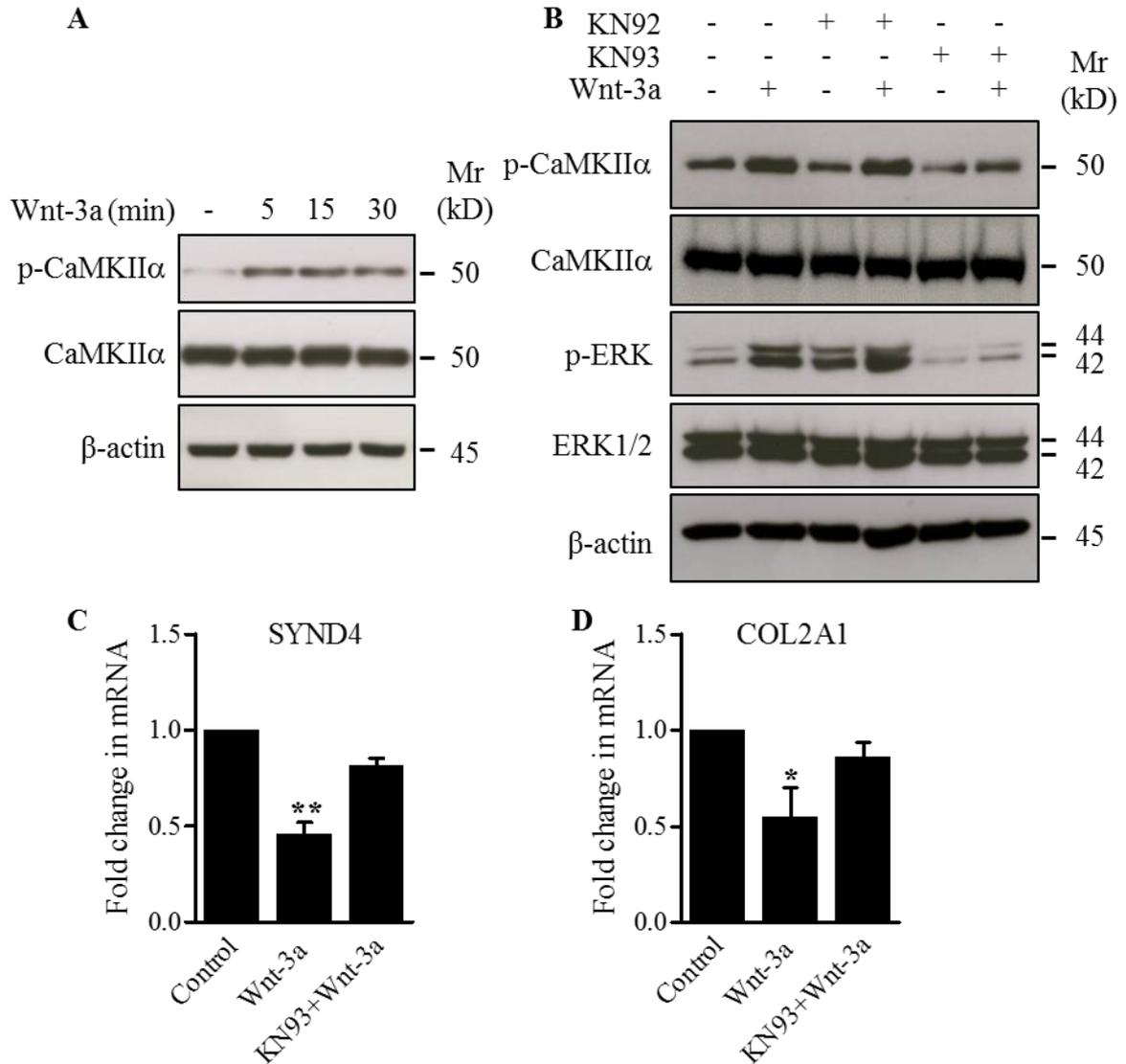


Figure 45. Wnt-3a-induced activation of ERK1/2 pathway is mediated through activation of CaMKII α

(A) Wnt-3a induces the phosphorylation of CaMKII α . Human primary chondrocytes were stimulated with Wnt-3a (100 ng/ml) or vehicle for indicated time, then cell lysates

were analysed by Western blotting with anti-p-CaMKII α and anti-CaMKII α antibodies. β -actin was used as loading control (n=3). (B) Wnt-3a-induced activation of ERK1/2 is blocked by inhibition of CaMKII α . Immunoblot analysis of total cell lysates of primary chondrocytes pre-treated with KN92, KN93 or vehicle (DMSO) for 12 h and stimulated with Wnt-3a or vehicle for 30 min, probed with anti-p-CaMKII α , anti-CaMKII α , anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control (n=3). (C and D) Inhibition of CaMKII α by KN93 rescued the down-regulation of (C) SYND4 and (D) COL2A1 by Wnt-3a, as evaluated by PCR (n=4). Data are representative of at least three experiments. Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

We showed above that B-raf mediates Wnt-3a-induced activation of ERK1/2 signaling pathway. To further investigate the role of CaMKII α on the activation of ERK1/2 pathway, we stimulated chondrocytes containing the active mutant V600E of B-raf with Wnt-3a in the presence of KN92 and KN93, respectively then analysed the activation of ERK1/2. As expected, expression of B-raf V600E mutant strongly activated ERK1/2, however treatment with Wnt-3a did not induce any significant increase in ERK1/2 phosphorylation (Figure 46). In addition, KN 93 did not reduce the phosphorylation of ERK1/2, indicating that when B-raf is activated CaMKII α does not mediate to the following ERK activation.

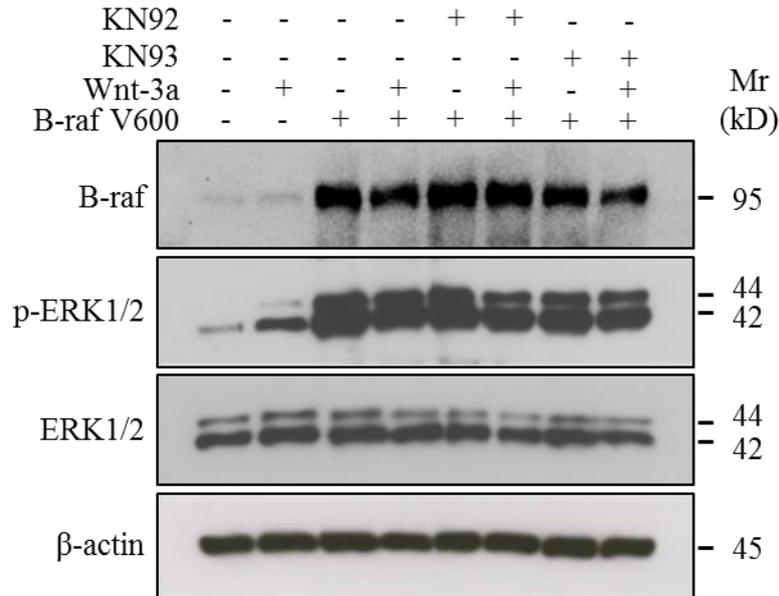


Figure 46. KN93 lost its effect on ERK1/2 when B-raf is activated

(A) *Wnt-3a* induces the phosphorylation of CaMKII α . Human primary chondrocytes Immunoblot analysis of total cell lysates of primary chondrocytes transfected with expression vector for active mutant of B-Raf (V600) pre-treated with KN92 or KN93, or DMSO for 12 h and stimulated with *Wnt-3a* or vehicle for 30 min, probed with anti-B-raf, anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control ($n=3$). Data are representative of at least three experiments.

G. DVL-2 is crucial for non-canonical Wnt activation of

CaMKII α

Our results showed that DVL-2 and SYND4 play a critical role in *Wnt-3a*-induced activation of non-canonical Wnt/ERK1/2 pathway. Given that activation of this pathway requires the activation of CaMKII α , we therefore hypothesized that DVL-2 and SYND4 may be involved in *Wnt-3a* activation of CaMKII α . To test this hypothesis, we stimulated chondrocytes that were knockdown for DVL-2 and SYND4, respectively with *Wnt-3a* then analysed the activation of CaMKII α and ERK1/2. As shown in figure 45, knockdown of DVL-2 strongly reduced the activation of CaMKII α and ERK1/2 by *Wnt-3a*, whereas knockdown of SYND4 decreased the basal activation of CaMKII α but did not prevent the phosphorylation of CaMKII α in response to *Wnt-3a*, arguing

for a dependence of CaMKII α activation by Wnt-3a on DVL-2 but not on SYND4. On the other hand, SYND4 knockdown abrogates the activation of ERK1/2 signaling by Wnt-3a without impairing CaMKII α activation (Figure 47), suggesting a model in which SYND4 functions downstream of DVL-2.

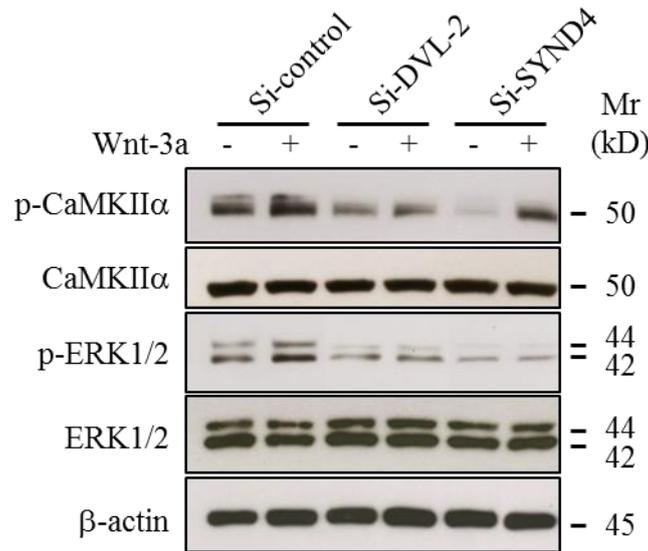


Figure 47. DVL-2 not SYND4 is crucial for non-canonical Wnt activation of CaMKII α

(A) Immunoblot of total cell lysates of primary chondrocytes transfected with siRNA specific to SYND4 (Si-SYND4), DVL-2 (Si-DVL-2) or siRNA control (Si-control) for 48 h and stimulated with Wnt-3a or vehicle for 12 h, probed with anti-p-CaMKII α , anti-CaMKII α , anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control (n=3). Data are representative of at least three experiments.

H. Syndecan 4 interact with CaMKII α and DVL-2

As SYND4 was required for the activation of ERK1/2 by Wnt-3a, we hypothesized that this HSPG may relay Wnt signaling through interaction with CaMKII α . Co-immunoprecipitation assays were performed using extracts from cells that have been transiently transfected with HA-SYND4. Co-immunoprecipitation indicated that HA-SYND4 physically interacts with CaMKII α (Figure 48). Interestingly, we found that the interaction between SYND4 and CaMKII α was significantly enhanced in the presence of Wnt-3a (Figure 48). These results indicated that although SYND4 is not

required for activation of CaMKII α by Wnt-3a, this HSPG interact with CaMKII α in Wnt-3a-dependent manner and suggested that SYND4 and CaMKII α interact to regulate non-canonical Wnt signaling in chondrocytes.

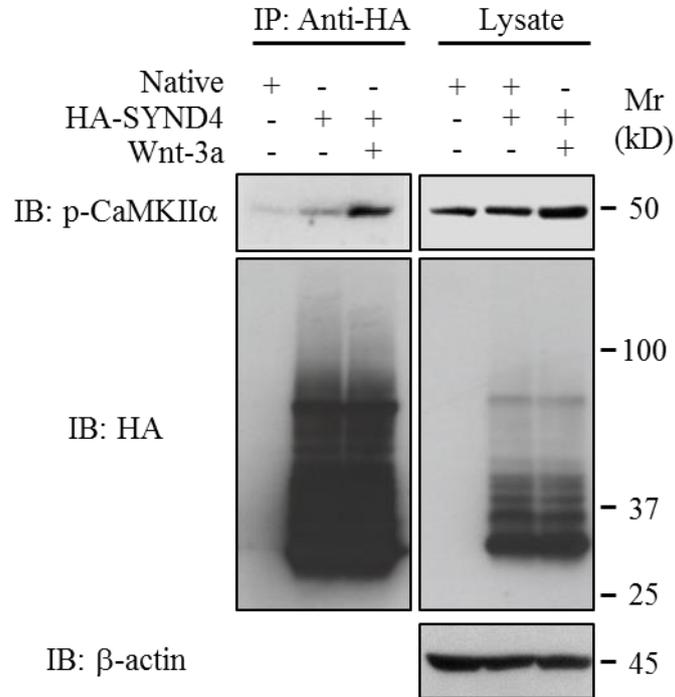


Figure 48. Syndecan 4 interacts with CaMKII α

Wnt-3 promotes the recruitment of p-CaMKII α by SYND4. Primary chondrocytes were transfected with empty vector (Native) or expression vector for HA-tagged SYND4 for 24 h, then treated with Wnt-3a (100 ng/ml) or vehicle for 1 h, assessed after immunoprecipitation with anti-HA by immunoblot analysis with anti-p-CaMKII α or anti-HA antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-p-CaMKII α and anti-HA antibodies. β -actin was used as loading control. SYND4 contains four glycosaminoglycan chains and as expected migrates as a broad smear of 30–200 kDa. Data are representative of at least three experiments.

SYND4 has been shown to interact with DVL (Munoz et al., 2006), a possible interaction between SYND4 and DVL-2 was examined using extracts from cells that have been transfected with Flag-DVL-2 and HA-SYND4. In agreement with the previous study, DVL-2 was co-immunoprecipitated with SYND4 (Figure 49). Taken together, our data indicate that SYND4 is a component of non-canonical Wnt pathway

that regulates the activation of this pathway through interaction with DVL-2 and CaMKII α .

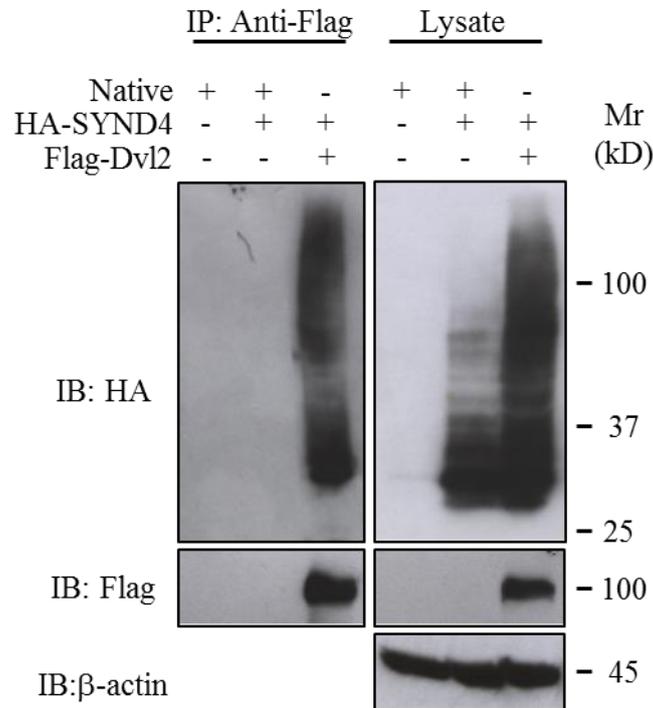


Figure 49. Syndecan 4 interacts with DVL-2

Primary chondrocytes were transfected with empty vector (Native) or expression vector for HA-tagged SYND4 or Flag-tagged DVL-2 for 24 h then assessed after immunoprecipitation with anti-Flag by immunoblot analysis with anti-HA and anti-Flag antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-HA and anti-Flag antibodies. β -actin was used as loading control. Data are representative of at least three experiments.

I. Frizzled 6 mediated non-canonical Wnt-3a signaling pathway

Frizzled (Fz) is a family of G protein-coupled receptor proteins that serves as receptors in the Wnt signaling pathway. To address the question of which of the known *FZD* genes could be involved in Wnt-3a activation of Wnt non-canonical pathway, we first performed qRT-PCR to monitor the expression pattern of all genes encoding Wnt receptors in human primary chondrocytes. As described before, Fz-6 and Fz-8 are predominantly expressed in chondrocytes compared to other Fz receptors. Noteworthy, Fz-6 has been associated with non-canonical Wnt signaling (Golan et al., 2004; Gray

et al., 2011; Guo et al., 2004). To evaluate the role of Fz-6 in Wnt-3a activation of non-canonical Wnt signaling in primary chondrocytes, we knocked down its expression and evaluated the impact of Fz-6 deficiency on Wnt-3a-induced down-regulation of *COL2A1* expression and on the phosphorylation of CaMKII α and ERK1/2. The knockdown of Fz-6 abrogated Wnt-3a-induced down-regulation of the non-canonical Wnt downstream target gene *COL2A1* (Figure 50A). Given that non-canonical Wnt-3a signal through activation of CaMKII α and ERK1/2, we analysed the phosphorylation status of CaMKII α and ERK1/2 in response to Wnt-3a in Fz-6-deficient chondrocytes. As shown in figure 50B, knockdown of Fz-6 reduced the phosphorylation of both kinases and abrogated their activation by Wnt-3a, indicating that in Fz-6-deficient chondrocytes the activation of non-canonical Wnt signaling is no more modulated by Wnt-3a. Taken together, these observations support the conclusion that Fz-6 mediates non-canonical Wnt-3a signaling in chondrocytes.

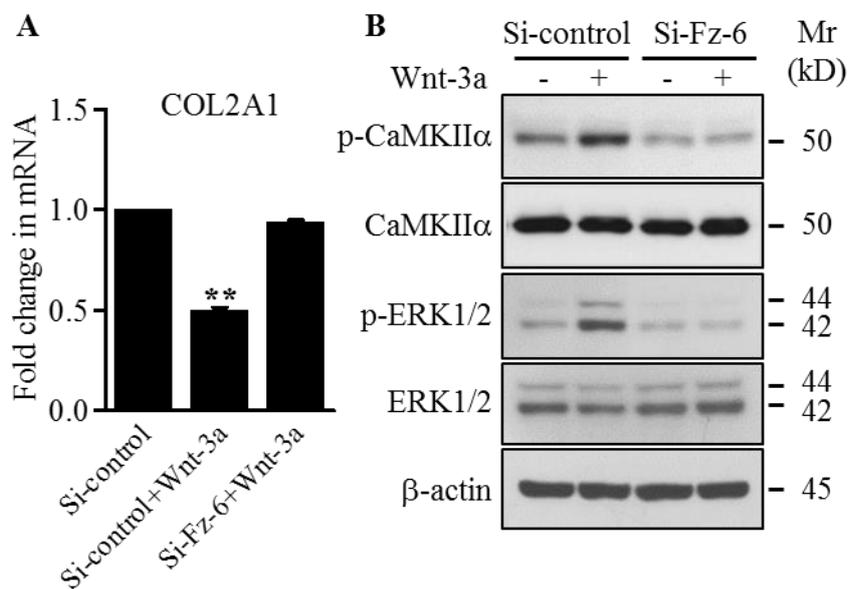


Figure 50. Frizzled6 mediates non-canonical Wnt-3a-induced loss of chondrocyte phenotype

(A and B) Fz-6 is essential for Wnt-3a-induced down-regulation of *COL2A1* and activation of non-canonical pathway. (A) Knockdown of Fz-6 suppressed Wnt-3a-induced down-regulation of *COL2A1* ($n=3$), as evaluated by Q-PCR. (B) Knockdown of Fz-6 prevented Wnt-3a-induced activation of CaMKII α and ERK1/2. Human

*primary chondrocytes were transfected with Si-Fz-6 or Si-control for 48 h and treated with Wnt-3a (100 ng/ml) or vehicle for a further 30 min and the protein level of p-CaMKII α , CaMKII α , p-ERK1/2 and ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (NS, statistically not significant; *, $p < 0.05$; **, $p < 0.01$).*

DISCUSSION

DISCUSSION

I. Part I - Wnt-3a regulates cartilage degeneration through the β -catenin dependent pathway

Since the level of β -catenin was found to be increased in chondrocytes of arthritic cartilage (Hwang et al., 2004), Wnt signaling was considered to be associated with cartilage destruction in osteoarthritis. Recently, accumulating evidences based on experimental animal models have shown that Wnt/ β -catenin signaling drives the expression of ADAMTS-4 (Oh et al., 2012; Yasuhara et al., 2010; Yuasa et al., 2008). Here, in remarkable contrast to what has been observed in animal models, we provide evidence that Wnt/ β -catenin signaling is a potent inhibitor of ADAMTS-4 in human primary chondrocytes and cartilage explants. Similar observation was made for the matrix metalloproteinase-13 (MMP13) which was down regulated by Wnt/ β -catenin pathway in human chondrocytes, whereas in animal chondrocytes it was up-regulated (Ma et al., 2012). These observations strongly argue for differential regulation of ADAMTS-4 by Wnt/ β -catenin signaling in human and animal chondrocytes.

The presence of four putative NF- κ B motifs in human ADAMTS4 gene promoter (Tian et al., 2013) suggests that it may be regulated by NF- κ B and that Wnt-3a may inhibit ADAMTS-4 by suppressing the activation of NF- κ B signaling. In agreement with this hypothesis, the knockdown of NF- κ B/p65/RelA strongly reduced basal and IL-1 β -induced expression of ADAMTS-4. Investigation of the mechanism involved in Wnt-3a down regulation of ADAMTS4 showed that Wnt-3a impaired the activation of NF- κ B signaling by reducing nuclear translocation of NF- κ B/p65. In line with this, Wnt-3a inhibited the expression of IL6 and IL-1 α , two established target genes of NF- κ B pathway in human chondrocytes.

In human chondrocytes it is well known that activation of NF- κ B signaling by proinflammatory cytokines such as IL-1 β potently induced cartilage matrix degradation.

Investigation of the effect of Wnt/ β -catenin signaling on the stimulatory effect of IL-1 β on ADAMTS-4 expression and activity revealed that Wnt/ β -catenin signaling counteracts in part these effects by reducing the nuclear translocation of NF- κ B/p65 induced by the cytokine. This suggests that Wnt/ β -catenin signaling constitute a feed-back loop counteracting NF- κ B signaling in cartilage and supports the notion that this signaling pathway may safeguard cartilage from degradation. In line with this, we showed that activation of β -catenin signaling in cultured human cartilage explant by Wnt-3a treatment reduced IL-1 β -mediated loss of proteoglycan.

It also suggest that increased levels of β -catenin observed in human OA cartilage may result from activation of Wnt/ β -catenin signaling in an attempt of human chondrocytes to counteract the catabolic effects of inflammatory cytokines such as IL-1 β to slow down matrix degradation. Accordingly, depletion of chondrocytes from β -catenin induced basal expression of ADAMTS-4 and potentiates the effect of IL-1 β on the expression of this important aggrecanase, indicating that β -catenin exerts an inhibitory effect on ADAMTS-4 expression. This revealed that β -catenin might have an anticatabolic role in human chondrocytes.

Because Fz proteins are serpentine receptors and belong to the major class of target proteins for currently available drugs, therefore addressing the question of which of the 10 known Fz receptors are required to regulate canonical Wnt signaling in human primary chondrocytes could also be relevant in terms of identifying novel therapeutic options to treat OA disorders (Overington et al., 2006; Wise et al., 2002). We found that Fz-6 and Fz-8 are the most expressed Fzs in human primary chondrocytes and bring evidence that Fz-8 mediates the activation of canonical Wnt/ β -catenin pathway. Similarly, Fz-8 was shown to mediates canonical Wnt signaling in *Xenopus* (Kühl et al., 2000), in lung cancer cells (Wang et al., 2012) and in osteoclasts during bone remodelling (Albers et al., 2013). Interestingly, we showed that expression of Fz-8 was down regulated by canonical Wnt signaling and this was prevented by the knockdown of β -catenin, therefore suggesting β -catenin exerted a negative feed-back loop control

on the canonical Wnt signaling mediated by Fz-8 receptor in human primary chondrocytes.

Relaying signal from receptor to downstream effectors, DVLs participate in both canonical and noncanonical pathways. We examined which of the three DVLs are required to regulate canonical Wnt signaling mediated by Wnt-3a in human primary chondrocytes. We found that DVL1 and DVL3 play a prominent role in this process compared to DVL2. In agreement with this, it has been shown that the activation of Wnt canonical signaling pathway by Wnt-3a in mouse F9 teratocarcinoma cells was most sensitive to changes in the abundance of either DVL3 or DVL1. Changes in expression of DVL2, the most abundant of the three isoforms, resulted in the least effect on canonical signaling (Lee et al., 2008). In these cells, DVL1 and DVL3 displayed similar patterns in plasma membrane and nuclear fractions in response to stimulation by Wnt-3a (Yokoyama et al., 2007). In addition, knockdown of DVL2 in Chinese hamster ovary cells markedly reduced Wnt-3a-dependent cell motility mediated by the activation of small GTPase RhoA through a β -catenin-independent signaling pathway (Endo et al., 2005).

In summary, the results of parte I reveal that activation of Wnt/ β -catenin signaling pathway by Wnt-3a in human primary chondrocytes is mediated through Fz-8 and DVL1/3 and results in β -catenin-dependent reduction of NF- κ B activity (Figure 51). Activation of this pathway counteracts IL-1 β -induced ADAMTS4 expression and activity and exerts a protective role on proteoglycan degradation induced by the cytokine in human cartilage. Therefore, we speculate that Wnt may have a protective role in OA. The higher level of β -catenin found in OA cartilage may be protective on NF- κ B activation and cartilage degradation induced by the proinflammatory cytokines.

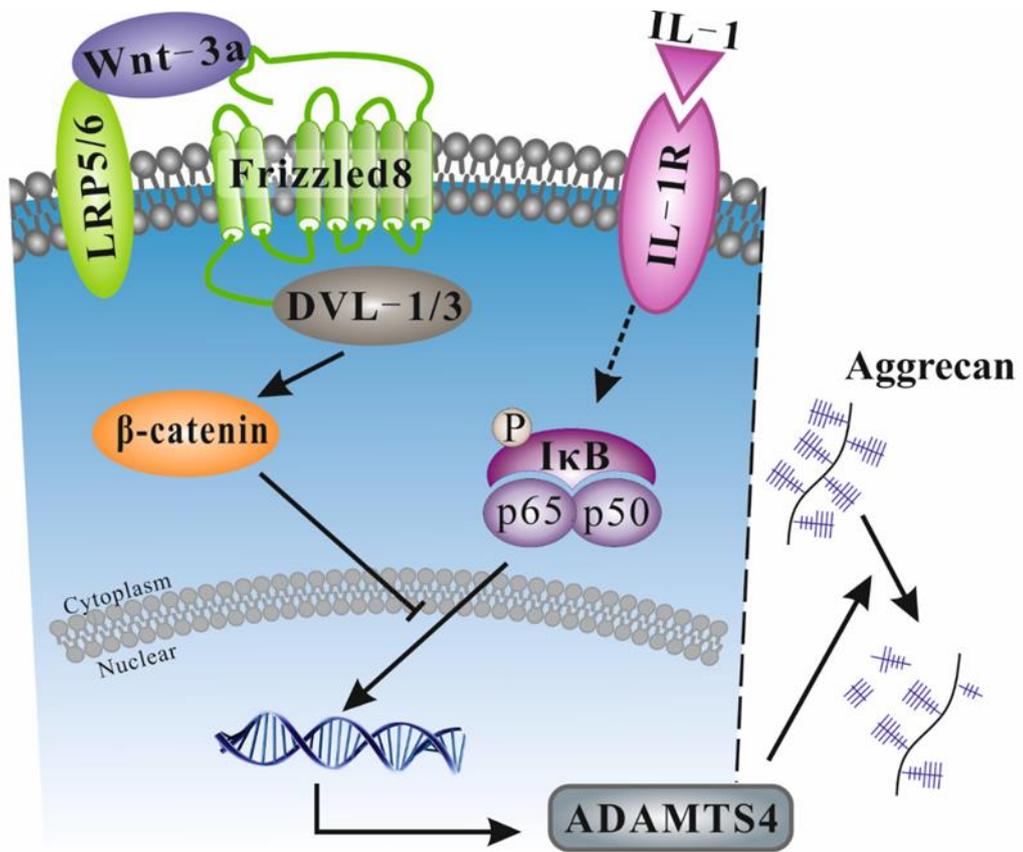


Figure 51. **Wnt canonical pathway in human chondrocyte**

Wnt-3a activates Fz-8 and DVL-1/3 then suppresses the expression of ADAMTS-4 via the inhibition of NFκB pathway.

II. Part - II Wnt-3a regulates chondrocytes de-differentiation through the noncanonical pathway

The key initiating events in OA are still unknown, but loss of phenotypic stability of articular chondrocytes has been proposed as an early event in the onset of the disease (Haag et al., 2008). De-differentiation of chondrocytes occurs through a complex programme with similarities to terminal differentiation of chondrocytes (hypertrophy), as can be found in the growth plate (Dreier, 2010). This triggers a disturbed cartilage homeostasis favoring degenerative changes (Dreier, 2010; Pap and Korb-Pap, 2015). Wnt signaling has a pivotal role in regulating chondrocyte differentiation during endochondral ossification and was also shown to play a regulative role in OA cartilage. Indeed, a role of non-canonical Wnt signaling in cartilage degeneration is suggested by repression of chondrocyte phenotypic marker gene expression by Wnt-5a (Ryu and Chun, 2006) and Wnt-3a (Nalesso et al., 2011), leading to chondrocyte hypertrophic differentiation. Here, we demonstrated that Wnt-3a-induced loss of chondrocyte phenotype is mediated by Fz-6 through CaMKII α -dependent activation of ERK1/2 signaling pathway. Indeed, knockdown of ERK1/2 or chemical inhibition of its activation prevented Wnt-3a-induced hypertrophic de-differentiation of chondrocytes, which is notoriously associated with down-regulation of COL2A1 (Lefebvre et al., 1998; Taschner et al., 2008), indicating that activation of ERK1/2 is a key event in Wnt-3a-induced loss of chondrocyte phenotype. Most important, we identified SYND4 and DVL-2 as key components of non-canonical Wnt/CaMKII α /ERK1/2 pathway that regulate the signaling by recruiting CaMKII α and B-raf, respectively. We demonstrated that SYND4 regulates, and is essential for non-canonical Wnt-induced loss of chondrocyte phenotype and activation of ERK1/2 signaling. On the other hand, our study revealed that Wnt-3a down-regulates the expression of SYND4 through activation of ERK1/2, therefore establishing a negative feed back loop to modulate the signaling.

SYND4 has been shown to regulate convergent and extension movements in *Xenopus* embryo through non-canonical Wnt pathway by interacting with DVL (Muñoz et al., 2006). We demonstrated here that DVL-2 relays Wnt-3a non-canonical signaling in primary chondrocytes, as its knockdown prevented Wnt-3a-induced activation of ERK1/2 pathway and down-regulation of COL2A1. In addition, as observed during PCP signaling in *Xenopus* (Muñoz et al., 2006), we found that DVL-2 interacts with SYND4. However, in contrast to SYND4, silencing of DVL-2 inhibited also Wnt-3a-induced activation of CaMKII α , in addition to that of ERK1/2 signaling. Noteworthy, deactivation of ERK1/2 by inhibition of CaMKII was previously reported in several cell types (Cipolletta et al., 2010; Liang et al., 2014; Salzano et al., 2012). Interestingly, inhibition of CaMKII α by KN93, a widely used CaMKII activity inhibitor, impaired the activation of ERK1/2 by Wnt-3a in primary chondrocytes, suggesting a cross-talk between CaMKII α and ERK1/2 pathway during non-canonical Wnt signaling. These data demonstrate that ERK1/2 phosphorylation induced by Wnt-3a is dependent on the activation of CaMKII α and that the MEK/ERK cascade is a key regulator that links the CaMKII signaling to loss of chondrocyte phenotype. In addition, given that DVL-2 but not SYND4 is critical for Wnt-3a activation of CaMKII α suggest that DVL-2 functions upstream of SYND4 in non-canonical Wnt signaling pathway.

An important issue we addressed here is the mechanism of activation of ERK1/2 pathway by non-canonical Wnt signaling, which promotes chondrocyte de-differentiation. It has been shown that SYND4 is highly expressed in hypertrophic chondrocytes during embryonic development and during OA, suggesting a role in chondrocyte de-differentiation. Interestingly, loss of SYND4 in mice protects from development of OA-like disease and that was associated with decreased ERK1/2 signaling (Echtermeyer et al., 2009). Accordingly, we showed that knockdown of SYND4 reduced basal activation of ERK1/2 signaling and impaired its activation by Wnt-3a in human primary chondrocytes. SYND4 has been shown to interact with different partners through its cytoplasmic domain (Elfenbein and Simons, 2013). Interestingly, co-immunoprecipitation studies revealed, for the first time, that SYND4

interacts with phosphorylated CaMKII α and that this interaction is strongly enhanced on Wnt-3a treatment. As SYND4 is required for activation of ERK1/2, we can speculate that interaction between SYND4 and CaMKII α might facilitate CaMKII-dependant activation of ERK1/2 signaling pathway. On the other hand, co-immunoprecipitation studies revealed that DVL-2 docks B-raf in Wnt-dependent manner. Further analysis showed that the C-terminal region of DVL-2 including the DEP domain, which is critical for membrane translocation of DVL-2, is also critical for B-raf docking. Therefore, in the presence of Wnt ligand, interaction between DVL-2 and SYND4 may promote assembly of CaMKII α and B-raf complex necessary to relay Wnt-3a activation of ERK1/2 signaling pathway. In this case, one can speculate that B-raf may directly be activated by CaMKII α and transduces signals to MEK and ERK1/2 MAPK. Of note, it has been shown that CaMKII binds Raf-1 and that is necessary for ERK1/2 activation by different stimuli and in different cell types (Illario et al., 2003; Monaco et al., 2009). Recently, Salzano et al reported that CaMKII directly phosphorylates Raf-1 (Salzano et al., 2012). However, an important issue remains to be explored is as to whether CaMKII α phosphorylates B-raf.

Fz-6 has been associated with non-canonical Wnt signaling (Abidin et al., 2015; Gray et al., 2011; Guo et al., 2004). Golan et al. (2004) showed that Fz-6 did not activate the canonical Wnt pathway in HEK293 cells following exposure to various Wnts, including Wnt-3a (Golan et al., 2004). Kühl et al. (2000) demonstrated that in *Xenopus* Fz-3, Fz-4 and Fz-6 activate CaMKII and promote ventral cell fate, whereas Fz-7 and Fz-8 activate the β -catenin pathway and induce axis duplication but do not activate CaMKII (Kühl et al., 2000). We have examined the role of the Wnt signaling receptor Fz-6 in canonical and non-canonical Wnt/CaMKII α /ERK1/2 signaling in primary chondrocytes and demonstrated that Fz-6 does not affect the activation of β -catenin pathway as monitored by stabilization of β -catenin and expression of the target gene *AXIN2*. In contrast, Fz-6 is critical for the activation of non-canonical pathway as demonstrated by the phosphorylation status of CaMKII α and ERK1/2, and the

expression of COL2A1. Knockdown of Fz-6 impaired the phosphorylation of both CaMKII α and ERK1/2 by Wnt-3a and prevented loss of chondrocyte phenotype.

In summary, we have identified SYND4 as an essential component of non-canonical Wnt/CaMKII α /B-raf/ERK1/2 pathway that regulate chondrocyte phenotype. In the presence of Wnt-3a, Fz-6 activates non-canonical signaling by triggering the docking of CaMKII α to SYND4 and that of B-Raf to DVL-2 as depicted in Figure 52. Interaction between SYND4 and DVL-2 may bring together CaMKII α and B-Raf probably to allow the activation of B-raf by CaMKII α and to transduce signals to MEK and ERK1/2 MAPK. In a feedback loop, non-canonical Wnt down-regulates the expression of SYND4 to negatively modulate the signaling. Our results may help provide clearer understanding of non-canonical Wnt CaMKII/ERK1/2 signaling pathway that is essential in both normal development and OA pathogenesis. Our finding may also provide new markers and targets for OA treatment.

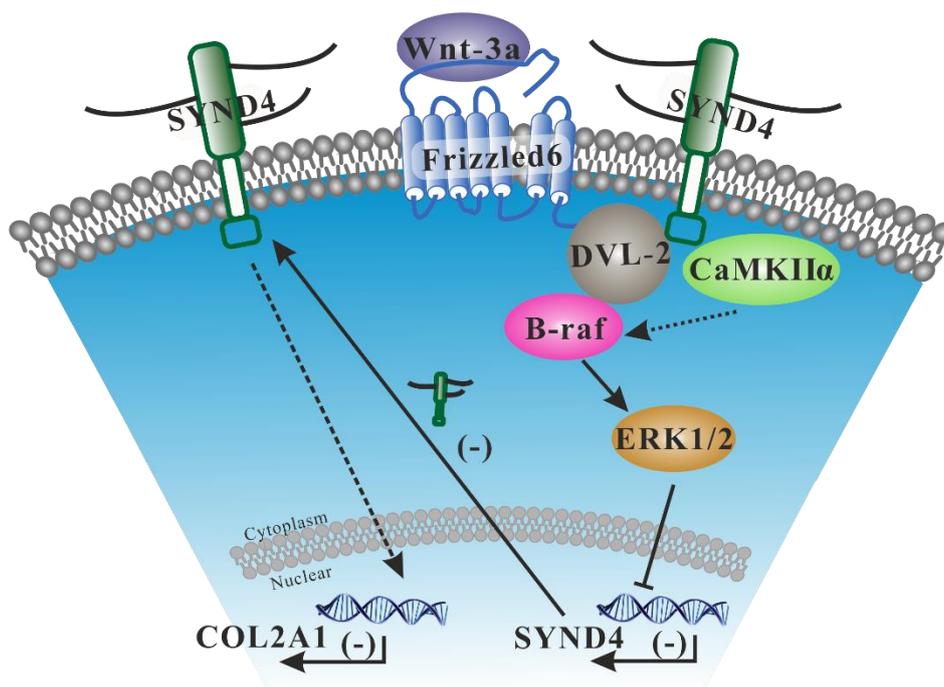


Figure 52. **A working model for Wnt nocanonical pathway**

The role of SYND4 in the regulation of non-canonical Wnt/CaMKII/B-raf/ERK1/2 pathway and chondrocyte phenotype.

CONCLUSION AND PERSPECTIVE

Conclusion and Perspective

The main objective of this thesis work was to elucidate the role of Wnt signaling pathways in cartilage degradation and chondrocytes de-differentiation. Focusing on the regulation of two representative genes expression, *ADAMTS-4* (most important aggrecanase in human cartilage degeneration) and *COL2A1* (phenotypic marker of chondrocytes). Exogenous Wnt-3a stimulates the accumulation of β -catenin through Fz-8 and DVL-1/3, then activation of this canonical cascade inhibits NF κ B pathway by suppressing the translocation of p65 into nucleus, therefore leading to downregulation of *ADAMTS-4* expression; On the other hand, Wnt-3a activates the noncanonical pathway through Fz-6 and triggering the docking of CaMKII to SYND4 and that of B-raf to DVL-2. The interaction of SYND4 with DVL-2 may facilitate the activation of B-raf/ERK1/2 pathway which down-regulates SYND4 and inactivates PCK α which is required for *COL2A1* expression. Interestingly, SYND4, the transmembrane heparin sulfate proteoglycan, is inhibited by Wnt-3a through ERK1/2 pathway, therefore forming a feedback loop to negatively regulate the signaling.

As the most common form of arthritis, OA affects especially the old people in a highly prevalent condition. However, only pain reduction and symptom control treatment strategies exist to remedy this disease. For instant, pharmaceutical treatments and bioengineering techniques aiming to halt disease progress or reverse the cartilage damage are not available yet.

As one of the signaling cascades triggered by the OA risk factors in the chondrocyte, Wnt signaling is an attractive potential target for the treatment. Our study demonstrates that activation of Wnt signaling has different influences on articular chondrocytes. The activation of β -catenin-dependent pathway attenuates the effect of NF κ B, and suppresses the expression of aggrecanase *ADAMTS-4* and inflammatory factors like IL-1 and IL-6. Others have demonstrated the inhibition of the collagenase induced by Wnt/ β -catenin pathway in human chondrocytes (Ma et al., 2012). As the NF κ B pathway

is activated and promotes the inflammation in OA chondrocytes, by inhibiting its activation, Wnt canonical pathway should play a protective role in the pathological process of OA. Inversely, Wnt noncanonical pathway seems inhibits the synthesis of the type II collagen, therefore induces the loss of chondrocytes phenotype. Activation of CaMKII and ERK1/2 which are effectors of Wnt noncanonical pathway reduces the mRNA level of COL2A1. Prospectively, the molecules or drugs which can inhibit the Wnt noncanonical pathways and/or enhance the canonical pathways may protect the cartilage from destruction. Interestingly, we showed that, Wnt canonical and noncanonical pathways are mediated by different Frizzled and DVL proteins, thus, our study revealed promising targets to develop new drugs against OA disease.

Current techniques used for repair of articular cartilage lesions include autologous chondrocyte transplantation, microfracture, and mosaicplasty, however they are limited to isolated areas of chondral loss and are less adaptable to the generalized degenerative changes. Nowadays, chondrocytes and mesenchymal stem cells (MSCs) based tissue engineering and repair are being actively explored, and how to mimics the native morphogenesis of cartilage then in order to satisfy the fundamental requirements of successful cartilage healing is under investigation (Makris et al., 2015). Therefore, in cell-based applications, a better understanding of how to maintain the chondrocytic phenotype of implanted chondrocytes and the chondrogenic differentiation of MSCs must be established. In view of the notable effect of the Wnt signaling pathway on the chondrogenic differentiation and de-differentiation, regulation of this signaling cascade will be a promising manner to control the phenotype of the chondrogenic cells. Since our study demonstrated the role of Wnt noncanonical pathway in chondrocytes de-differentiation, several drugs such as antagonists of Fz-6 or DVL-2, and potential inhibitors of CaMKII α may stabilize chondrocyte phenotype in cell-based bioengineering.

Although animal experiments are essential to elucidate the regulation of cartilage biology and pathology, caution need to be exercised when apply these findings and knowledge to human. In animal models, the basal activation of β -catenin supports the

chondrocyte survival and regenerative potential (Yasuhara et al., 2010; Yuasa et al., 2008). However, in human chondrocyte Wnt/ β -catenin signaling reduces the expression of destructive enzymes and counteracts the effect of IL-1 β . Likewise, syndecan-4 is highly expressed in the human OA chondrocytes and in animal models also. In mice, the deficiency of syndecan4 significantly impaired the activation of ADAMTS-5 and protect the cartilage from OA development (Echtermeyer et al., 2009). However, in human chondrocytes, inhibition of the syndecan4 notably reduced the synthesis of collagen type II and increased the expression of aggrecanases and collagenases, and amplifies the destructive effect of IL-1 β . Thus, diversity and complexity in regard to human biology should be considered to be much larger than in animals.

In this thesis we described a complex model of the Wnt signaling pathways in human chondrocytes, and elucidated the function of different effectors of Wnt signaling, however many details deserve to be further explored. Lower nuclear p65 level following β -catenin accumulation in the cells may be explained by the post-translational modification of p65. Even though we excluded the possibility that Wnt-3a inhibits the acetylation of p65 at Lys310 or phosphorylation at Ser468 or Ser510, whether acetylation at Lys122 (Stokes et al., 2001) or phosphorylation at Ser276 (King et al., 2011) is changed by Wnt still need to be investigated. In the case of Wnt noncanonical pathway, the prediction of interaction between the CaMKII and B-raf is essential for noncanonical-pathway-induced loss of chondrocyte phenotype, therefore as many inhibitors or regulators of the activation of these two proteins exist, it is interesting to test them as anti-OA drugs.

Our study used monolayer culture of human chondrocytes, it is therefore important to further confirm these results in vivo studies. Inhibitors and other technology which can regulate the Wnt signaling pathway deserve to be tested in vivo to explore their potential effect in treating OA or clinical trial to investigate the side effects.

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RESUME EN FRANÇAIS

Résumé en français

I. Situation du sujet

L'arthrose est une maladie caractérisée par la destruction du cartilage. La prévalence de cette pathologie est importante chez les sujets âgés et est responsable d'une dégradation importante de la qualité de vie. Il est de ce fait très essentiel d'étudier les mécanismes et les facteurs impliqués afin d'identifier de nouvelles cibles pour le traitement de cette maladie. Les causes et la physiopathologie de l'arthrose ne sont pas bien connues. Plusieurs voies de signalisation participent à ce processus, telles que la voie MAPK, NFκB, TGF/BMP et plus particulièrement Wnt.

Wnt est une famille de glycoprotéines sécrétées, caractérisées par plusieurs résidus cystéines conservés. Depuis 40 ans, différentes études ont montré une implication de la famille Wnt dans le développement, la croissance et l'homéostasie de différents tissus et organes y compris l'os et le cartilage (Chun et al., 2008; Nalesso et al., 2011). Wnt exerce ses effets à travers différentes voies de signalisation (Macdonald et al., 2007), la plus caractérisée étant la voie Wnt/β-caténine nommée voie canonique. Dans cette voie, la liaison des ligands sur le récepteur Frizzled (Fz) et le corécepteur LRP5/6 conduit à l'accumulation de la β-caténine puis à sa translocation dans le noyau où elle interagit avec les facteurs de transcription TCF/LEF, induisant la transcription de plusieurs gènes cibles (Macdonald et al., 2007). D'autres voies de signalisation nommées non-canoniques, indépendantes de la β-caténine, ont été découvertes chez la Drosophile (Amerongen et al., 2008). Malgré le rôle joué par les récepteurs Fz et les protéines dishevelled (DVL) dans la voie non-canonique Wnt/PCP, il n'existe aucune preuve de l'implication de la β-caténine, du récepteur LRP ou des facteurs de transcription TCF/LEF dans la régulation de cette voie. Le récepteur Fz est aussi impliqué dans la voie Wnt/Ca²⁺, une autre voie non-canonique (Kühl et al., 2000a; Miller et al., 1999).

De nombreuses études ont montré que les protéines de la voie de signalisation de Wnt sont modifiées dans le cartilage arthrosique. L'accumulation de la β -caténine induit l'expression de gènes cataboliques comme les MMPs (Matrix metalloproteinases) et les ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs) dans les chondrocytes (Yuasa et al., 2008). La surexpression de WISP1 augmente l'expression et l'activité des MMPs et des aggrecanases, puis aboutit à la destruction du cartilage chez la souris (Blom et al., 2009). En effet, la voie de signalisation Wnt est également impliqués dans la dédifférenciation des chondrocytes (cellules du cartilage) matures en chondrocytes hypertrophiés exprimant la MMP13 et le facteur de transcription Runx2, perdant ainsi leur phénotype chondrocytaire caractérisé par l'expression de marqueurs comme le collagène de type II (COL2), le facteur de transcription SOX9 et l'aggrecane (AGN) (Hwang et al., 2005; Nalesso et al., 2011).

Récemment, il a été montré qu'un protéoglycane membranaire exprimé à la surface des cellules, le syndecan-4 (SYND4), est impliqué dans la voie de signalisation de Wnt. La relation entre Wnt et le SYND4 a été établie chez le Xenope (Muñoz et al., 2006); le SYND4 interagit avec le récepteur Fz-7 et la R-Spondin3 (agoniste de Wnt) et régule la voie non-canonique de Wnt. En fait, le SYND4 assure la médiation de nombreux processus cellulaires, il fonctionne comme un co-récepteur pour les facteurs de croissance ou mime la liaison du ligand activant des voies de signalisation de façon indépendante. Différentes études ont montré que le SYND4 est induit dans les chondrocytes hypertrophiés et que son expression augmente dans le cartilage arthrosique (Barre et al., 2000; Echtermeyer et al., 2009). Des études dans un modèle de souris d'arthrose montre que le SYND4 augmente l'activation de l'ADAMTS-5 (Elfenbein and Simons, 2013). Néanmoins, le lien entre Wnt et le SYND4 dans l'arthrose n'a pas encore été élucidé.

Wnt-3a est un membre de la famille de WNT qui active à la fois la voie canonique et la voie non-canonique de Wnt. Une étude récente a montré que Wnt-3a stimule la prolifération des chondrocytes et inhibe l'expression des marqueurs phénotypiques du

chondrocyte (*Col2A1*, *SOX9* et *Aggrecan*) via la voie canonique et non-canonique (Nalesso et al., 2011). Ces effets sont essentiels dans la physiopathologie de l'arthrose. Ainsi, Wnt-3a active la voie canonique et les voies non-canoniques simultanément, nous avons utilisé la protéine recombinante Wnt-3a afin d'étudier les effets de ce ligand sur les chondrocytes articulaires humains. Nous avons montré lors de ces travaux comment Wnt-3a régule l'expression de l'ADAMTS-4 (l'aggrécanase surexprimé dans l'arthrose) et du COL2 (marqueur du phénotype chondrocytaire), ainsi que le rôle du SYND4 dans cette régulation.

II. Objectifs du travail

- Etudier l'effet de Wnt-3a sur l'expression de l'ADAMTS-4 et du COL2 dans les chondrocytes et dans les explants de cartilage humains en culture et déterminer les facteurs et les mécanismes moléculaires impliqués.
- Etudier le rôle du protéoglycane syndécan-4 (SYND4) dans la signalisation de Wnt-3a, élucider les mécanismes et identifier les facteurs impliqués.
- Etudier le rôle potentiel de Wnt-3a dans l'arthrose chez l'homme.

III. Méthodologie générale

Les chondrocytes ont été préparés à partir d'échantillons de cartilage provenant de pièces opératoires issues de remplacements chirurgicaux de genoux de patients atteints d'arthrose. Les chondrocytes sont obtenus par digestion enzymatique séquentielle (Protéase pendant 2 heures et puis collagénase B toute la nuit). Les chondrocytes fraîchement isolés sont mis en culture en monocouche (stade P0). Une fois la confluence atteinte les cellules sont amplifiées (stade P1) pour les études ultérieures. Pour les explants du cartilage, les morceaux sont disséqués à partir de condyles fémoraux et un soin extrême a été pris pour éviter l'os sous-chondral, le cartilage abîmé et le fibrocartilage.

L'utilisation d'inhibiteurs pharmacologiques et des ARN interférents (siRNA) nous a permis respectivement de bloquer spécifiquement une voie de signalisation ou

d'examiner la fonction d'une protéine distincte. Ainsi, le DKK1 et le si-CTNNB1 sont utilisés pour bloquer la voie canonique de Wnt. Le siRNA si-p65 a été utilisé pour bloquer la voie NFκB, si-MAPK1 et si-MAPK3 ont été utilisés pour bloquer la voie ERK1/2, et l'inhibiteur KN93 pour empêcher la phosphorylation de la kinase CaMKII. Certains siARNs ont été utilisés afin d'inhiber spécifiquement l'expression des protéines, comme si-SYND4, si-DVLs, si-Fz. D'autre part, la surexpression de certaines protéines a été effectuée par transfection du plasmide codant pour le gène d'intérêt sous le contrôle du promoteur du gène CMV.

L'expression du gène a été évaluée par réaction en chaîne par polymérase en temps réel (RT-PCR), et la quantité de protéine a été estimée par Immunoblot. L'activation de la voie de signalisation a été étudiée par évaluation de l'expression de la protéine cible ou par l'état de sa phosphorylation. La technique d'immunofluorescence a également été utilisée pour étudier l'expression et la localisation subcellulaire de la protéine. La régulation de l'expression des gènes telle que celle du SYND4 ou l'activation de la voie de signalisation telle que celle de NFκB a été évaluée par essai luciférase. L'interaction des protéines a été évaluée par immunoprécipitation.

L'activation de l'ADAMTS-4 a été mesurée par sa capacité de libérer le 5-FAM du substrat 5-FAM/TAMRA FRET. Les glycosaminoglycanes (GAGs) radiomarqués par le ³⁵Soufre ont été précipités au chlorure de cétypyrimidium (CPC) pour estimer la quantité de protéoglycanes présents dans le milieu des chondrocytes et des explants de cartilage.

Chaque expérience a été répétée au moins trois fois de façon indépendante. Les données quantitatives sont exprimées en moyenne \pm *écart-type*. L'expression des ARNm dans différents échantillons a été comparée en utilisant le test de Student, et les effets ont été considérés statistiquement significatifs quand $*P < 0,05$. Un immunoblot représentatif de trois expériences indépendantes a été montré dans les résultats.

IV. Résultats obtenus

A. Wnt-3a inhibe l'expression de l'ADAMTS-4 via la voie

canonique

Dans les chondrocytes primaires et les explants de cartilage humains en culture, Wnt-3a inhibe l'expression des ARNm d'ADAMTS-4 mais sans influencer celle de l'ADAMTS-5. L'inhibition de la voie canonique par les siRNA dirigés contre la β -caténine (si-CTNNB1) ou par l'inhibiteur biologique DKK-1 empêche l'inhibition de l'expression des ARNm de l'ADAMTS-4 par Wnt-3a indiquant que Wnt-3a régule l'expression de ces gènes par la voie canonique, Wnt/ β -caténine. Chez les mammifères, il existe trois isoformes de dishevelled (DVL), DVL-1, 2 et 3 qui sont des produits de gènes paralogues. L'inhibition de DVL-1 et de DVL-3 prévient la diminution de l'expression de l'ADAMTS-4 par Wnt-3a. Parmi les 10 isoformes du récepteur Frizzled (Fz), Fz-6 et Fz-8 sont les plus exprimés dans les chondrocytes primaires humains. La transfection des chondrocytes par le si-Fz-8 mais pas celle de si-Fz-6, empêche l'accumulation de la β -caténine et l'inhibition de l'expression de l'ADAMTS-4. Ces résultats montrent que Wnt-3a active la voie canonique et inhibe l'expression de l'ADAMTS-4 via le DVL-1/3 et le récepteur Fz-8.

B. Wnt-3a inhibe la voie NF κ B

Nous avons montré que le traitement des chondrocytes par Wnt-3a inhibe l'expression des gènes IL-1 α et IL-6 qui sont des cibles du facteur transcriptionnel NF κ B. L'essai luciférase réalisé avec le rapporteur de NF κ B montre que l'activation de la voie NF κ B est inhibée par le traitement de Wnt-3a. De plus, les expériences d'immunoblot et d'immunofluorescence montrent que la translocation de p65 du cytosol vers le noyau est réduite par Wnt-3a et que ce processus est indépendant d'I κ B α . Dans les chondrocytes, la suppression de la voie NF κ B par le knock-out de p65 inhibe l'expression basale de l'ADAMTS-4 suggérant que Wnt-3a inhibe l'expression de l'ADAMTS-4 par inhibition de l'activité de NF κ B.

Nous avons montré que Wnt-3a s'oppose à la stimulation de l'expression de l'ADAMTS-4 et à la dégradation du cartilage induite par l'IL-1 β . Le traitement par Wnt-3a empêche partiellement l'augmentation de l'expression des ARNm de l'ADAMTS-4 et son activité dans les chondrocytes. Dans le cartilage humain, Wnt-3a inhibe partiellement l'effet de l'IL-1 β sur la synthèse et la libération des protéoglycanes dans le milieu de culture.

C. Wnt-3a régule l'expression du collagène II et d'autres marqueurs chondrocytaires dans les chondrocytes humains.

Nos résultats montrent que Wnt-3a inhibe l'expression du collagène de type II (*COL2A1*), de *Sox9* ainsi que de l'*aggrécane* et induit l'expression de *Runx2* dans les chondrocytes et le cartilage. Cependant, l'inhibition du *COL2A1* par le Wnt-3a n'est pas empêchée par le traitement par DKK1 ou la transfection du si-CTNNB1, indiquant que cette inhibition est β -caténine-indépendant (non-canonique).

D. Wnt-3a inhibe le COL2A1 via l'inhibition du syndécan4

Le knock-out du syndécan4 (*SYND4*) n'empêche pas l'accumulation de la β -caténine ni l'augmentation de l'*AXIN2* induite par Wnt-3a, mais inhibe le *COL2A1* et s'oppose à l'inhibition de ce gène par Wnt-3a. De façon intéressante, la surexpression du *SYND4* bloque l'inhibition du *COL2A1* par Wnt-3a.

Des tests menés dans les chondrocytes et les explants de cartilage montrent que Wnt-3a inhibe l'expression du *SYND4*. De plus, nous avons pu confirmer que le promoteur du *SYND4* est inhibé par le traitement par Wnt-3a par des test de gène rapporteur. Cependant, la suppression de la voie canonique par DKK1 et si-CTNNB1 ne permet pas d'empêcher l'inhibition du *SYND4* induite par Wnt-3a. Par ailleurs, nous avons montré que Wnt-3a peut induire la phosphorylation d'ERK1/2. Nous avons également montré que le blocage de la voie ERK1/2 par l'inhibiteur U0126 ou la transfection du si-MAPK1 ou si-MAPK3 inhibe l'effet de Wnt-3a sur le *SYND4* et le *COL2A1*. Ces résultats suggèrent que Wnt-3a inhibe l'expression du *SYND4* et du *COL2A1* par l'activation de la voie ERK1/2.

E. DVL-2 et B-raf sont les médiateurs de l'inhibition du COL2A1

par Wnt-3a

DVL-3 est le DVL le plus abondant dans les chondrocytes primaires, tandis que DVL-1 est le moins exprimé. Cependant, seul le knock-out de DVL-2 abolit l'inhibition du COL2A1 et du SYND4 et l'activation d'ERK1/2 suggérant ainsi que DVL-2 participe à l'activation de la voie non canonique de Wnt-3a.

Des études de co-immunoprécipitation montrent que DVL-2 interagit avec B-raf en présence de Wnt-3a, mais que cette interaction nécessite la présence du domaine DEP. La surexpression du mutant inactif de B-raf supprime l'activation d'ERK1/2 induite par Wnt-3a. L'ensemble de ces résultats montre que l'interaction entre DVL-2 et B-raf est essentielle pour la phosphorylation d'ERK1/2 et l'inhibition du COL2A1.

F. L'activation de la CaMKII est cruciale pour la, phosphorylation d'ERK1/2 par Wnt-3a

Nous avons montré que Wnt-3a induit la phosphorylation de CaMKII. Quand cette phosphorylation est inhibée par l'inhibiteur KN-93, la phosphorylation d'ERK1/2 est également bloquée. Les résultats obtenus par RT-PCR confirment que le KN-93 peut empêcher l'inhibition du SYND4 et du COL2A1 induite par Wnt-3a. Néanmoins, le KN-93 n'inhibe pas la phosphorylation d'ERK1/2 induite par l'expression de B-raf constitutivement actif.

D'autre part, le knock-out du SYND4 empêche la phosphorylation d'ERK1/2, mais il n'inhibe pas la phosphorylation de CaMKII induite par Wnt-3a. Des expériences de co-immunoprécipitation indiquent que le SYND4 interagit avec la pCaMKII et ce de manière plus importante en présence de Wnt-3a. Lorsque le DVL-2 et le SYND4 sont co-transfectés dans les cellules, on observe une interaction entre les deux protéines. L'ensemble de ces résultats indique que le SYND4 est un composant de la voie non-canonique du Wnt et qu'il régule l'activation de cette voie par interaction avec DVL-2 et CaMKII.

G. Le récepteur Frizzled 6 est responsable de l'activation de la voie non-canonique de Wnt-3a

Nous avons montré que le knock-out de Fz-6 n'empêche pas l'accumulation de la β -caténine et la stimulation de l'expression de l'AXIN2 induites par Wnt-3a, mais il inhibe l'effet de Wnt-3a sur l'expression du COL2A1. De façon intéressante, l'inhibition de l'expression de Fz-6 bloque l'activation de la CaMKII et d'ERK1/2 ce qui suggère que Fz-6 participe à l'activation de la voie non-canonique de Wnt.

V. Discussion

Les voies de signalisation de Wnt jouent un rôle essentiel dans la pathologie arthrosique. Dans les chondrocytes articulaires de lapin, Wnt-5a et Wnt-7a sont induits par l'IL- β et produisent une inhibition du COL2A1 (Hwang et al., 2004). D'autre part, le traitement par Wnt-3a et l'activation de la β -caténine entraînent une stimulation importante de l'expression de MMP-3, MMP-13, ADAMTS-4 et ADAMTS-5 (Yuasa et al., 2008). Bien que dans les modèles animaux l'activation de la voie canonique de Wnt induit l'expression d'enzymes cataboliques, l'effet de cette voie dans les chondrocytes humains est encore controversée. L'activation de la voie Wnt/ β -caténine par le LiCl ou le knock-out du LEF-1 augmente l'expression des MMP-9, 13, 14 et d'ADAMTS-5 (Papathanasiou et al., 2012). Cependant, dans une autre étude, dans laquelle les effets de Wnt-3a sur les chondrocytes humains et animaux sont comparés, les résultats montrent que Wnt-3a inhibe l'expression des MMPs dans les chondrocytes humaines ce qui est à l'opposé de ce qui se passe dans les chondrocytes animaux (Ma et al., 2012). Nos résultats vont dans le sens de cette dernière étude, Wnt-3a inhibe l'ADAMTS-4 par la voie canonique, et cette inhibition est médiée par l'inactivation de la voie NF κ B. Par conséquent, Wnt-3a jouerait un rôle protecteur contre la dégradation du cartilage humain induit par IL-1 β .

Les voies canonique et non-canonique de Wnt coexistent dans les chondrocytes

humains, et les récepteurs Fz ainsi les protéines DVL interviennent dans les deux types de cascades. Cependant, la spécificité de chacun des Fz et DVL dans l'activation d'une voie donnée n'est pas encore établie. Nous avons montré que Fz-6 et Fz-8 sont les récepteurs les plus exprimés dans les chondrocytes primaires humains. Nous avons montré que Fz-8 est indispensable pour l'activation de la voie canonique et Fz-6 est essentiel pour l'activation de la voie non-canonique CaMKII/ERK1/2 par Wnt3-a. Chez le Xenope, Fz-3, Fz-4 et Fz-6 activent la voie non-canonique CaMKII, alors que Fz-7 et Fz-8 activent la voie β -caténine (Kühl et al., 2000b). Nos études portant sur les DVLs montrent que seul DVL-2 participe à l'activation de CaMKII et d'ERK1/2. L'ensemble de nos résultats suggère que l'activation des voies canonique et non-canonique par Wnt-3a est médiée par des récepteurs Fzs et des protéines DVLs spécifiques.

Le SYND4 est impliqué dans la transduction du signal de plusieurs voies de signalisation (Elfenbein and Simons, 2013), y compris celle de Wnt. D'après nos résultats, le SYND4 joue deux rôles dans la régulation de l'expression du COL2A1 par Wnt-3a. D'une part, même si le knock-out du SYND4 ne bloque pas l'activation de la CaMKII comme c'est le cas de DVL-2 et Fz-6, ce protéoglycane est indispensable pour l'activation de la voie ERK1/2 par Wnt-3a. Les expériences d'immunoprécipitation montrent que le SYND4 interagit avec DVL-2, et de ce fait favorise probablement l'activation de B-raf par la CaMKII. Le SYND4 et DVL-2 font le lien entre Fz6 et la voie CaMKII/ERK1/2. Il est à noter que le SYND4 est indispensable pour l'activation d'ERK1/2 mais qu'il est aussi inhibé par cette signalisation, dans une boucle de rétrocontrôle.

VI. Conclusions et perspectives

Notre étude met en lumière la signalisation globale de Wnt dans les chondrocytes humains. Wnt-3a active la voie non-canonique *via* le récepteur Fz-6 qui induit l'activation de la CaMKII *via* DVL2 et l'interaction entre DVL2 et B-raf d'une part et entre le SYND4 et pCaMKII d'autre part, ceci conduit à l'activation la voie ERK1/2

responsable de l'inhibition de l'expression du COL2A1. Au même temps, Wnt-3a induit l'accumulation de la β -caténine par Fz-8 via DVL-1/3, cette voie canonique inhibe l'activité de NF κ B et réduit fortement l'expression de l'ADAMTS-4. Ces résultats sont importants d'un point de vue pharmacologique. Son rôle étant mieux compris, la signalisation de Wnt devient une cible potentielle intéressante dans le traitement de l'arthrose. Les molécules ou médicaments qui seraient capables d'inhiber les voies non-canoniques de Wnt et d'activer les voies canoniques pourrait protéger le cartilage contre la dégradation lors de l'inflammation et la dégénérescence.

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ANNEX

ANNEX

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Non-canonical Wnt induces chondrocyte de-differentiation through Frizzeld 6 and CaMKII α /syndecan4 axis

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Abstract:

Dysregulation of Wnt signaling has been implicated in developmental defects and in the pathogenesis of many diseases. Non-canonical Wnt induces loss of chondrocyte phenotype which is considered as an early event in osteoarthritis; however, the underlying mechanisms are poorly understood. Here, we show that non-canonical Wnt induces chondrocyte de-differentiation through Fz-6/DVL-2/SYND4/CaMKII α /B-raf/ERK1/2 cascade. We also revealed that in response to Wnt-3a, Fz-6 activates non-canonical signaling by triggering the docking of CaMKII α to SYND4 and that of B-raf to DVL-2 leading to the activation of B-raf by CaMKII and transduction of signals to ERK1/2 MAPK. Collectively, our results revealed a new pathway that regulates chondrocyte phenotype and provides new potential markers and targets which may have therapeutic potential in osteoarthritis.

Introduction

WNT is a family of secreted glycoproteins, characterized by several cysteines conserved residues. Wnts signal through multiple pathways (1, 2), the best characterized of which is the Wnt/ β -catenin signaling, also known as the “canonical” pathway. Canonical Wnt signaling is initiated by binding of Wnt proteins to a Frizzled-LRP5/6 receptor complex (3). This leads to activation of the protein dishevelled (Dsh/DVL) and inhibition of phosphorylation events that otherwise target β -catenin for proteasomal destruction, resulting in its stabilization in the cytoplasm. The stabilized β -catenin accumulate and translocate to the nucleus, where it interacts with transcription factors of the T cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family to activate the transcription of target genes (4, 5). In contrast to the above mechanism, Wnts can instead activate other signaling pathways referred as “non-canonical”, which does not involve stabilization of β -catenin such as the planar cell polarity pathway (PCP) (6) and the Ca^{2+} /CaMKII pathways (2, 7).

Wnts play a pivotal role in development and disease, and understanding their complex signaling mechanisms and biological roles is of wide interest (8, 9). Various studies have shown the involvement of the Wnt family in the development, growth and homeostasis of various tissues and organs including the joint, bone and cartilage (10–12). A growing number of studies connects Wnt signaling with bone disease (13), osteoarthritis (OA) (11, 14) and cancer (15, 16).

Besides Wnts, the syndecans (SYND) are a family of four transmembrane proteoglycans (PGs), which control cell proliferation, differentiation, adhesion and migration (17, 18). Syndecans not only act as co-receptors for growth factors they can also transduce signals via their intracellular domain, by interacting with numerous effectors (18). Of the four mammalian SYND family members, SYND4 activates intracellular signaling cascades through activation of PKC α (19). Its ability to bind and activate the PKC α allows the

regulation of the GTPases pathway of the Rho family, including RhoG, Rac1 and RhoA (20), responsible for the remodeling of the actin cytoskeleton, thereby regulating cell polarity and mobility (21). In addition, SYND4 has been shown to regulate the non-canonical Wnt signaling in *Xenopus* by interacting with the receptor Frizzled7 (Fz-7) and its signal transducer DVL (22) and with R-Spondin3, a ligand that acts as a Wnt agonist (23). SYND4 also regulates several intracellular signaling pathways which operate in parallel through the activation of common effectors. These downstream effectors influence many cellular processes that regulate migration, polarity and adhesion as well as cellular homeostasis and inflammation (22, 24).

In recent years, a growing number of evidences has accumulated on the relationship between SYND4 and OA. In cartilage, SYND4 seems to be induced specifically in hypertrophic chondrocytes and its expression is elevated in human OA cartilage and in animal models of OA (25, 26). In addition, SYND4-null mice develop less severe OA-like cartilage destruction through marked decrease in disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) activity by preventing the phosphorylation of ERK1/2 (26).

In this study, we showed for the first time that non-canonical Wnt-3a signaling induced loss of chondrocyte phenotype through activation of CaMKII α /B-raf/ERK1/2 cascade and revealed that SYND4 regulates the signaling and is an essential component of the pathway. To fulfil these functions SYND4 docks CaMKII α in Wnt-dependent manner and interacts with DVL-2 which docks B-raf leading to the activation of ERK1/2 signaling pathway that induces loss of chondrocyte phenotype.

Results

Non-canonical Wnt mediates loss of chondrocyte phenotype

Wnt/ β -catenin signaling has been recognized as key regulator of cartilage development and chondrocyte function (27, 28). Wnt-3a is expressed in cartilage and has an established role in osteoarthritis (29). To gain insight into the mechanism by which Wnt-3a mediates loss of chondrocyte phenotype, we first investigated the expression of chondrocyte-lineage markers *COL2A1*, *SOX9* and *aggrecan* in human primary chondrocytes and articular cartilage explants treated with either Wnt-3a or vehicle (control). In agreement with other studies (11), the expression of chondrocyte-lineage markers *COL2A1*, *SOX9* and *aggrecan* was decreased by Wnt-3a in primary chondrocytes. We also found that the expression of the hypertrophy marker *RUNX2* was increased (Fig. 1A). Similar results were obtained with human cartilage explants (Fig. 1B), indicating that Wnt-3a activates de-differentiation of articular chondrocytes.

Wnt-3a induced both canonical and non-canonical Wnt pathways. To determine whether Wnt-3a mediates loss of chondrocyte phenotype through canonical Wnt pathway, we investigated the effect of β -catenin knockdown on Wnt-3a-induced down-regulation of the chondrocyte phenotypic marker *COL2A1*. Treatment of primary chondrocytes with Wnt-3a resulted in β -catenin accumulation (Fig. 1C). However, knockdown of β -catenin (Fig S1A) failed to rescue the Wnt-3a down regulation of *COL2A1* (Fig. 1D), indicating that Wnt-3a regulates *COL2A1* through β -catenin-independent pathway. This was further confirmed using DKK1, a specific inhibitor of canonical Wnt signaling pathway (Fig. S1B). Co-treatment with DKK1 could not prevent Wnt-3a-induced down-regulation of *COL2A1* (Fig. 1E), therefore suggesting that down-regulation of *COL2A1* expression by Wnt-3a is mediated through non-canonical Wnt signaling pathway in human primary chondrocytes.

It has been shown that Wnt-3a also signal through β -catenin-independent mechanisms to mediate distinct physiologically relevant responses. Indeed, Wnt-3a induces proliferation of NIH3T3 fibroblasts and regulates inflammation in primary microglia cells through activation of ERK1/2 MAPK (30, 31). To determine whether non-canonical Wnt/ERK1/2 pathway mediates Wnt-3a-induced down-regulation of *COL2A1*, we first analyzed whether Wnt-3a activates this pathway in primary chondrocytes. The activation status of ERK1/2 kinases was determined in primary chondrocytes following Wnt-3a treatment. The p-ERK level was increased in a time-dependent manner starting within 5 min of the treatment and last over 2 h (Fig. 1F), indicating that Wnt-3a activates ERK1/2 pathway in primary chondrocytes. Co-treatment with the MEK inhibitor, U0126 abrogated the activation of ERK1/2 pathway by Wnt-3a (Fig. S2A). We next analyzed the effect of inhibition of Wnt-3a-induced activation of ERK1/2 on *COL2A1* expression. Inhibition of ERK1/2 pathway rescued Wnt-3a-induced down-regulation of *COL2A1* (Fig. 1G), thus indicating that Wnt-3a regulates the expression of *COL2A1* through activation of ERK1/2 pathway. Consistent with this finding, knockdown of ERK2 (Fig. S2B) reverted the effect of Wnt-3a on the expression of *COL2A1* (Fig. 1H), confirming the key role of ERK1/2 in Wnt-3a-induced down-regulation of *COL2A1*. Altogether, these results indicated that Wnt-3a-induced activation of ERK1/2 signaling pathway negatively regulates the expression of *COL2A1*.

DVL-2 mediates non-canonical Wnt down-regulation of COL2A1

DVL is key component of both canonical and non-canonical Wnt signaling pathways and, depending on the cellular context, specifically activates each Wnt signaling branch (32). Mammalian DVLs include three isoforms, DVL-1, DVL-2 and DVL-3 which display differential expression and are not truly “redundant“ with respect to function (33–35). Analysis of the expression DVL-1, DVL-2 and DVL-3 by Western blotting showed that

DVL3 is the most abundant DVL expressed in primary chondrocytes, whereas DVL1 is the less abundant (Fig. 2A). Considering of DVL's role of hub in Wnt signaling and in order to determine which of the DVLS mediates Wnt-3a-induced down-regulation of *COL2A1*, we knocked down each of DVLS individually (Fig. 2B-D) and evaluated the effect on Wnt-3a-induced decrease in the expression of *COL2A1*. DVL-2 knockdown not only prevented down-regulation of *COL2A1* by Wnt-3a but, promoted the expression of this chondrocyte phenotype marker (Fig. 2E). The knockdown of DVL-1 and DVL-3 did not prevent Wnt-3a-induced down-regulation of *COL2A1* instead, silencing of DVL-1 potentate it (Fig. 2E). These results indicate that DVL-2 mediates Wnt-3a down-regulation of *COL2A1* and suggests that, in chondrocytes, non-canonical Wnt pathway signal through DVL-2. These results also revealed that DVL-2 exerts a repressive effect on the expression of *COL2A1* in chondrocytes.

We showed above that Wnt-3a-induced down-regulation of *COL2A1* is mediated through activation of ERK1/2 pathway. Since knockdown of DVL-2 suppresses Wnt-3a-induced down-regulation of *COL2A1*, we asked whether DVL-2 silencing affects activation of ERK1/2 in response to Wnt-3a. Although Wnt-3a provoked the activation of ERK1/2 signaling, it failed to stimulate this pathway in chondrocytes made deficient in DVL-2 (Fig. 2F). The knockdown of DVL-2 provoked a decline, rather than increase in ERK1/2 activation in response to Wnt-3a, indicating that DVL-2 play a critical role in Wnt-3a-induced activation of ERK1/2 pathway that is linked to non-canonical Wnt signaling.

DVL-2 docks B-raf to activate ERK1/2 signaling

DVL-2 is the scaffold protein that relays Wnt signaling by bridging receptor and adaptor proteins in both canonical and non-canonical Wnt pathways (36, 37). As DVL-2 was required for the activation of ERK1/2 by Wnt-3a, we hypothesized that B-raf which transduces signals to MEK and ERK1/2 MAPK may relay Wnt signaling through interaction with DVL-2. To

test whether DVL-2 interacts with B-raf, co-immunoprecipitation assays were performed. Human primary chondrocytes were treated or not with Wnt-3a and cells extracts were immunoprecipitated with anti-B-raf specific antibodies. Co-immunoprecipitation showed that DVL-2 associates with B-raf and this occurs only in the presence of Wnt-3a (Fig. 3A), therefore revealing that B-raf interacts with DVL-2 in Wnt-dependent manner. To determine whether recruitment of DVL-2 to the membrane is required for DVL-2 interaction with B-raf, we used a DVL-2 mutant lacking the DEP domain (Flag-DVL-2 Δ 361-736) that is important for membrane recruitment (38). We stimulated cells containing the Flag-tagged wild-type or Δ 361-736 mutant of DVL-2 with Wnt-3a and then immunoprecipitated B-raf with specific antibodies. As shown in Figure 3B, the Wnt-3a-dependent binding between DVL-2 and B-raf was observed only in the presence of Wnt-3a in cell expressing the wild-type DVL-2, but not the DVL-2 (Δ 361-736) mutant. These results indicate that DVL-2 interaction with B-raf requires the presence of the DEP domain and suggest that recruitment of DVL-2 to the membrane is critical for DVL-2 docking of B-raf. Taken together, these data strongly suggest that activation of ERK1/2 pathway by Wnt-3a is regulated through recruitment of DVL-2 to the plasma membrane and docking of B-raf.

To further investigate the role of B-raf in Wnt-3a-induced activation of ERK1/2 signaling pathway, we stimulated chondrocytes containing the wild-type or D594V kinase-dead mutant of B-raf with Wnt-3a then analyzed the phosphorylation of ERK1/2. As shown in Figure 3C, forced expression of wild-type B-raf increased the phosphorylation of ERK1/2 which was increased further in response to Wnt-3a. In contrast, expression of D594V mutant significantly reduced basal activation of ERK1/2 and abrogated the activation of ERK1/2 by Wnt-3a (Fig. 3D), indicating that B-raf mediates Wnt-3a-induced activation of ERK1/2 signaling pathway.

Ca²⁺/CaMKII pathway mediates Wnt-3a-induced activation of ERK1/2

It has been reported that Wnt-3a activates the non-canonical Ca²⁺/CaMKII-dependent pathway in chondrocytes (11). In agreement with this study, we found that treatment of chondrocytes with Wnt-3a induced the phosphorylation of CaMKII α in a time-dependent manner, likewise, the phosphorylation of B-raf has also been observed (Fig. 4A). Noteworthy, CaMKII has been shown to mediate the Ca²⁺-induced activation of ERK1/2 in rat aortic VSM cells (39). We then hypothesized that CaMKII α may mediate Wnt-3a-induced activation of B-raf/ERK1/2 in chondrocytes. To test this hypothesis, primary chondrocytes were pre-treated or not with the specific CaMKII inhibitor KN-93 or with KN92, an analogue of KN-93 which does not inhibit CaMKII, then stimulated with Wnt-3a. Analysis of cell extracts showed that not only KN-93 prevented Wnt-3a-induced activation of CaMKII α but almost completely abrogated the activation of B-raf/ERK1/2 signaling by Wnt-3a (Fig. 4B). In contrast, KN92 did not prevent the activation by Wnt-3a of either CaMKII α or ERK1/2 (Fig. 4B). These results indicate that the activation of non-canonical Wnt/ERK1/2 signaling pathway by Wnt-3a requires the activation of CaMKII α . In line with this, inhibition of CaMKII α prevented down-regulation of the expression of COL2A1 (Fig. 4C) by Wnt-3a.

We showed above that B-raf mediates Wnt-3a-induced activation of ERK1/2 signaling pathway. To further investigate the role of CaMKII α on the activation of ERK1/2 pathway, we stimulated chondrocytes containing the active mutant V600E of B-raf with Wnt-3a in the presence of KN92 and KN93, respectively then analysed the activation of ERK1/2. As expected, expression of B-raf V600E mutant strongly activated ERK1/2, however treatment with Wnt-3a did not induce any significant increase in ERK1/2 phosphorylation (Fig. 4D). In addition, KN 93 did not reduce the phosphorylation of ERK1/2, indicating that when B-raf is activated CaMKII α does not mediate to the following ERK activation.

To explore whether calcium mobilization is involved in the activation of CaMKII α and ERK1/2 pathway by Wnt-3a, intracellular Ca²⁺ accumulation was monitored in primary chondrocytes treated or not with Wnt-3a. The results clearly showed that Wnt-3a induced Ca²⁺ mobilization in human primary chondrocytes which was efficiently blocked by pertussis toxin (PTX), a G protein inhibitor (Figure 4E). Western blot analysis of the phosphorylation status of CaMKII α and ERK1/2 showed that PTX prevented Wnt-3a-induced activation of both kinases (Figure 4F). In line with this, PTX prevented down-regulation of COL2A by Wnt-3a (Figure 4G). These results indicate that Wnt-3a induces loss of chondrocyte phenotype by activation of Ca²⁺/CaMKII α /ERK1/2 signaling pathway in a G protein-dependent manner.

Syndecan4 docks CaMKII α and mediates Wnt-3a-induced activation of ERK1/2

SYND4 has been shown to regulate the non-canonical Wnt signaling in *Xenopus* embryos by interacting with the receptor Frizzled7 (Fz-7) and its signal transducer DVL (22). To determine the role of SYND4 in noncanonical Wnt pathway in primary chondrocytes, we performed loss-of-function (Fig. 5A) and analysed the effect on Wnt-3a-induced activation of CaMKII α and ERK1/2 signaling. As shown in figure 5B, knockdown of SDC4 abrogates the activation of ERK1/2 signaling by Wnt-3a but did not prevent the phosphorylation of CaMKII α in response to Wnt-3a. These results indicate that activation of CaMKII α by Wnt-3a is not SDC4 depend. This also, suggests that activation of CaMKII α is not *per se* sufficient for the activation of ERK1/2 signaling pathway by Wnt-3a but requires SYND4 for relaying the signal to ERK1/2.

As for SDC4, we showed above that knockdown of DVL2 abrogated Wnt-3a-induced activation of ERK1/2 pathway. Given that activation of this pathway requires the activation of CaMKII α , we investigated whether DVL2 is involved in Wnt-3a-induce activation of

CaMKII α . Knockdown of DVL-2 strongly reduced the activation of CaMKII α and ERK1/2 by Wnt-3a (Fig. 5B) arguing for a dependence of CaMKII α activation by Wnt-3a on DVL-2 but not on SDC4 and suggesting a model in which SDC4 functions downstream of DVL2.

SDC4 interacts with diverse intracellular partners, as it is required for the activation of ERK1/2 by Wnt-3a, we hypothesized that this HSPG may relay Wnt signaling to ERK1/2 through interaction with CaMKII α . Co-immunoprecipitation assays were performed using extracts from cells that have been transiently transfected with HA-SYND4. Co-immunoprecipitation indicated that HA-SYND4 physically interacts with CaMKII α (Fig. 5C). Interestingly, we found that the interaction between SYND4 and CaMKII α was significantly enhanced in the presence of Wnt-3a (Fig. 5C). These results indicated that although SYND4 is not required for activation of CaMKII α by Wnt-3a, this HSPG interacts with CaMKII α in a Wnt-3a-dependent manner and suggested that SYND4 and CaMKII α interact to regulate non-canonical Wnt signaling in chondrocytes.

On the other hand, SYND4 has been shown to interact with DVL (Munoz et al., 2006), a possible interaction between SYND4 and DVL-2 was examined using extracts from cells that have been transfected with Flag-DVL-2 and HA-SYND4. In agreement with the previous study, DVL-2 was co-immunoprecipitated with SYND4 (Fig. 5D). Taken together, our data indicate that SYND4 is a component of non-canonical Wnt pathway that regulates the activation of this pathway through interaction with DVL-2 and CaMKII α .

To gain more insight in the role of SDC4 in the regulation of noncanonical Wnt signaling, we investigated the effect of Wnt-3a on the expression of this HSPG. Interestingly, treatment of chondrocytes with Wnt-3a significantly decreased the expression of SYND4 (Fig. 5E). Similar results were observed in human cartilage explants (Fig. 5F), indicating that Wnt-3a down-regulates the expression of SDC4 in human cartilage. Next, we cloned the proximal promoter sequence of human *SYND4* gene and monitored the effect of Wnt-3a on its

transcriptional activity using a luciferase reporter assay. *SYND4* gene promoter showed significant basal activity in primary chondrocytes. Importantly, treatment with Wnt-3a significantly reduced the activity of the promoter, indicating that Wnt-3a down-regulates the expression of *SYND4* by inhibiting the transcriptional activity of the gene promoter (Fig. 5G). We can therefore hypothesize that down-regulation of *SYND4* by Wnt-3a may constitute a negative feedback loop that may attenuate the non-canonical Wnt signaling.

Frizzled 6 mediated non-canonical Wnt-3a signaling pathway

Frizzled (Fz) is a family of G protein-coupled receptor proteins that serves as receptors in the Wnt signaling pathway. To address the question of which of the known *FZD* genes could be involved in Wnt-3a activation of Wnt non-canonical pathway, we first performed qRT-PCR to monitor the expression pattern of all genes encoding Wnt receptors in human primary chondrocytes. The result obtained showed that Fz-6 and Fz-8 are predominantly expressed in chondrocytes compared to other Fz receptors (Fig. S3). Noteworthy, Fz-6 has been associated with non-canonical Wnt signaling (40–42). Given that non-canonical Wnt-3a is characterized by the activation of CaMKII α and ERK1/2 and the inhibition of *COL2A1* in the human chondrocytes, we evaluated the impact of Fz-6 deficiency to further confirmed our Wnt noncanonical signaling model. The knockdown of Fz-6 efficiently decreased its expression (Fig. 6A) and did not impair Wnt-3a-induced upregulation of the canonical Wnt/ β -catenin downstream target gene *AXIN2* (Fig. 6B) or stabilization of β -catenin (Fig. 6C), suggesting that Fz-6 is not involved in Wnt-3a-induced canonical signaling in chondrocytes. In contrast, knockdown of Fz-6 abrogated Wnt-3a-induced down-regulation of the non-canonical Wnt downstream target gene *COL2A1* (Fig. 6D). As shown in Fig. 6E, knockdown of Fz-6 reduced the phosphorylation of both kinases and abrogated their activation by Wnt-3a, indicating that in Fz-6-deficient chondrocytes the activation of non-canonical Wnt signaling characterized by

the phosphorylation of CaMkII α and ERK1/2 is no more modulated by Wnt-3a. Taken together, these observations support the conclusion that Fz-6 mediates non-canonical Wnt CaMkII α and ERK1/2 signaling in chondrocytes.

Discussion

The key initiating events in OA are still unknown, but loss of phenotypic stability of articular chondrocytes has been proposed as an early event in the onset of the disease (43). De-differentiation of chondrocytes occurs through a complex programme with similarities to terminal differentiation of chondrocytes (hypertrophy), as can be found in the growth plate (44). This triggers a disturbed cartilage homeostasis favoring degenerative changes (44, 45). Wnt signaling has a pivotal role in regulating chondrocyte differentiation during endochondral ossification and was also shown to play a regulative role in OA cartilage. Indeed, a role of non-canonical Wnt signaling in cartilage degeneration is suggested by repression of chondrocyte phenotypic marker gene expression by Wnt-5a (46) and Wnt-3a (11), leading to chondrocyte hypertrophic differentiation. Here, we demonstrated that Wnt-3a-induced loss of chondrocyte phenotype is mediated by Fz-6 through Ca^{2+} /CaMKII α -dependent activation of B-raf/ERK1/2 signaling pathway. Indeed, knockdown of ERK1/2 or chemical inhibition of its activation prevented Wnt-3a-induced hypertrophic de-differentiation of chondrocytes, which is notoriously associated with down-regulation of COL2A1 (47, 48), indicating that activation of ERK1/2 is a key event in Wnt-3a-induced loss of chondrocyte phenotype. Most important, we identified SYND4 and DVL-2 as key components of non-canonical Wnt/CaMKII α /ERK1/2 pathway that regulate the signaling by recruiting CaMKII α and B-raf, respectively. We demonstrated that SYND4 regulates, and is essential for non-canonical Wnt-induced activation of ERK1/2 signaling.

SYND4 has been shown to regulate convergent and extension movements in *Xenopus* embryo through non-canonical Wnt pathway by interacting with DVL (22). We demonstrated here that DVL-2 relays Wnt-3a non-canonical signaling in primary chondrocytes, as its knockdown prevented Wnt-3a-induced activation of ERK1/2 pathway and down-regulation of COL2A1. In addition, as observed during PCP signaling in *Xenopus* (22), we found that

DVL-2 interacts with SYND4. However, in contrast to SYND4, silencing of DVL-2 inhibited also Wnt-3a-induced activation of CaMKII α , in addition to that of ERK1/2 signaling. Noteworthy, deactivation of ERK1/2 by inhibition of CaMKII was previously reported in several cell types (49–51). Interestingly, inhibition of CaMKII α by KN93, a widely used CaMKII activity inhibitor, impaired the activation of ERK1/2 by Wnt-3a in primary chondrocytes, suggesting a cross-talk between CaMKII α and ERK1/2 pathway during non-canonical Wnt signaling. These data demonstrate that ERK1/2 phosphorylation induced by Wnt-3a is dependent on the activation of CaMKII α and that the MEK/ERK cascade is a key regulator that links the CaMKII signaling to loss of chondrocyte phenotype. In addition, given that DVL-2 but not SYND4 is critical for Wnt-3a activation of CaMKII α suggest that DVL-2 functions upstream of SYND4 in non-canonical Wnt signaling pathway.

An important issue we addressed here is the mechanism of activation of ERK1/2 pathway by non-canonical Wnt signaling, which promotes chondrocyte de-differentiation. It has been shown that SYND4 is highly expressed in hypertrophic chondrocytes during embryonic development and during OA, suggesting a role in chondrocyte de-differentiation. Interestingly, loss of SYND4 in mice protects from development of OA-like disease and that was associated with decreased ERK1/2 signaling (26). Accordingly, we showed that knockdown of SYND4 reduced basal activation of B-raf/ERK1/2 signaling and impaired its activation by Wnt-3a in human primary chondrocytes. SYND4 has been shown to interact with different partners through its cytoplasmic domain (18). Interestingly, co-immunoprecipitation studies revealed, for the first time, that SYND4 interacts with phosphorylated CaMKII α and that this interaction is strongly enhanced on Wnt-3a treatment. As SYND4 is required for activation of ERK1/2, we can speculate that interaction between SYND4 and CaMKII α might facilitate CaMKII-dependant activation of ERK1/2 signaling pathway. On the other hand, co-immunoprecipitation studies revealed that DVL-2 docks B-raf

in Wnt-dependent manner. Further analysis showed that the C-terminal region of DVL-2 including the DEP domain, which is critical for membrane translocation of DVL-2, is also critical for B-raf docking. Therefore, in the presence of Wnt ligand, interaction between DVL-2 and SYND4 may promote assembly of CaMKII α and B-raf complex necessary to relay Wnt-3a activation of ERK1/2 signaling pathway. In this case, one can speculate that B-raf may directly be activated by CaMKII α and transduces signals to MEK and ERK1/2 MAPK. Of note, it has been shown that CaMKII binds Raf-1 and that is necessary for ERK1/2 activation by different stimuli and in different cell types (52, 53). Recently, Salzano et al reported that CaMKII directly phosphorylates Raf-1 (49). However, an important issue remains to be explored is as to whether CaMKII α phosphorylates B-raf.

Wnt-3a is known to activate both canonical and non-canonical Wnt signaling. One of the key questions is as to whether individual Fz receptors can activate both pathways, or if the simultaneous activation of canonical and CaMKII α /ERK1/2 pathways is mediated through Fz receptors with different signaling specificity. We found that Fz-6 and Fz-8 are the most expressed Fz receptors in human chondrocytes. Fz-6 has been associated with non-canonical Wnt signaling (40, 41, 54). Golan et al. showed that Fz-6 did not activate the canonical Wnt pathway in HEK293 cells following exposure to various Wnts, including Wnt-3a (42). Kühl et al. demonstrated that in *Xenopus* Fz-3, Fz-4 and Fz-6 activate CaMKII and promote ventral cell fate, whereas Fz-7 and Fz-8 activate the β -catenin pathway and induce axis duplication but do not activate CaMKII (55). We have examined the role of the Wnt signaling receptor Fz-6 in canonical and non-canonical Wnt/CaMKII α /ERK1/2 signaling in primary chondrocytes and demonstrated that Fz-6 does not affect the activation of β -catenin pathway as monitored by stabilization of β -catenin and expression of the target gene *AXIN2*. In contrast, Fz-6 is critical for the activation of non-canonical pathway as demonstrated by the phosphorylation status of CaMKII α and ERK1/2, and the expression of *COL2A1*.

Knockdown of Fz-6 impaired the phosphorylation of both CaMKII α and ERK1/2 by Wnt-3a and prevented loss of chondrocyte phenotype.

In summary, we have identified SYND4 as an essential component of non-canonical Wnt/Ca²⁺/CaMKII α /B-raf/ERK1/2 pathway that regulate chondrocyte phenotype. In the presence of Wnt-3a, Fz-6 activates non-canonical signaling by triggering the docking of CaMKII α to SYND4 and that of B-Raf to DVL-2 as depicted in Figure 6F. Interaction between SYND4 and DVL-2 may bring together CaMKII α and B-Raf probably to allow the activation of B-raf by CaMKII α and to transduce signals to MEK and ERK1/2 MAPK. Our results may help provide clearer understanding of non-canonical Wnt Ca²⁺/CaMKII/ERK1/2 signaling pathway that is essential in both normal development and OA pathogenesis. Our finding may also provide new markers and targets for OA treatment.

Material and method

Chondrocytes and cartilage explants isolation and culture

Human primary chondrocytes were isolated from cartilage obtained from patients who underwent joint replacement for knee OA. Cartilage tissue was dissected from preserved areas of the femoral condyles and patellar groove avoiding subchondral bone and fibrocartilage. Cartilage was washed twice with DMEM F12 complete medium (2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 10% (v/v) fetal bovine serum) and aliquot was then digested with pronase (1 mg/ml) for 30 min à 37 °C, followed by an overnight digestion in collagenase B (1 mg/ml) in similar medium as above. The chondrocytes recovered from the digestion were seeded at a density of 25,000 cells/cm² in DMEM F12 complete medium at 37°C in a humidified atmosphere supplemented with 5% CO₂. All experiments were performed using confluent P0 cells.

For cartilage explant isolation, cartilage specimens were dissected within 1 h of operation, and full-thickness cartilage was removed from preserved areas of the femoral condyles using a 6 mm biopsy punch. Extreme care was taken to avoid subchondral bone, osteophytic cartilage and fibrocartilage. Explants were washed with DMEM F12 complete medium and cultured in 24-well plates in the same medium and condition as chondrocytes for 48 h before use.

Aliquot of cartilage was used for histological scoring for features of OA as described previously(56). Only cells and explants obtained from samples with a Mankin score < 4 were used for subsequent experiments. This study was approved by our local research institution (Commission de la Recherche Clinique; registration no. UF 9757, CPRC 2004, Cellules souches et chondrogénèse). The protocol conforms to the ethical guidelines of the Declaration of Helsinki, and written informed consent has been obtained from each patient.

Cell and cartilage explant treatments

Human primary chondrocytes were seeded onto six-well plates at 5×10^5 cells/well and allowed to attach overnight in standard culture conditions. The cells were then treated for 12 h with 100 ng/ml of recombinant Wnt-3a (R&D Systems, Minneapolis, MN USA) or vehicle (0.1% BSA in PBS) in DMEM F12 medium (2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 2% (v/v) fetal bovine serum) prior to gene or protein expression analyses. When co-treatments with inhibitors and Wnt-3a are indicated, cells were pre-treated for 30 min with the inhibitor *ie.* U0126 (10 µM; Cell signaling, Danvers), DKK1 (100 ng/ml; R&D Systems, Minneapolis, MN USA) KN93 and KN92 (10 µM; R&D Systems, Minneapolis, MN USA) prior to treatment with Wnt-3a or vehicle.

Gene expression analysis

Total RNA from chondrocytes and cartilage explants was extracted using TRIzol (Lifetech, Carlsbad, CA) and purified with RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The reverse transcription was performed using 500 ng (primary chondrocytes) or 200 ng (cartilage explants) of total RNA from each sample with SuperScript® VILO™ Master Mix (Lifetech, Carlsbad, CA). Quantitative PCR was performed with iTaq™ Universal SYBER Green Supermix kit (BIO-RAD, Hercules, CA) using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Primer sequences are listed in Table S1.

Plasmids and siRNA

B-raf vector was a gift from Dustin Maly (University of Washington), Flag-DVL-2 and Flag-DVL-2(Δ361-736) vectors were a gift from Jeff Wrana (University of Toronto, Canada) and TOP-FLASH-firefly reporter plasmid was a gift from Randall Moon (University of Washington). B-raf V600E and B-raf D594V were generated by site directed mutagenesis

using B-raf as template. HA-SYND4 was generated by PCR and cloned into EcoRI and BamHI sites of pCMV empty vector (Stratagene, Valencia, CA). All the vectors were verified by sequencing.

Small interfering RNA (siRNA) targeting SYND4, MAPK1, MAPK3, DVL-1, DVL-2, DVL-3, CTNNB1, Fz-6 and control siRNA were purchased from QIAGEN and are listed in Table S2.

Transfection

Subconfluent human primary chondrocytes in 6-well plates were transfected in triplicate with 2 µg of plasmid using lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. For siRNA transfection, cells were transfected with 25 nM siRNAs using DharmaFECT transfection reagent (Thermo Scientific, Waltham, MA) according to manufacturer's instructions. 24 h after transfection the medium was replaced and at 48 h posttransfection, cells were stimulated for 12 h with recombinant Wnt-3a 100 ng/ml (R&D systems) or vehicle at 37°C and 5% CO₂.

Western Blotting

Total protein from chondrocytes was extracted using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Cell lysates were sonicated on ice and protein concentration of the samples was determined by the Bradford method. Proteins (50 µg/lane) were separated on 10% SDS-PAGE gels, transferred to a PVDF membrane (Millipore, Eschborn, Germany), and subsequently blocked in PBS-Tween 20 containing 5% nonfat milk or 5% BSA. Membranes were then incubated overnight with primary antibodies directed against p44/42 MAPK, phospho-p44/42, β-catenin, phospho-

B-raf, DVL-2, DVL-3, CaMKII α (diluted 1:1000; Cell signaling, Danvers, USA), DVL-1, B-raf, phospho-CaMKII α (diluted 1:1000 Santa Cruz, Texas), Flag (diluted 1:1000, Sigma, Saint Louis MO) or HA (diluted 1:1000, Biologend, San Diego, CA) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (diluted 1:2000, Cell signaling). Antibodies were diluted in 5% nonfat milk/0.01% tween 20 in PBS. In the case of p44/42 MAPK, phospho-p44/42, DVL-2, DVL-3 antibodies were diluted in 5% BSA/0.01% tween 20 in PBS. The blots were then developed using Clarity Western ECL substrate (BIO-RAD, Hercules, CA) according to the instructions of the manufacturer.

Immunoprecipitation

For immunoprecipitation, subconfluent primary chondrocytes in 10-cm plates were treated with Wnt-3a (100 ng/ml) or vehicle for 1 h and were lysed on ice with TNE buffer (50 mM Tris pH 8, 1% IGEPAL[®]CA-630, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche Diagnostics). The lysates were sonicated and centrifuged and the resultant supernatants were used to recover B-raf, Flag-tagged and HA-tagged proteins by incubating overnight at 4°C with anti-B-raf, anti-Flag and anti-HA antibodies, respectively followed by incubation with protein A beads (Millipore, Eschborn, Germany) for 3 h at room temperature. After washing with lysis buffer, bound proteins were eluted with loading buffer and analysed by immunoblotting. Immunoblotting was performed as described previously with antibodies against Flag, HA, B-raf, DVL-2 and phospho-CaMKII α .

Calcium mobilization assay

Fluo-4 NW Calcium Assay Kit (Invitrogen) was used to evaluate calcium mobilization according to the manufacturer's manual. After removing the medium, the chondrocytes were labelled with Fluo-4 NW dye loading solution containing Probenecid for 1 h (30 min at 37°C

and 30 min at room temperature). After the treatment, fluorescence was measured using a fluorometer (Varioskan flash, Thermo Scientific) with settings appropriate for excitation at 494 nm and emission at 516 nm. The fluorescence was expressed as relative fluorescence units.

Data Analysis and Statistical Procedures

Each experiment was repeated at least three times independently. Quantitative data were expressed as mean \pm S.D. Statistical analysis was performed with an unpaired two-tailed Student's t-test, and effects were considered statistically significant at $*P < 0.05$. One representative immunoblot of three independent experiments was shown in results.

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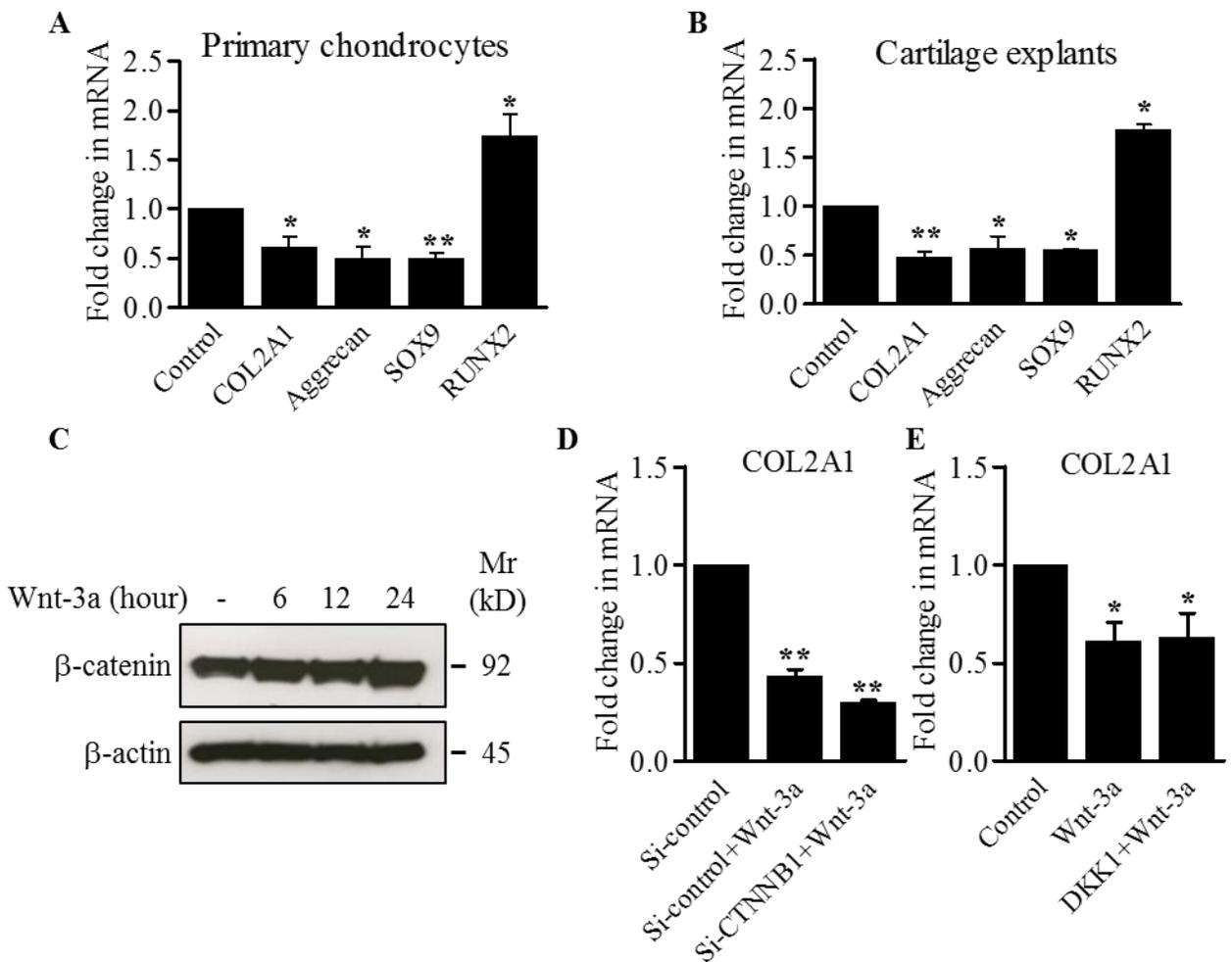
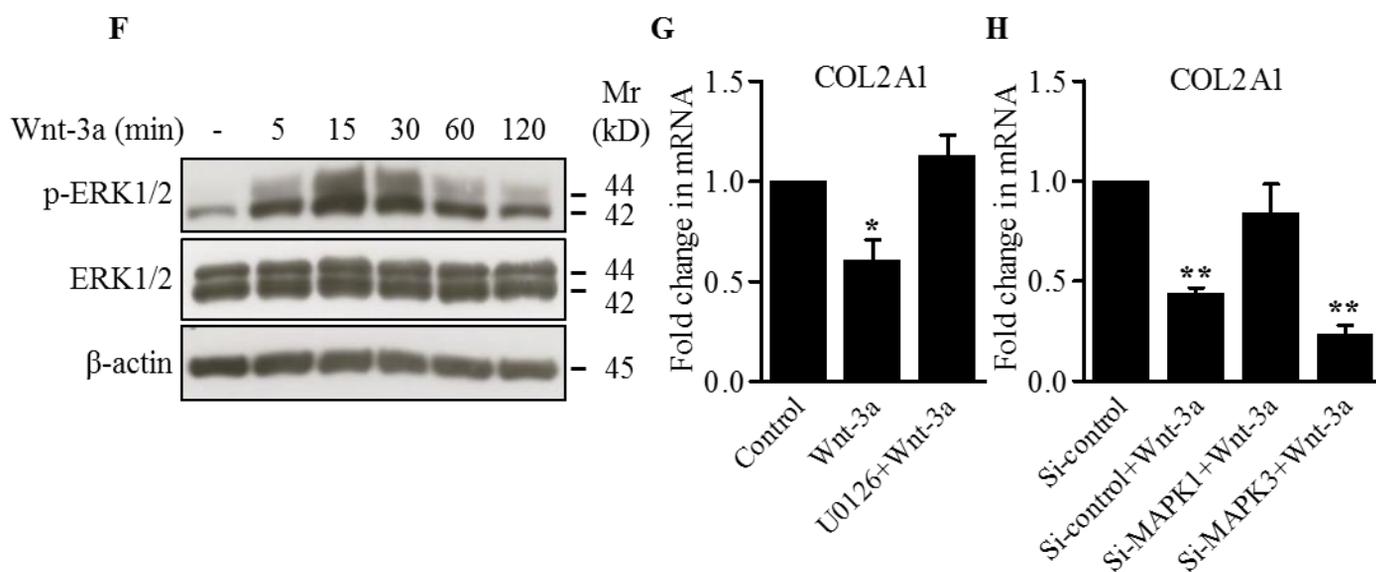


Figure 1. Wnt-3a induces loss of chondrocyte phenotype through β -catenin-independent pathway. (A and B) Wnt-3a inhibited the expression of chondrocyte phenotype markers. Wnt-3a(100 ng/ml) down-regulated the expression of *COL2A1*, *Aggrecan* and *SOX9* and up-regulated that of *RUNX2* in human chondrocytes (A, n=5) and cartilage explants (B, n=5) as evaluated by Q-PCR. Values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. (C) Wnt-3a activates canonical Wnt pathway in primary chondrocytes. Western blotting for β -catenin indicated time dependent accumulation of β -catenin. Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle (control) for 6 h, 12 h and 24 h and the protein level of β -catenin was determined by Western blotting, β -actin was used as loading control (n=3). (D and E) Inhibition of β -catenin-dependent pathway could not rescue Wnt-3a-induced down-regulation of the chondrocyte phenotypic gene marker *COL2A1*. (D) Silencing of β -catenin or (E) blockage of the Wnt canonical pathway by DKK1 failed to rescue the Wnt-3a-induced down regulation of *COL2A1* (n=3).



(F-H) Wnt-3a down-regulation of *COL2A1* is mediated by ERK1/2 pathway. (F) Wnt-3a rapidly activates ERK1/2 signaling in primary chondrocytes. Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for different time points and the protein level of ERK1/2 and p-ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). (G) Pharmacological inhibition of ERK1/2 activation prevented Wnt-3a-induced down-regulation of *COL2A1*. Human primary chondrocytes were treated with Wnt-3a (100 ng/ml) in combination or not with the MEK1/2 inhibitor U0126 (1 μ M) for 12 h and the expression of *COL2A1* was evaluated by Q-PCR (n=4). (H) Inhibition of ERK1/2 signaling by the knockdown of ERK1 or ERK2 prevented down-regulation of SYND4 by Wnt-3a. Primary chondrocytes were transfected with Si-MAPK1, Si-MAPK2 or Si-control for 48 h and treated with Wnt-3a (100 ng/ml) or vehicle for a further 12 h (n=4) and expression of SYND4 was analysed by Q-PCR. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

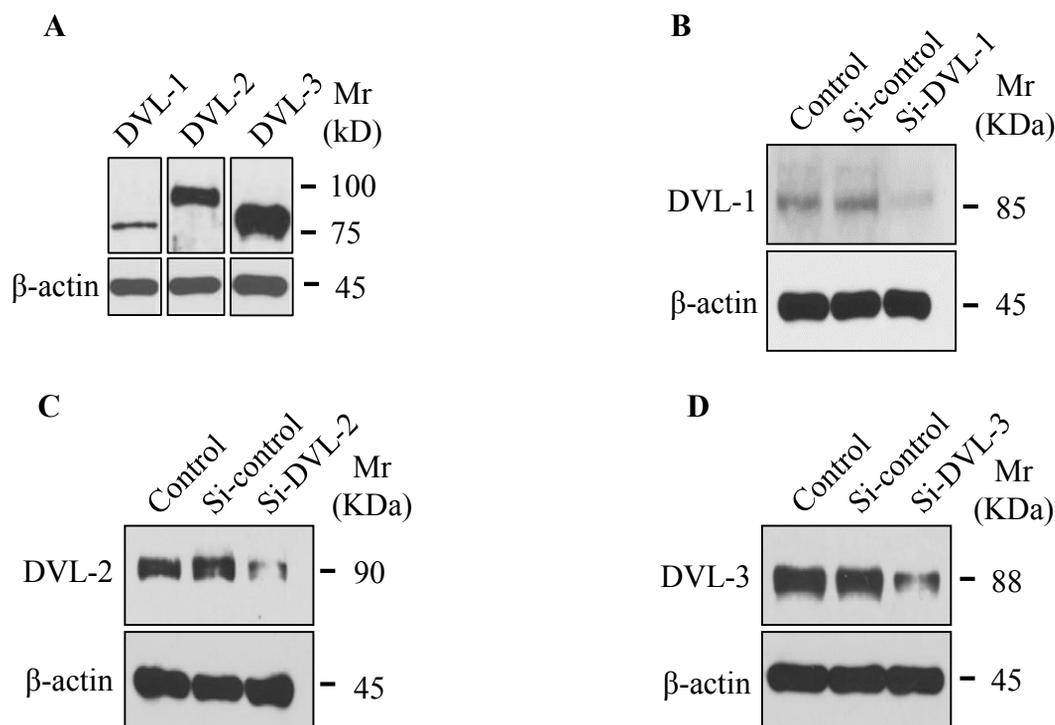
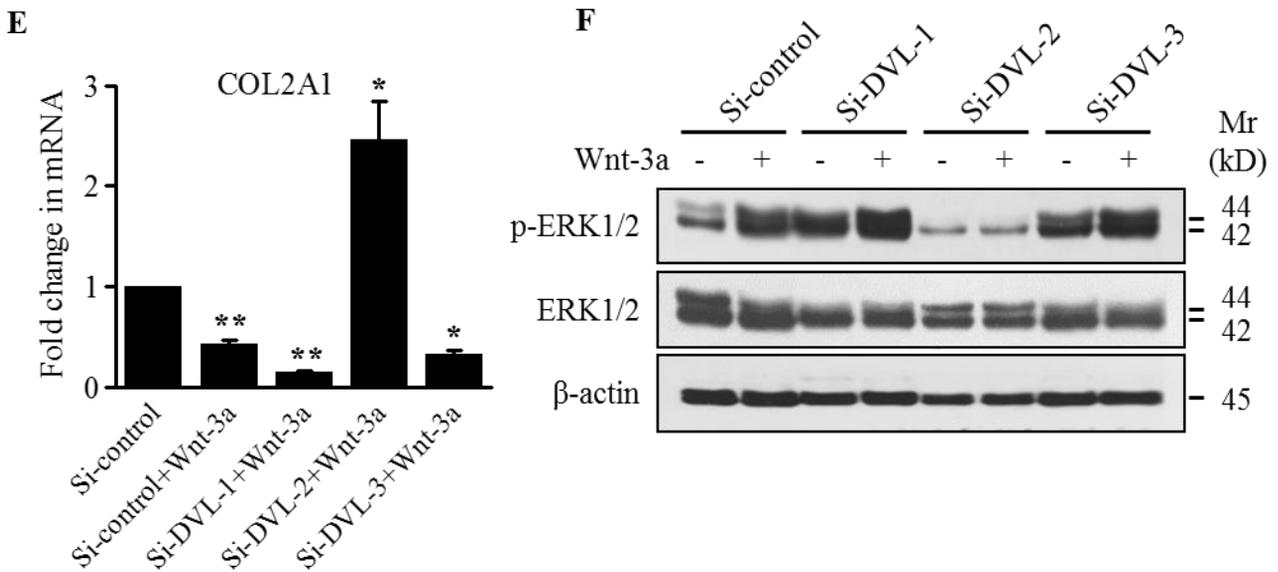


Figure 2. DVL-2 mediates the down-regulation of COL2A1 induced by non-canonical Wnt signaling. (A) Expression levels of different subtypes of DVL are significantly different in primary chondrocytes. Western blotting for DVLs indicated that DVL-3 is the most expressed DVL protein in human chondrocytes, while DVL-1 is the least expressed (n=3). (B-D) siRNA efficiency on the expression of DVLs in human primary chondrocytes. Knockdown of DVL-1, DVL-2 and DVL-3 efficiently decreased the corresponding protein level. Primary chondrocytes were transfected with siRNA specific to (B) DVL-1 (Si-DVL-1), (C) DVL-2 (Si-DVL-2), (D) DVL-3 (Si-DVL-3), or siRNA control (Si-control) for 48 h and the level of DVL-1, DVL-2 and DVL-3 was determined by Western blotting, β-actin was used as loading control (n=3).



(E) DVL-2 is essential for Wnt-3a-induced down-regulation of *COL2A1*. Knockdown of DVL-2 reversed the inhibition by Wnt-3a of *COL2A1* as evaluated by PCR (n=3). (D) Knock down of DVL-2 but not of DVL-1 or DVL-3 inhibited Wnt-3a-induced activation of ERK1/2 signaling. Primary chondrocytes were transfected with siRNA specific to DVL-1, DVL-2, DVL-3 or siRNA control for 48 h then treated with Wnt-3a (100 ng/ml) or vehicle for 30 min and the protein level of ERK1/2 and pERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$)

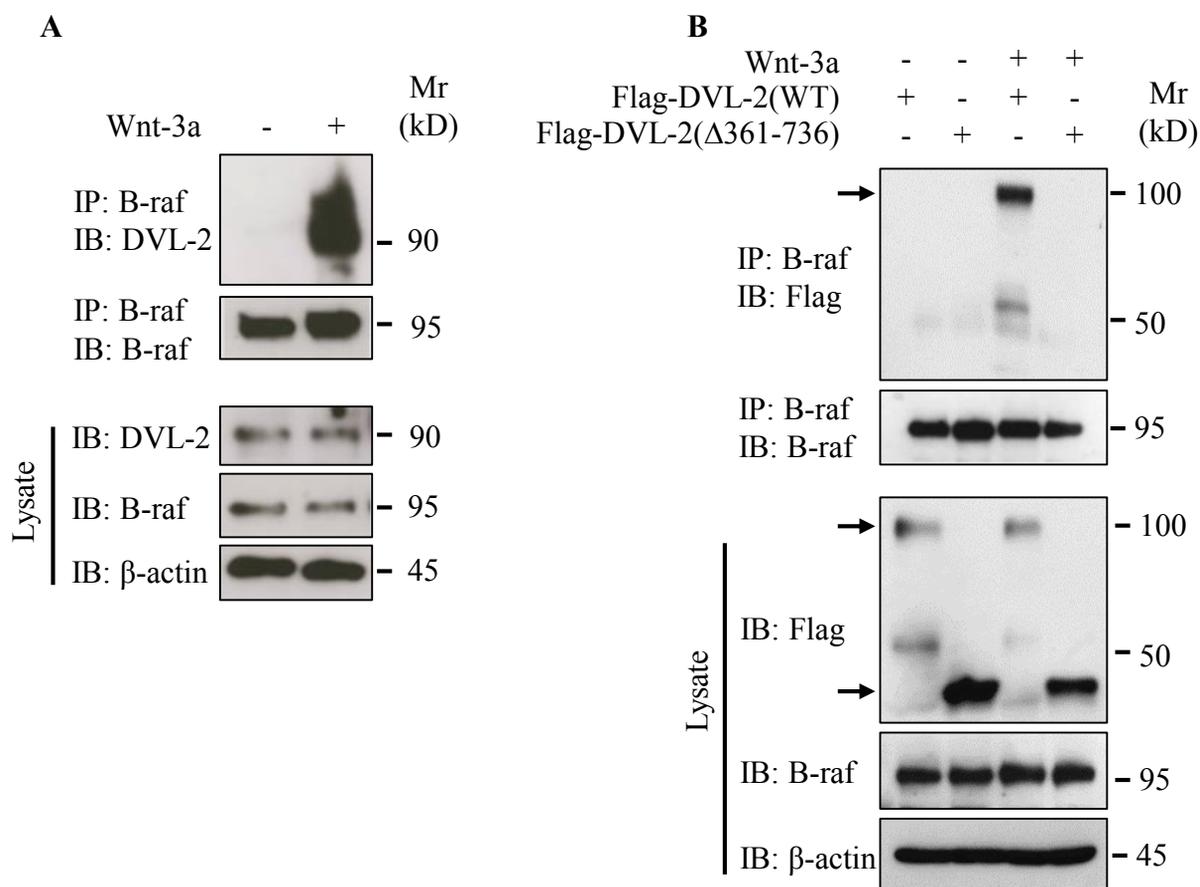
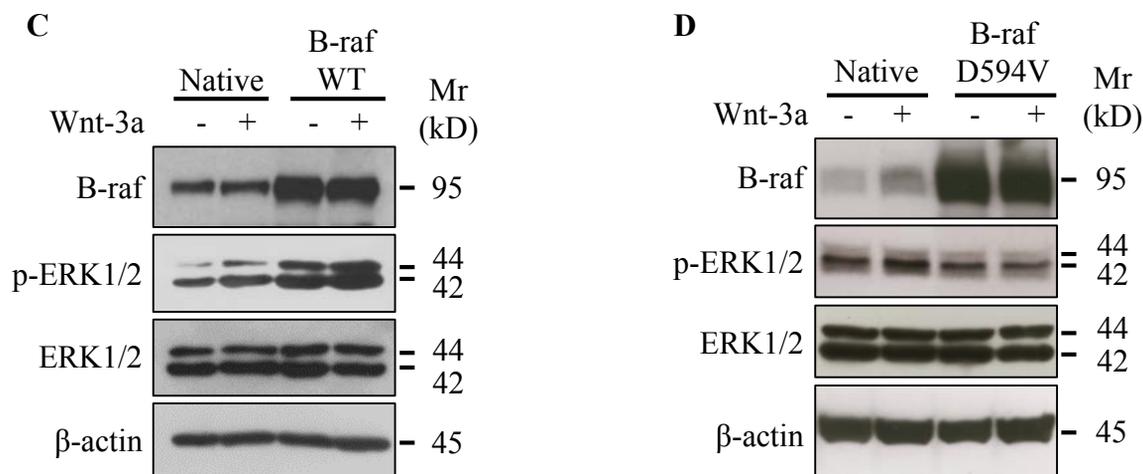


Figure 3. DVL-2 docks B-raf in Wnt-dependent manner. DVL-2 interacts with B-raf through the C-terminal domain. (A) Primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for 1 h and cell lysates were assessed after immunoprecipitation with anti-B-raf by immunoblot analysis with anti-DVL-2 or anti-B-raf antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-DVL-2 and anti-B-raf antibodies. β -actin was used as loading control. (B) Primary chondrocytes were transfected with expression vector for Flag-tagged wildtype DVL-2 or Flag-DVL-2(Δ 361-736) for 24 h, then treated with Wnt-3a (100 ng/ml) or vehicle for 1 h, assessed after immunoprecipitation with anti-B-raf by immunoblot analysis with anti-Flag or anti-B-raf antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-Flag and anti-B-raf antibodies. β -actin was used as loading control.



(C and D) B-Raf is essential for Wnt-3a activation of ERK1/2 signaling. Immunoblot analysis of lysates of primary chondrocytes transfected (C) with empty vector (Native) or expression vector for wild-type B-raf, or (D) with empty vector (Native) or expression vector for dead mutant of Braf (D594V), and treated with Wnt-3a (100 ng/ml) or vehicle for 12 h, probed with anti-B-raf, anti-p-ERK1/2 or anti-ERK1/2. β -actin was used as loading control (n=3). One representative blot of three independent experiments is shown.

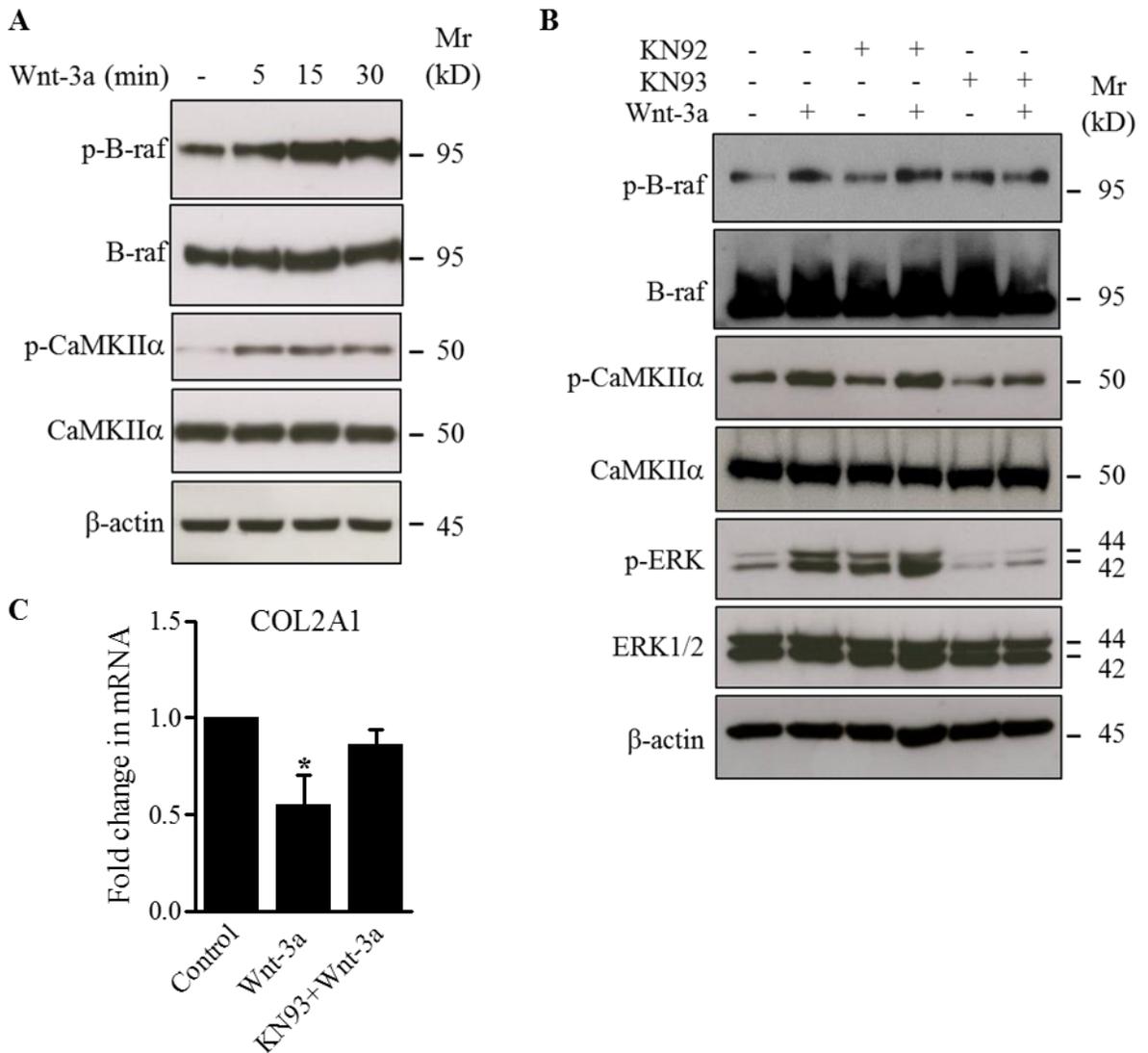
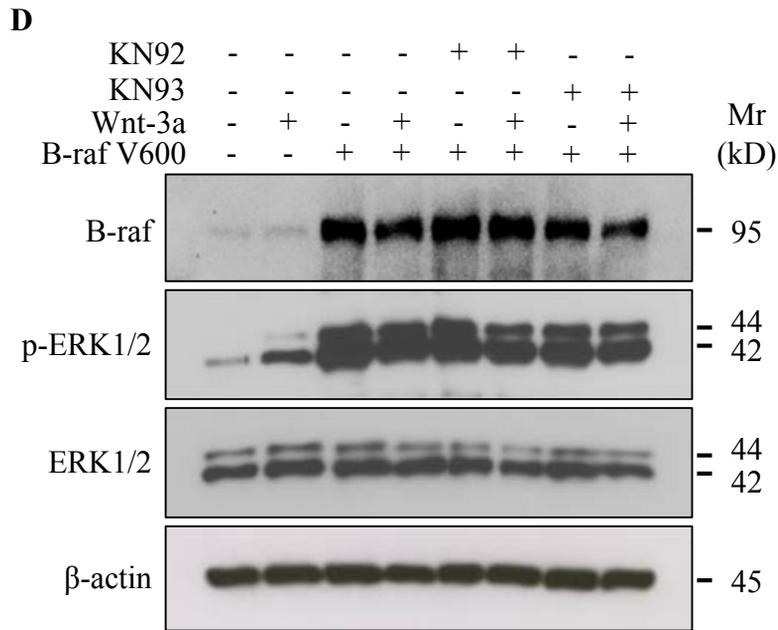
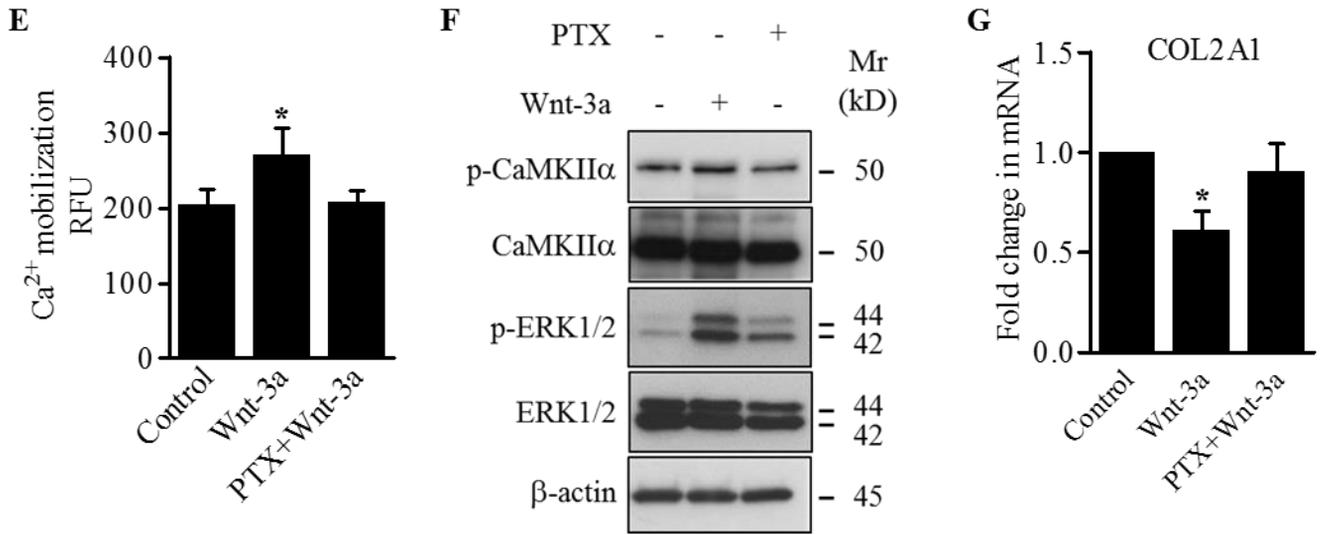


Figure 4. Wnt-3a-induced activation of B-raf/ERK1/2 pathway is mediated through activation of CaMKII α . (A) Wnt-3a induces the phosphorylation of CaMKII α . Human primary chondrocytes were stimulated with Wnt-3a (100 ng/ml) or vehicle for indicated time, then cell lysates were analysed by Western blotting with anti-B-raf, anti-p-B-raf, anti-p-CaMKII α and anti-CaMKII α antibodies. β -actin was used as loading control (n=3). (B) Wnt-3a-induced activation of B-raf/ERK1/2 is blocked by inhibition of CaMKII α . Immunoblot analysis of total cell lysates of primary chondrocytes pre-treated with KN92, KN93 or vehicle (DMSO) for 12 h and stimulated with Wnt-3a or vehicle for 30 min, probed with anti-p-CaMKII α , anti-CaMKII α , anti-B-raf, anti-p-B-raf, anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control (n=3). (C) Inhibition of CaMKII α by KN93 rescued the down-regulation of COL2A1 by Wnt-3a, as evaluated by PCR (n=4).



(D) Immunoblot analysis of total cell lysates of primary chondrocytes transfected with expression vector for active mutant of B-Raf (V600) pre-treated with KN92 or KN93, or DMSO for 12 h and stimulated with Wnt-3a or vehicle for 30 min, probed with anti-B-raf, anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control (n=3).



(E) Calcium mobilization induced by Wnt-3a depend on the activation of G protein. Human primary chondrocytes were pre-treated overnight by 1 μ g/ml G protein inhibitor PTX or vehicle then treated with Wnt-3a for 5 min. PTX prevented calcium mobilization induced by Wnt-3a (n=3).

(F) Phosphorylation of CaMKII α and ERK1/2 induced by Wnt-3a depend on the activation of G protein. Chondrocytes were pre-incubated overnight with PTX or vehicle then treated by Wnt-3a for 30 min, PTX abolished the activation of CaMKII α /ERK1/2 pathway by Wnt-3a.

(G) Inhibition of G protein by PTX rescued the down-regulation of COL2A1 by Wnt-3a, as evaluated by PCR (n=4). Data are representative of at least three experiments. Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$)

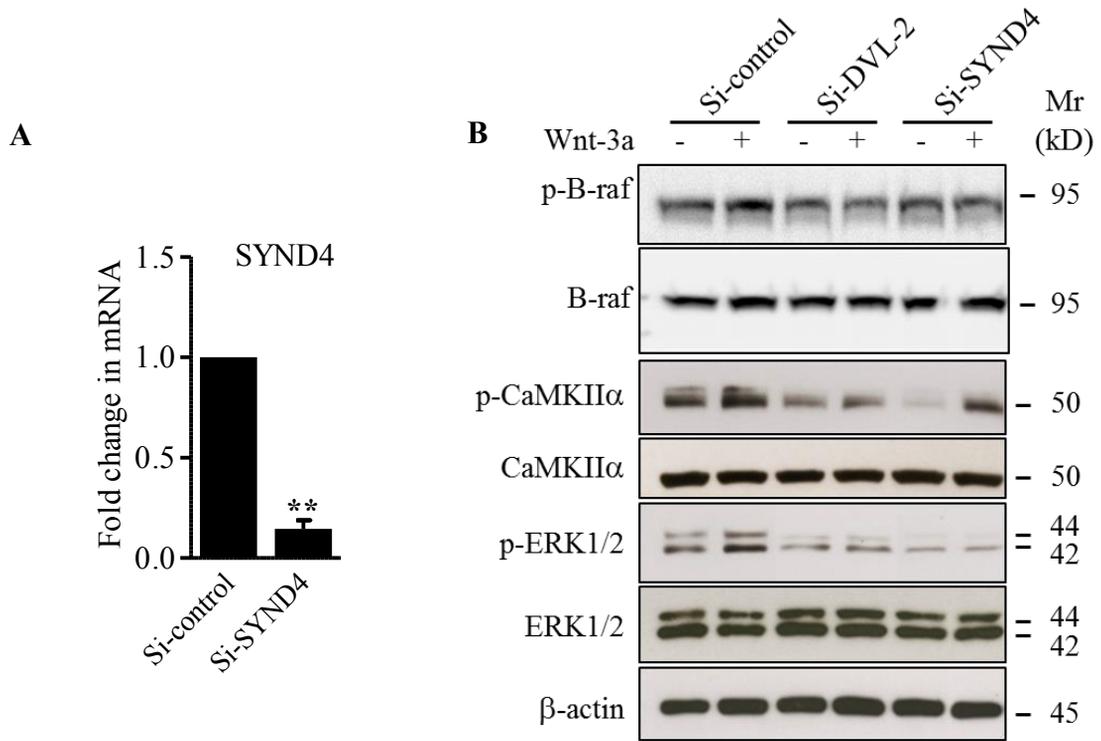
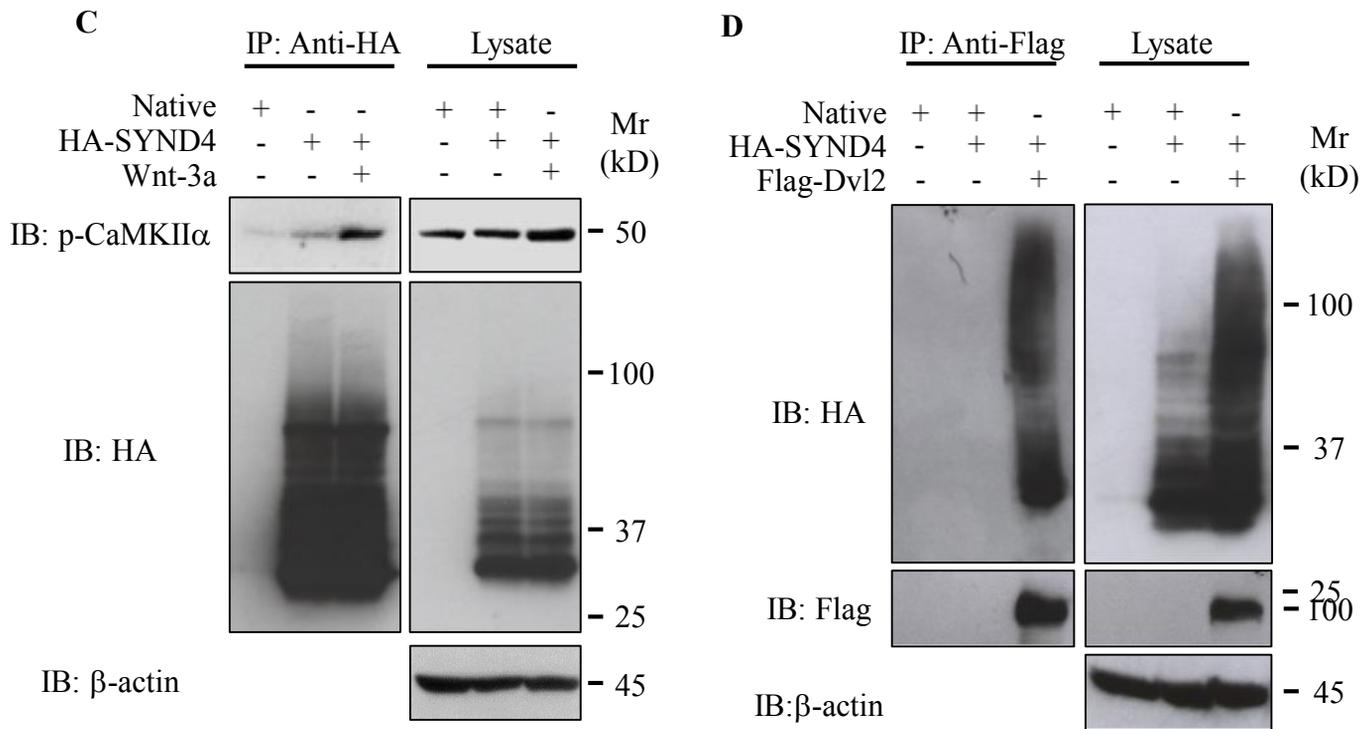
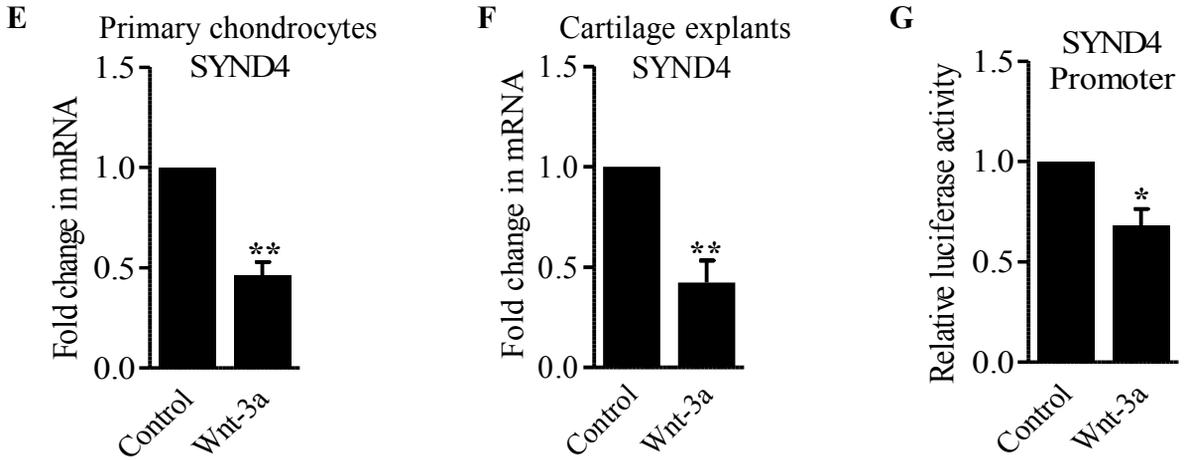


Figure 5. Syndecan 4 interacts with CaMKII α and DVL-2. Knockdown of SYND4 inhibited Wnt-3a-induced activation of B-raf/ERK1/2 but not that of CaMKII α , whereas knockdown of DVL-2 inhibited the activation of B-raf/ERK1/2 and CaMKII α by Wnt-3a. (A) Transfection of chondrocytes with si-SYND4 strongly reduced its expression as evaluated by Q-PCR (n=4). (B) Immunoblot of total cell lysates of primary chondrocytes transfected with siRNA specific to SYND4 (Si-SYND4), DVL-2 (Si-DVL-2) or siRNA control (Si-control) for 48 h and stimulated with Wnt-3a or vehicle for 12 h, probed with anti-p-B-raf, anti-B-raf, anti-p-CaMKII α , anti-CaMKII α , anti-p-ERK1/2 or anti-ERK1/2 antibodies. β -actin was used as loading control (n=3).



(C) Wnt-3 promotes the recruitment of p-CaMKII α by SYND4. Primary chondrocytes were transfected with empty vector (Native) or expression vector for HA-tagged SYND4 for 24 h, then treated with Wnt-3a (100 ng/ml) or vehicle for 1 h, assessed after immunoprecipitation with anti-HA by immunoblot analysis with anti-p-CaMKII α or anti-HA antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-p-CaMKII α and anti-HA antibodies. β -actin was used as loading control. SYND4 contains four glycosaminoglycan chains and as expected migrates as a broad smear of 30–200 kDa (D) Primary chondrocytes were transfected with empty vector (Native) or expression vector for HA-tagged SYND4 or Flag-tagged DVL-2 for 24 h then assessed after immunoprecipitation with anti-Flag by immunoblot analysis with anti-HA and anti-Flag antibodies (n=3). Total cell lysates were analysed by immunoblot with anti-HA and anti-Flag antibodies. β -actin was used as loading control. Data are representative of at least three experiments.



(E-G) Wnt-3a inhibited the expression of SYND4. Wnt-3a (100 ng/ml) downregulated the expression of SYND4 in human chondrocytes (E, n=5) and cartilage explants (F, n=5), as evaluated by Q-PCR. (G) SYND4 promoter activity was significantly inhibited by Wnt-3a in human primary chondrocytes (n=4). Primary chondrocytes were transfected with SYND4prom-firefly reporter vector or pGL3-Basic (empty vector, control) and pRL-TK vector (used as internal control) and treated with Wnt-3a (100 ng/ml) or vehicle for 24 h. Relative luciferase activity was calculated as fold of activation over that of pGL3-Basic after normalization. Q-PCR results were normalized as described previously. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

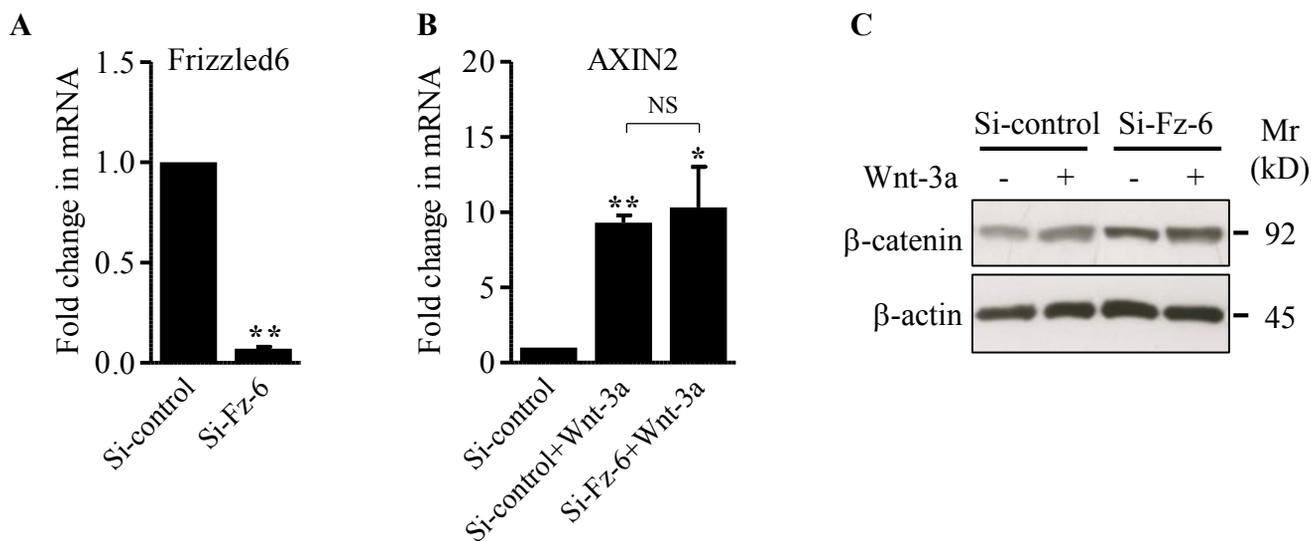
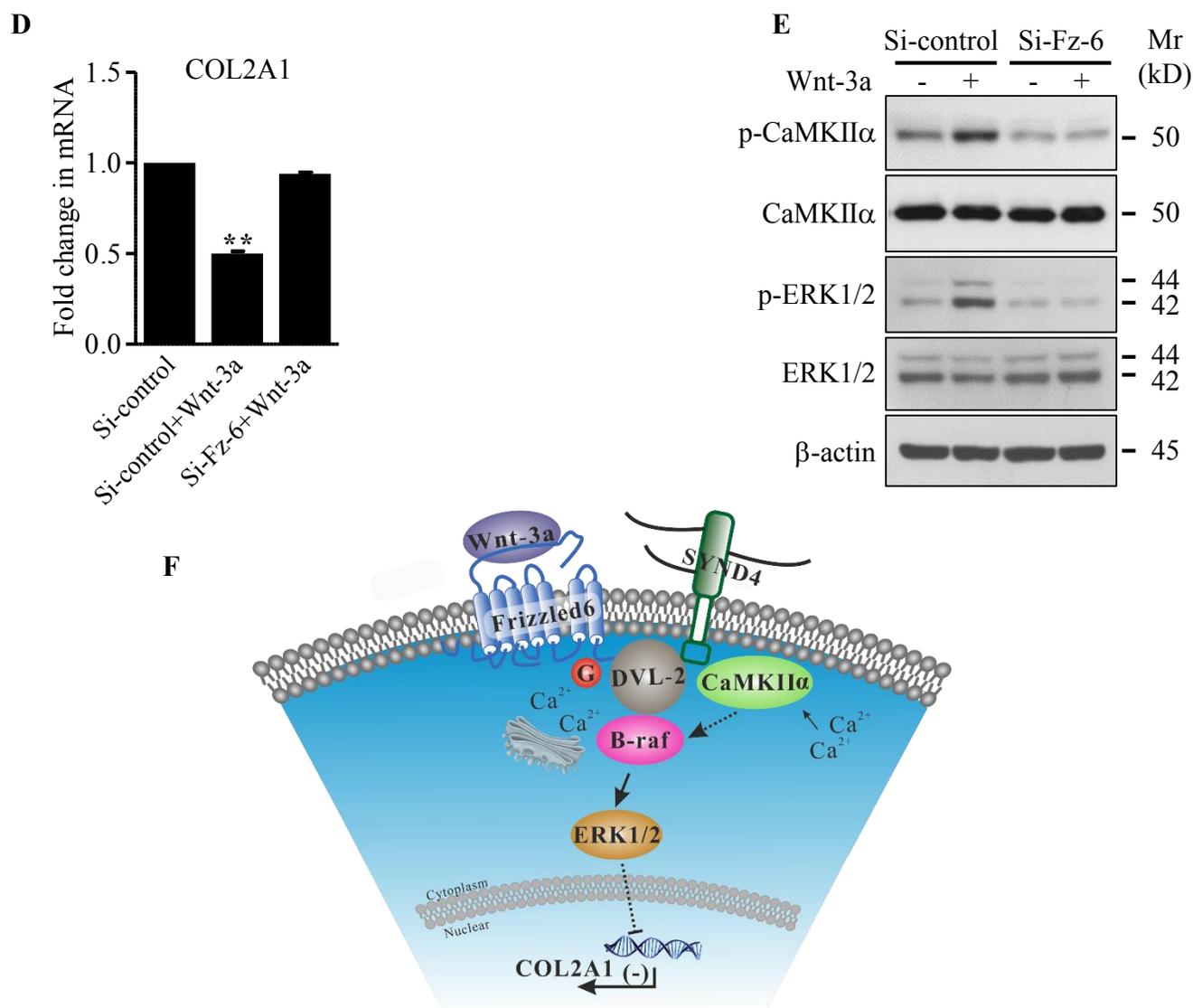


Figure 6. Frizzled6 mediates non-canonical Wnt-3a-induced loss of chondrocyte phenotype.

(A-C) Fz-6 is not required for Wnt-3a activation of canonical Wnt pathway in primary chondrocytes. (A) Knockdown efficiency of Fz-6 in human primary chondrocytes (n=5). (B) Knockdown of Fz-6 did not block Wnt-3a-induced up-regulation of Wnt canonical target gene AXIN2 in primary chondrocytes (n=3), as evaluated by Q-PCR. Human primary chondrocytes were transfected with siRNA specific to Fz-6 (Si-Fz-6) or siRNA control (Si-control) for 48 h then treated with Wnt-3a (100 ng/ml) or vehicle for 12 h and the expression of AXIN2 was determined by Q-PCR. (C) Wnt-3a-induced accumulation of β -catenin was not impaired by Fz-6 knockdown. Western blotting for β -catenin in chondrocytes transfected with Si-Fz-6 or Si-control for 48 h and treated with Wnt-3a (100 ng/ml) or vehicle for a further 12 h, β -actin was used as loading control (n=3).

Figure 6



(D and E) Fz-6 is essential for Wnt-3a-induced down-regulation of COL2A1 and activation of noncanonical pathway. (D) Knockdown of Fz-6 suppressed Wnt-3a-induced down-regulation of COL2A1 (n=3), as evaluated by Q-PCR. (E) Knockdown of Fz-6 prevented Wnt-3a-induced activation of CaMKII α and ERK1/2. Human primary chondrocytes were transfected with Si-Fz-6 or Si-control for 48 h and treated with Wnt-3a (100 ng/ml) or vehicle for a further 30 min and the protein level of p-CaMKII α , CaMKII α , p-ERK1/2 and ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). (F) A working model for the role of SYND4 in the regulation of non-canonical Wnt/CaMKII/B-raf/ERK1/2 pathway and chondrocyte phenotype. See text for details. Q-PCR values were normalized for the housekeeping gene ribosomal protein S29 and are expressed as relative expression compared with control. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (NS, statistically not significant; *, $p < 0.05$; **, $p < 0.01$).

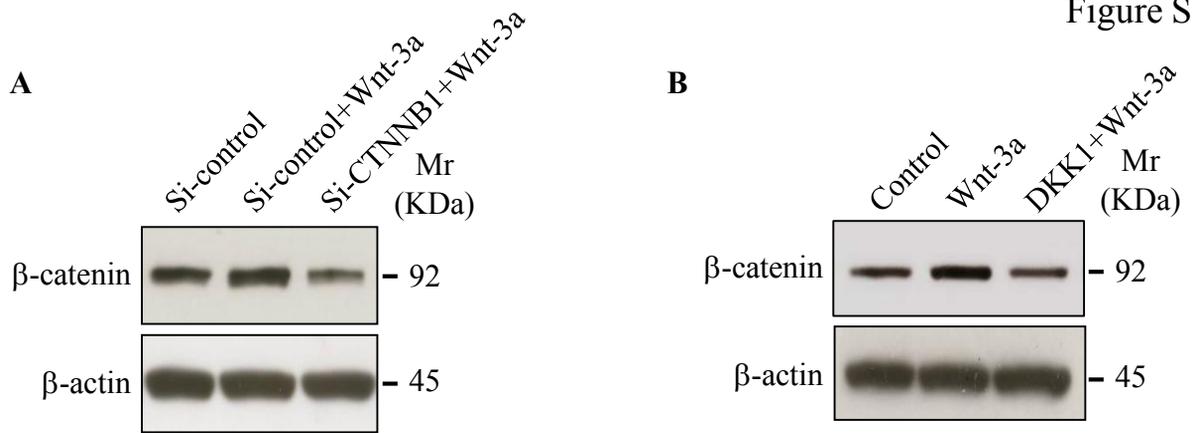


Figure S1. **siRNA and inhibitors efficiency on Wnt canonical signaling pathways.** (A) Knockdown of β -catenin by specific siRNA prevents its accumulation induced by Wnt-3a. Western blotting for β -catenin in chondrocytes transfected with siRNA to β -catenin (Si-CTNNB1) or siRNA control (Si-control) for 48 h then stimulated with Wnt-3a (100 ng/ml) or vehicle for 12 h, β -actin was used as loading control (n=3).

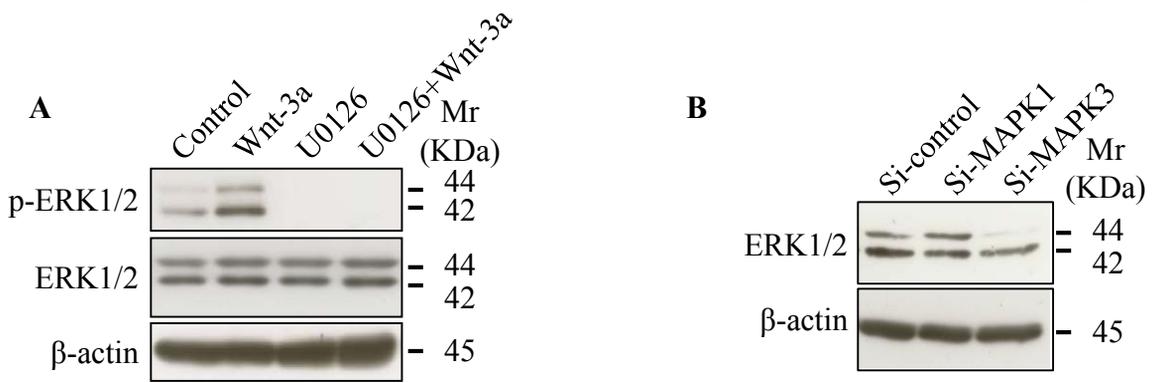


Figure S2. **siRNA and inhibitors efficiency on ERK1/2 signaling pathways.** (A) Pharmacological inhibition of MEK1/2 prevented activation of ERK1/2 by Wnt-3a. Primary chondrocytes were treated with Wnt-3a (100 ng/ml) or vehicle for 12 h, in presence and absence of MEK1/2 inhibitor U0126 and the protein level of p-ERK1/2 and ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3). (B) Knockdown of MAPK1 and MAPK3 efficiently decreased their expression. Primary chondrocytes were transfected with siRNA specific to MAPK1 (Si-MAPK1), MAPK3 (Si-MAPK3) or siRNA control (Si-control) for 48 h and the level of ERK1/2 was determined by Western blotting, β -actin was used as loading control (n=3).

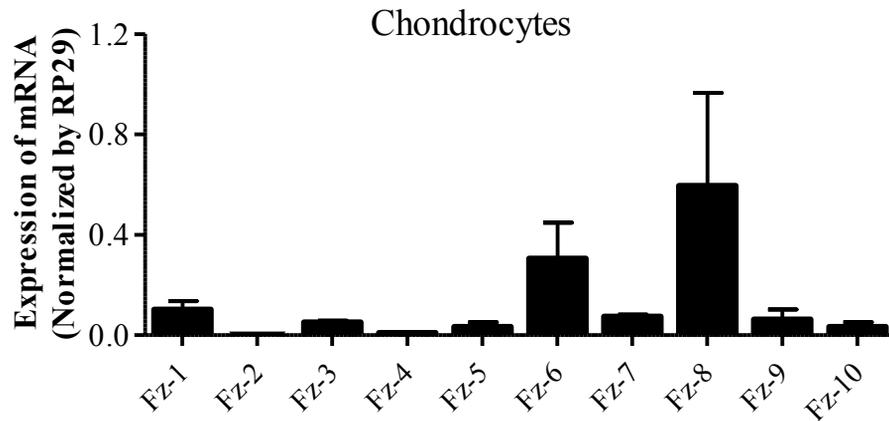


Figure S3. **The relative expression of Fz receptors in human primary chondrocytes.** Fz-6 and Fz-8 receptors are predominantly expressed in human primary chondrocytes. Expression of different Fz receptors was evaluated by absolute Q-PCR. Specific DNA segment for each of the ten Fz receptors (standards) was amplified by RT-PCR, purified on agarose gel, quantified and used as template for Q-PCR. Absolute quantification was determined compared to standards that are run in parallel. Data are expressed as mean \pm S.D. of 3 separate experiments

Supplementary Tables

Table S1. **Human primers sequences used in the experiments**

Gene name	Primer sequence	Gene name	Primer sequence
RP29 F	5'-TTCAAACCGGCACGGTCTGA-3'	Fz-1 F	5'-GGCTTTGTGTCGCTCT-3'
RP29 R	5'-TGCCGTA CTGACGGAAACAC-3'	Fz-1 R	5'-GGGATAGCGTAGCTCTT-3'
COL2A1 F	5'-CTGCTCGTCGCCGCTGTCCTT-3'	Fz-2 F	5'-GAGCGTGATTGTGCTG-3'
COL2A1 R	5'-AAGGGTCCCAGGTTCTCCATC-3'	Fz-2 R	5'-GCTCTGGGTAGCGGAA-3'
Aggrecan F	5'-GTTGTCATCAGCACCAGCATC-3'	Fz-3 F	5'-GCTTACAGTGAGTGTTTCG-3'
Aggrecan R	5'-ACCACACAGTCCTCTCCAGC-3'	Fz-3 R	5'-CACGCACATGCAGAAA-3'
Sox9 F	5'-GAACGCACATCAAGACGGAG-3'	Fz-4 F	5'-GCTACAACGTGACCAAG-3'
Sox9 R	5'-TCTCGTTGATTTTCGCTGCTC-3'	Fz-4 R	5'-TCAGGACGGGYCACA-3'
RUNX2 F	5'-GCTGTTATGAAAAACCAAGT-3'	Fz-5 F	5'-CTGTCGCTAAACTTTCCG-3'
RUNX2 R	5'-GGGAGGATTTGTGAAGAC-3'	Fz-5 R	5'-CGTCCAAAGATAAACTGCT-3'
SYND4 F	5'-CGAGAGACTGAGGTCATCGAC-3'	Fz-6 F	5'-TTCCCTAATCTGATGGGTC-3'
SYND4 R	5'-GCGGTAGA ACTCATTGGTGG-3'	Fz-6 R	5'-TTCAAGCTCCTCAGGC-3'
Axin2 F	5'-AGTGTGAGGTCCACGGAAAC-3'	Fz-7 F	5'-GACGTGCAAGAGCTATG-3'
Axin2 R	5'-CTGGTGCAAAGACATAGCCA-3'	Fz-7 R	5'-TGCTGCTGTGGCTAAGT-3'
DVL-1 F	5'-TTATCTACCACATGGACGAGGA-3'	Fz-8 F	5'-ACCCGAGATTTGGAGC-3'
DVL-1 R	5'-CAAAGATCTCCTCCTCACCAC-3'	Fz-8 R	5'-GGCATTTCCTGGTCA-3'
DVL-2 F	5'-CGTCACAGATTCACAATGTCT-3'	Fz-9 F	5'-AAGGTCAGGCAAGTGAG-3'
DVL-2 R	5'-TCGTTGCTCATGTTCTCAAAGT-3'	Fz-9 R	5'-CTTCCAGAGGAACGCAA-3'
DVL-3 F	5'-CATGTC ACTCAACATCATCACG-3'	Fz-10 F	5'-GAACGCCTCAACATGGA-3'
DVL-3 R	5'-ACAACATATCTCCTGGCTCGAT-3'	Fz-10 R	5'-CTTTAACCTACGGCTGC-3'

F: Forward, R: Reverse

Table S2. **siRNA sequences used in the experiments**

Gene	siRNA sequence
SYND4	5'-UACGGGAAUCUCUGUGGUAUA-3'
CTNNB1	5'-CUCGGGAUGUUCACAACCGAA-3'
MAPK1	5'-AACACUUGUCAAGAAGCGUUA-3'
MAPK3	5'-CUCCUGACCCGUCUAAUAUA-3'
DVL-1	5'-CAGCGUUUAGAUGGUUACAUA-3'
DVL-2	5'-GGUUCCUCCUCCAUGAGCACCAU-3'UA
DVL-3	5'-CUCCUGGUUACUUCAUCACAA-3'
Fz-6	5'-GGUUCCACCUUGUCGUAAA-3'

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Title

Wnt/ β -catenin signaling counteracts interleukine-1-induced ADAMTS-4 expression and activity in human articular chondrocytes

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Keywords: Wnt-3a, osteoarthritis, β -catenin, NF κ B, cartilage degradation, ADAMTS-4

Highlights

- Wnt-3a inhibits the expression of ADAMTS-4 through the β -catenin dependent pathway
- Wnt canonical pathway was mediated by Dvl1, Dvl3 and Fzd8 in human chondrocytes
- Wnt-3a inhibits the expression of ADAMTS-4 through the inhibition of NF κ B pathway
- Wnt-3a counteracts IL-1 β -induced up-regulation of ADAMTS-4 and cartilage degradation

Abstract

Wnt signaling inhibited the expression of MMPs via canonical Wnt/ β -catenin-dependent pathway in human chondrocytes. However, the effect of Wnt on ADAMTS-4, the only aggrecanase regulated by IL-1 β in human chondrocytes and playing a key role in cartilage degradation, has not been elucidated yet. Here, we showed that Wnt/ β -catenin pathway inhibits ADAMTS-4 in human chondrocytes and demonstrated that it is mediated by Fz-8 and Dvl1/3. Analysis of the molecular mechanism involved showed that activation of the Wnt/ β -catenin pathway inhibits the activity of NF κ B. Furthermore, we showed that Wnt/ β -catenin pathway counteracts, to some extent, the IL-1 β -induced expression and activity of ADAMTS-4. This indicates that Wnt canonical pathway might safeguard against OA progression.

Introduction

Osteoarthritis (OA) disease is characterized by inflammation and extracellular matrix (ECM) degradation. OA affects more than half of the world population over 65 years-old [1]. In OA pathophysiology, extracellular matrix homeostasis in articular cartilage is interfered by mechanical and inflammatory stresses. One of the most well-studied proinflammatory cytokines to play a prominent role in OA is IL-1 β . It activates many mediators such as NF κ B, p38 and c-Jun N-terminal kinase (JNK) to repress the expression of essential ECM components (i.e. aggrecan and type II collagen), and to induce a spectrum of proteolytic enzymes including collagenases and aggrecanases in chondrocytes, then shifts the balance towards degradation [2].

Since higher level of β -catenin was found in chondrocytes within the areas of degenerative cartilage [3], Wnt signaling was considered to participate in the pathological development of OA. In addition to the differential expression patterns of Wnt proteins and their inhibitors in normal and OA cartilages [4], the Wnt signaling was reported to promote the dedifferentiation of chondrocytes [5,6] or to mediate the chondrocytes dedifferentiation induced by IL-1 β [7]. However, little is known on Wnt's effects on the expression of proteolytic enzymes which seems to be different between human and animal models. Indeed, it has been show that activation of Wnt/ β -catenin signaling in rabbit and mouse chondrocytes induced the expression of cartilage matrix-degrading MMPs [8,9]. In contrast, Wnt/ β -catenin signaling was demonstrated to act as a potent inhibitor of MMP-1, MMP-3, and MMP-13 expression in human chondrocytes [10].

On the other hand, ADAMTS-4, as the only aggrecanase regulated by IL-1 β in human chondrocytes, is considered to be essential in cartilage destruction. But its regulation by Wnt was reported in few works. In bovine chondrocytes, the expression of ADAMTS-4 was induced by Wnt-3a through the β -catenin dependent pathway [11]. Wnt-3a even enhanced the catabolic effect of IL-1 β in the rabbit chondrocytes [9]. In contrast, we showed here that in human cartilage explant and chondrocytes Wnt-3a inhibited the expression and activity of ADAMTS-4. Investigation of the mechanism involved indicated that Wnt/ β -catenin pathway inhibits the activity of NF κ B.

Furthermore, we showed that IL-1 β -induced expression of ADAMTS-4 was impaired by Wnt-3a. These results revealed for the first time that Wnt canonical pathway may play a protective role in the aggrecan catabolism in osteoarthritis

Materials and Methods

Primary chondrocytes were isolated from macroscopically normal areas of human cartilage as described previously [12]. This study was approved by our local research institution (Commission de la Recherche Clinique; registration no. UF 9757, CPRC 2004, Cellules souches et chondrogénèse). Chondrocytes were cultured in DMEM-F12 medium supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin. For cartilage explant culture, human cartilage specimens were dissected within 1 h of operation, and full-thickness cartilage was removed from the femoral condyle and cut with 6 mm biopsy punch. Each explant was then equally divided into two pieces and cultured in the same medium as chondrocytes for 48 h before further use. Chondrocytes and cartilage explants were treated or not with Wnt-3a and/or IL-1 β at a concentration of 100 ng/ml and 10 ng/ml, respectively in DMEM-F12 serum-free medium.

ADAMTS-4 activity assay

ADAMTS-4 activity was measured using SensoLyte[®]520 Aggrecanase-1 Assay Kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Briefly, human chondrocytes were treated or not with Wnt-3a and/or IL-1 β in serum-free medium for 48 h, then the conditioned medium was collected and centrifuged for 15 min at 1000 x g at 4°C. 50 μ l of the supernatant was incubated with the aggrecanase substrate (5-FAM/TAMRA FRET) solution for 1 h. The fluorescence of 5-FAM is quenched by TAMRA and upon the cleavage of the FRET peptide by ADAMTS-4, the fluorescence of 5-FAM is recovered and can be monitored at excitation/emission = 490 nm/520 nm. The concentration of 5-FAM was determined using a standard curve and the increase in FAM concentration is correlated to ADAMTS-4 activity.

Real-time Quantitative PCR

Total RNA from human primary chondrocytes was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the recommendation of the manufacturer. For cartilage explants, total RNA was extracted with TRIzol (lifetech, Carlsbad, CA)

and further purified with the RNeasy column (Qiagen, Hilden, Germany). Complementary DNAs were synthesized from 500 ng total RNA using SuperScript® VILO™ Master Mix (lifestech, Carlsbad, CA). Quantitative PCR was performed with StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) using relative standard curve. Gene expression was determined in triplicate in three separate experiments and normalized using the housekeeping gene ribosomal protein S29. Analyses and fold differences were determined using the comparative CT method. The primers for each gene were obtained from Qiagen.

Small interfering RNA (siRNA) transfection

Human primary chondrocyte cells were seeded onto six-well plates at 5×10^5 cells/ml in DMEM/F12 and allowed to grow to 80% confluence over 24 h. Then, the cells were transfected with the siRNA (25 nM) for CTNNB1 (CUCGGGAUGUUCACAACCGAA), DVL1 (CAGCGUUUAGAUGGUUAACAU), DVL2 (GGUUCCUCCUCCAUGAGCACCAUUA), DVL3 (CUCCUGGUUACUUCAUCACAA), Fz-6 (GGUUCCACCUUGUCGUAAA), Fz-8 (AAGACAGGCCAGAU CGCUAAC) or siRNA control (Qiagen) using the DharmaFECT Transfection Reagent (Thermo Scientific, Waltham, MA) in DMEM-F12 medium supplemented with 10% FBS and 1% glutamine according to manufacturer's instructions. At 48 h post-transfection, cells were treated or not with Wnt-3a (100 ng/ml) and/or IL-1 β (10 ng/ml) for 12 h. The expression level of CTNNB1, DVL-1, DVL-2, DVL-3 Fz-6 and Fz-8 genes was analyzed by quantitative real-time PCR.

Western blot

Total protein from human primary chondrocytes was extracted using RIPA buffer (Cell signaling, Danvers, USA) containing 1 μ M PMSF. Nuclear and cytoplasmic proteins were isolated using Nuclear Extract Kit according to the recommendations of the supplier (Carlsbad, Active Motif, CA). Briefly, after treatment, chondrocytes were harvested by scrapping and washed in ice cold PBS. The cell pellet was incubated with hypotonic buffer in ice, then treated with detergent and homogenized with a Dounce homogenizer to lyse cells and release nuclei. After centrifuge for 30 s at 14,000 x g, the nuclear pellet was dissolved in complete lysis buffer and analyzed by Western blot. 30 μ g of protein was separated by SDS-PAGE and transferred onto the PVDF immobilon membrane (Millipore, Darmstadt, Germany) then incubated overnight at 4°C with appropriate primary antibodies. The protein bands were visualized by chemiluminescence using chemiluminescence luminol reagent (Bio-Rad, Hercules, CA). Densitometry analysis was performed using Image J software (version 1.46r, National Institute of health, Bethesda, MD, USA).

Luciferase reporter assay

Human primary chondrocyte cells were seeded in 24-well plates and grown to 80% confluence. Cells were then transfected with 2 μ g of NF κ B reporter construct (Clontech) and 100 ng of pRL-TK vector (Promega) using Lipofectamine 2000 (Invitrogen, MA USA). The corresponding empty vector was used as control. Twenty-four hours after transfection, cells were treated or not with Wnt-3a (100 ng/ml) for 24 h, then firefly and Renilla luciferase activities were measured in cell extracts using the Dual-Luciferase Assay System kit (Promega) and a Berthold luminometer (Bad Wildbad, Germany). Luciferase activities were normalized to pRL-TK vector activity and were expressed relative to the basal activity of empty vector. The data presented were mean values (\pm S.D.) of triplicates repeated in three independent experiments.

Metabolic labelling of proteoglycans

Cartilage explants were incubated with 10 $\mu\text{Ci/ml}$ ^{35}S -sulfate (PerkinElmer Life Sciences, MA USA) for 16 h to pre-label the proteoglycans. After being washed three times with PBS, cartilage explants were treated with Wnt-3a, IL-1 β or both in the DMEM-F12 medium supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin for 48 h. ^{35}S -labeled proteoglycans in the medium were digested with papain (1 mg/ml), precipitated by cetylpyridinium chloride then dissolved in scintillation fluid (Ultima Gold, PerkinElmer Life Science, MA USA). The radioactivity associated with GAGs was measured by liquid scintillation counting (Packard, Rungis, France). Total DNA content of each well of the chondrocytes or wet weight of each cartilage explants were recorded and used for normalisation.

Immunofluorescence

Chondrocytes were seeded onto the coverslips in 24-well plates and allowed to grow for 24 h, then treated or not with Wnt-3a and/or IL-1 β for 20 min. After the treatment, cells were washed with PBS and fixed with 4% (w/v) para-formaldehyde solution and permeabilized with 0.1% Triton-X100 then blocked in PBS containing 0.2% fish skin gelatin. Next, cells were incubated with anti-NF κ B/p65 antibodies (1/400, Cell signaling), then washed with PBS and incubated with fluorophore-conjugated secondary antibodies. The nuclei were stained with DAPI. Digital images were captured with an inverted microscope, Leica DMI3000 B (Leica Microsystems, Germany).

Statistical analysis

Data for replicate experiments are expressed as the means \pm SD and analyzed by parametric 2 tailed Student's t test for pairwise comparisons between groups. Statistical significance were as follows: n.s. no significance, * P <0.05, ** P <0.01, *** P <0.001.

Results

Wnt-3a down-regulates the expression of ADAMTS-4 through the canonical Wnt/ β -catenin pathway

Activation of Wnt/ β -catenin in mice articular chondrocytes resulted in OA-like phenotypes with progressive cartilage degradation [13]. To gain insight into the mechanism involved in Wnt-3a-induced cartilage degradation, we investigated whether Wnt-3a regulates the expression of the aggrecan-degrading aggrecanases ADAMTS-4 and ADAMTS-5 in human primary chondrocytes and cartilage explants. Unexpectedly, activation of Wnt signaling by Wnt-3a strongly decreased (50%) the expression of ADAMTS-4 in primary chondrocytes and cartilage explants, whereas no significant changes in the expression of ADAMTS-5 were observed (Fig. 1A and B). ADAMTS-4 was also significantly down regulated (30%) by Wnt-3a in bone marrow-derive mesenchymal stem cells (Figure. 1C), indicating that the inhibitory response of Wnt signaling on ADAMTS-4 expression is conserved during mesenchymal stem cells differentiation and cartilage development.

To determine whether Wnt-3a mediates down-regulation of ADAMTS-4 through canonical Wnt pathway, primary chondrocytes were treated with Wnt-3a alone or in combination with the canonical Wnt-antagonist DKK1. As expected, DKK1 prevented the increase of β -catenin induced by Wnt-3a (Supplementary figure 1A). Interestingly, Wnt-3a failed to down regulate the expression of ADAMTs-4 in presence of DKK1 (Figure. 1D), indicating that Wnt-3a inhibits the expression of ADAMTS-4 through canonical Wnt signaling. To further confirm this result, siRNA knockdown of β -catenin was performed. Silencing of β -catenin efficiently decreased its expression (Supplementary figure 1B) and unexpectedly strongly induced ADAMTS-4 expression (Figure. 1E), suggesting that β -catenin exerts an inhibitory effect on the basal expression of ADAMTS-4 in human primary chondrocytes. In addition, knockdown of β -catenin not only rescued Wnt-3a-induced decrease of ADAMTS-4 expression but promoted it (Figure. 1F), indicating that Wnt-3a regulation of ADAMTS-4 expression in human primary chondrocytes is β -catenin-dependant.

DVL-1/3 and Frizzled-8 mediate canonical Wnt signaling and down-regulation of ADAMTS-4 in human primary chondrocytes

In order to determine which of the DVLs mediates Wnt-3a-induced down-regulation of ADAMTS-4, we knocked down each of DVLs individually and evaluated the effect on Wnt-3a-induced down-regulation of ADAMTS-4. The knockdown of each DVL efficiently decreased the expression of the corresponding mRNA without affecting other DVLs (Supplementary figure 2A-C). Knockdown of DVL-1 and DVL-3 well reduced the accumulation of β -catenin induced by Wnt-3a (Figure 2A) and the expression of the Wnt/ β -catenin-dependent targeted gene *AXIN2* (Figure 2B), suggesting that canonical Wnt pathway signal mainly through DVL-1/3 in human primary chondrocytes. Importantly, knockdown of DVL-1 and DVL-3 prevented Wnt-3a-induced down-regulation of ADAMTS-4 and even, in the case of DVL-1, promoted its expression (Figure 2C), indicating that Wnt-3a-induced down-regulation of ADAMTS-4 is mediated by DVL-1 and DVL-3. These results also implied that DVL-1 exerted a repressive effect on basal expression of ADAMTS-4 in human primary chondrocytes.

Frizzled (Fz) is a family of G protein-coupled receptor proteins that serves as receptors in the Wnt signaling pathway. To address which of the 10 known Fz genes are involved in Wnt-3a-induced activation of the canonical pathway, we first performed qRT-PCR to monitor the expression pattern of all genes encoding Wnt receptors in human primary chondrocytes. The result obtained showed that in chondrocytes Fz-6 and Fz-8 were predominant (Supplementary figure S3 A). To explore their function in Wnt signaling and in Wnt-3a-induced down-regulation of ADAMTS-4 in human primary chondrocytes, siRNA knockdown of Fz-6 and Fz-8 was performed (Supplementary figure 3 B and C). The knockdown of Fz-8 but not that of Fz-6 prevented Wnt-3a-induced accumulation of β -catenin (Figure 2D) and the augmentation of Axin2 (Figure 2E), indicating that Fz-8 is essential for Wnt-3a activation of canonical Wnt signaling. Importantly, knockdown of Fz-8 but not that of Fz-6 prevented Wnt3a-induced down-regulation of ADAMTS-4 (Figure 2F). Altogether, these results indicate that Wnt-3a-induced activation of canonical Wnt

signaling in human primary chondrocytes is mediated by Fz-8 receptor and by DVL-1/3, then led to down-regulation of the expression of ADAMTS-4.

Wnt-3a down-regulates the expression of ADAMTS-4 by inhibiting NF- κ B signaling

ADAMTS-4 expression has been shown to be regulated through NF- κ B-dependent pathway [14,15]. So NF- κ B/p65/RELA was knocked down (Supplementary figure 4) and the expression of ADAMTS-4 was evaluated. The Silencing of NF- κ B/p65/RELA efficiently reduced expression of ADAMTS-4 (Figure 3A), indicating that the NF- κ B signaling plays an essential role in maintaining basal expression of ADAMTS-4 and suggested that Wnt-3a may regulates NF- κ B pathway. Of note, Wnt-3a down regulated the expression of IL6 and IL-1 α , two established target genes of NF- κ B pathway in human chondrocytes (Figure 3B). The effect of Wnt-3a on NF- κ B activity was examined using NF- κ B responsive promoter construct confirmed the down-regulatory effect of Wnt-3a on NF- κ B signaling. As shown in figure 3C, Wnt-3a significantly reduced NF- κ B reporter activity, clearly indicating that Wnt-3a inhibits NF- κ B signaling.

As shown in figure 3D, Wnt-3a did not produce any significant alteration of I κ B levels, indicating that Wnt-3a reduced NF- κ B activation through a mechanism that is independent of I κ B degradation. Activation of the NF- κ B transcription factor is associated with nuclear translocation of the p65 component of the complex. We then examined the translocation of NF- κ B/p65 into the nucleus in chondrocytes treated or not with Wnt-3a. NF- κ B/p65 was detected in nuclear extract of control cells, indicating the presence of basal NF- κ B activity in human primary chondrocytes (Figure 3E). Interestingly, treatment with Wnt-3a reduced the translocation of NF- κ B/p65 into the nucleus (Figure 3E). Epifluorescence microscopy was employed to further explore the translocation of NF- κ B/p65 into the nucleus. Significant staining of NF- κ B/p65 was observed in the nucleus of unstimulated cells, while in Wnt-3a stimulated cells it was not (Figure 3F). These results strongly suggested that Wnt-3a reduces the activation of NF- κ B pathway by blocking the translocation of

NF- κ B/p65 into the nucleus. Given that loss of β -catenin abolished the effect of Wnt-3a on ADAMTS-4 (Figure 3G), these data support the notion that inhibition of NF- κ B pathway by Wnt-3a is β -catenin-dependent. In line with this, silencing of β -catenin not only prevented the inhibition of the NF- κ B regulated gene IL-1 α by Wnt-3a but even promoted its expression (Figure 3G). Altogether, these results demonstrated that Wnt-3a down regulates the expression of ADAMTS-4 by attenuating NF- κ B signaling through β -catenin-dependent pathway.

Wnt-3a counteracts IL-1 β -induced up-regulation of ADAMTS-4 by reducing nuclear translocation of NF κ B/p65

Depletion of aggrecan from articular cartilage is an early and critical pathophysiological event in OA [16,17]. In human chondrocytes, the aggrecanase ADAMTS-4 but not ADAMTS-5 is induced by IL-1 β , the main cytokine incriminated in OA pathogenesis, through activation of NF κ B signaling pathway [18–21], therefore suggesting that induction of ADAMTS-4 is involved in the pathogenesis of OA. In line with these studies, treatment of human primary chondrocytes with IL-1 β strongly increased the expression of ADAMTS-4 and did not significantly affect that of ADAMTS-5 (Figure. 4A). In addition, the knockdown of NF- κ B/p65/RELA reduced by 80% the expression of ADAMTS-4 induced by IL-1 β in primary chondrocytes (Figure 4B). Given that Wnt-3a reduced the activation of NF κ B signaling pathway, the ability of Wnt-3a to counteract IL-1 β -induced up-regulation of ADAMTS-4 was tested. As expected, co-treatment with Wnt-3a attenuated by about 60% the induction of ADAMTS-4 by IL-1 β (Figure 4C), indicating that Wnt-3a is able to antagonize the effect of IL-1 β on ADAMTS-4 expression. Similarly, analysis of ADAMTS-4 activity indicated co-treatment with Wnt-3a reduced by 30% the IL-1 β -induced activity of ADAMTS-4 (Figure 4D). Altogether, these results clearly demonstrate that Wnt-3a display an ability to counteract IL-1 β -induced stimulation of the expression and activity of ADAMTS-4 in human primary chondrocytes.

SAPK/JNK and NF κ B signaling pathways have been suggested to be involved in the regulation of the expression of ADAMTSs by IL-1 β . To gain insight into the

mechanism by which Wnt-3a reduced IL-1 β -induced expression of ADAMTS-4, we tested whether IL-1 β -induced activation of SAPK/JNK and NF κ B signaling pathways was affected by Wnt-3a. Analysis of the phosphorylation status of SAPK/JNK kinases and I κ B indicated similar activation by IL-1 β either in absence or in presence of Wnt-3a (Figure 4E). However, analysis of nuclear protein revealed that co-treatment with Wnt-3a partly interrupted IL-1 β induced nuclear translocation of NF- κ B/p65, but not the acetylation of NF- κ B/p65 (Figure 4F). Epifluorescence microscopy was used to further explore the translocation of NF- κ B/p65 into the nucleus. Interestingly, co-treatment with Wnt-3a reduced the level of NF- κ B/p65 staining in the nucleus compared to the treatment with IL-1 β alone (Figure 4G), suggesting that Wnt-3a counteracts IL-1 β -induced translocation of NF- κ B/p65 into the nucleus. These results indicated that Wnt-3a counteracts IL-1 β -induced NF- κ B activity by reducing the nuclear translocation of NF- κ B/p65. In line with this, Wnt-3a reduced by about 5-fold the IL-1 β -induced up-regulation of the NF- κ B responsive gene, IL1 α (Figure 4H). Thus, we conclude that Wnt-3a negatively modulates NF- κ B activity and therefore reduced IL-1 β -induced stimulation of the expression of ADAMTS-4 in human primary chondrocytes.

Wnt-3a counteracts IL-1 β -induced inhibition of proteoglycan synthesis

Proteoglycan loss is a major event in the process of OA that leads to cartilage alteration and loss of joint function. Proteoglycan loss occurs early in OA and can be induced by IL-1 β . To examine whether Wnt-3a could counteract loss of proteoglycans induced by IL-1 β , proteoglycan in human cartilage explants were prelabelled for 16 h by the ³⁵S-sulfate, then cartilage explants were treated with vehicle, Wnt-3a or a combination of IL-1 β and Wnt-3a. As shown in figure 5A, Wnt-3a reduced 50% basal proteoglycan release and cut half of the proteoglycan release induced by IL-1 β , which indicated that Wnt-3a attenuated IL-1 β -induced loss of proteoglycans in human cartilage. Altogether, these results revealed that Wnt-3a might have a protective effect against the development of human OA.

Discussion

WNT signaling was considered to be associated with arthritic cartilage destruction, since the level of β -catenin was found to be increased in chondrocytes of arthritic cartilage [22]. Recently, accumulating evidences based on experimental animal models have shown that Wnt/ β -catenin signaling drives the expression of ADAMTS-4 [8,9,23]. Here, in remarkable contrast to what has been observed in animal models, we provide evidence that Wnt/ β -catenin signaling is a potent inhibitor of ADAMTS-4 in human primary chondrocytes and cartilage explants. Similar observation was made for the matrix metalloproteinase-13 (MMP13) which was down regulated by Wnt/ β -catenin pathway in human chondrocytes, whereas in animal chondrocytes it was up-regulated [10]. These observations strongly argue for differential regulation of ADAMTS-4 by Wnt/ β -catenin signaling in human and animal chondrocytes.

The presence of four putative NF- κ B motifs in human ADAMTS4 gene promoter [24] suggests that it may be regulated by NF- κ B and that Wnt-3a may inhibit ADAMTS-4 by suppressing the activation of NF- κ B signaling. In agreement with this hypothesis, the knockdown of NF- κ B/p65/RelA strongly reduced basal and IL-1 β -induced expression of ADAMTS-4. Investigation of the mechanism involved in Wnt-3a down regulation of ADAMTS4 showed that Wnt3a impaired the activation of NF- κ B signaling by reducing nuclear translocation of NF- κ B/p65. In line with this, Wnt-3a inhibited the expression of IL6 and IL-1 α , two established target genes of NF- κ B pathway in human chondrocytes.

It is well known that activation of NF- κ B signaling by proinflammatory cytokines such as IL-1 β potently induced cartilage matrix degradation. Investigation of the effect of Wnt/ β -catenin signaling on the stimulatory effect of IL-1 β on ADAMTS-4 expression and activity revealed that Wnt/ β -catenin signaling counteracts these effects by reducing the nuclear translocation of NF- κ B/p65 induced by the cytokine. This suggests that Wnt/ β -catenin signaling counteracts NF- κ B signaling in cartilage and may safeguard cartilage from degradation. In line with this, we showed that activation of β -catenin signaling in cultured human cartilage explant by Wnt-3a treatment reduced IL-1 β -mediated loss of proteoglycans.

It also suggest that increased levels of β -catenin observed in human OA cartilage may result from activation of Wnt/ β -catenin signaling in an attempt of human chondrocytes to counteract the catabolic effects of inflammatory cytokines such as IL-1 β to slow down matrix degradation. Accordingly, depletion of chondrocytes from β -catenin induced basal expression of ADAMTS-4 and potentiates the effect of IL-1 β on the expression of this important aggrecanase, indicating that β -catenin exerts an inhibitory effect on ADAMTS-4 expression. This revealed that β -catenin might have an anticatabolic role in human chondrocytes.

Because Fz proteins are serpentine receptors and therefore belong to the major class of target proteins for currently available drugs, therefore addressing the question of which of the 10 known Fz receptors is involved in the regulate canonical Wnt signaling in human primary chondrocytes could also be relevant in terms of identifying novel therapeutic options to treat OA disease [25,26]. We bring evidence that Fz-8 mediates the activation of canonical Wnt/ β -catenin pathway. Similarly, Fz-8 was shown to mediates canonical Wnt signaling in *Xenopus* [27], in lung cancer cells [28] and in osteoclasts during bone remodelling [29]. Relaying signal from receptor to downstream effectors, DVLs participate in both canonical and noncanonical pathways. We found that DVL-1 and DVL-3 play a more prominent role in this process compared to DVL-2. In agreement with this, it has been shown that the activation of Wnt canonical signaling pathway by Wnt-3a in mouse F9 teratocarcinoma cells was most sensitive to changes in the abundance of either DVL-3 or DVL-1. Changes in expression of DVL-2, the most abundant of the three isoforms, resulted in the least effect on canonical signaling [30].

In summary, our study revealed that activation of Wnt/ β -catenin signaling pathway by Wnt-3a in human primary chondrocytes is mediated through Fz-8 and DVL-1/3 and resulted in β -catenin-dependent reduction of NF- κ B activity (Figure 5B). Activation of this pathway counteracts IL-1 β -induced ADAMTS4 expression and activity and exerts a protective role on proteoglycan degradation induced by the cytokine in human cartilage. Therefore, we speculate that Wnt may have a protective role in OA.

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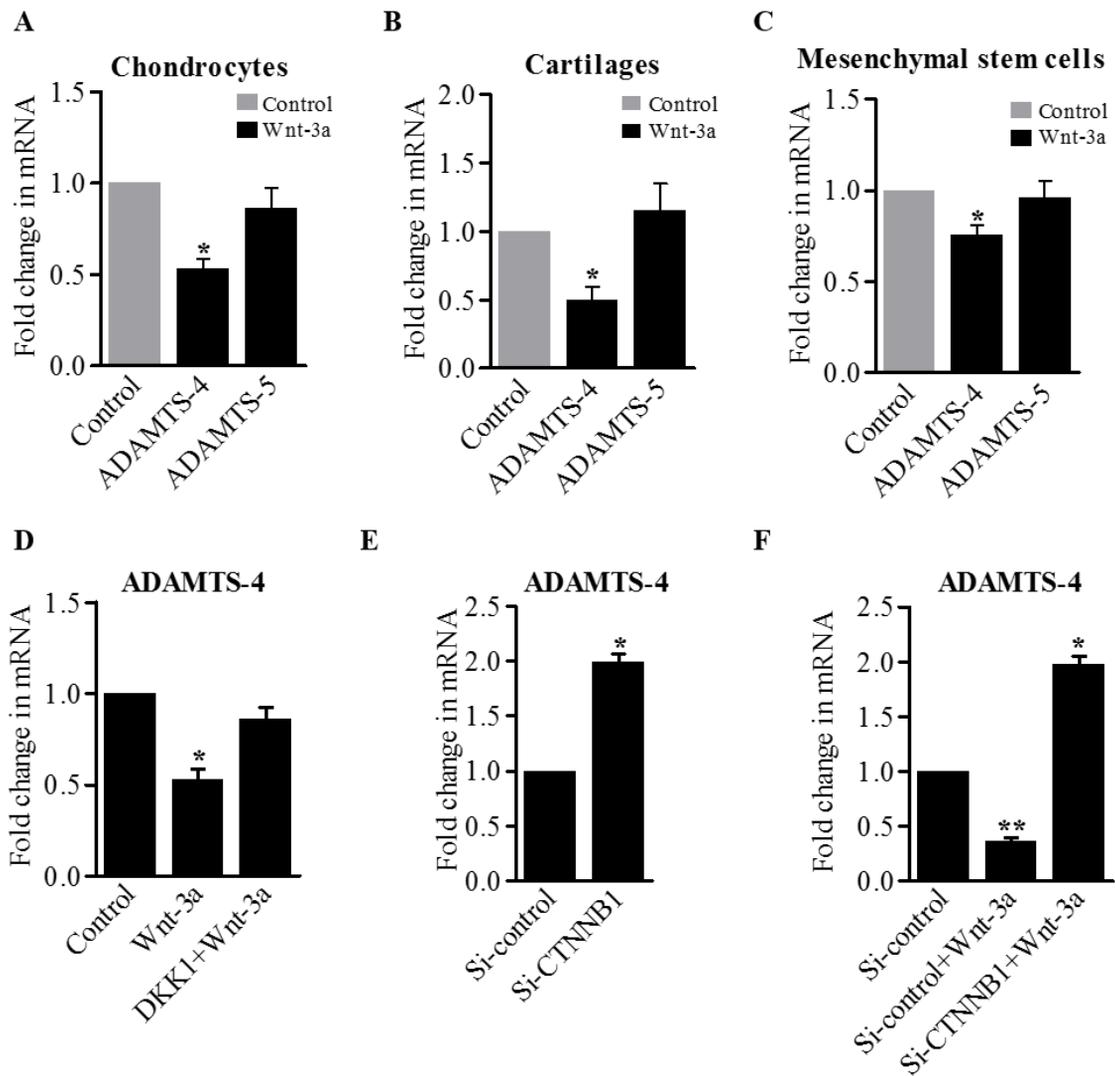


Figure 1. Wnt-3a regulates the expression of ADAMTS-4 through canonical pathways

Chondrocytes (A, n=5), cartilage explants (B, n=5) and Bone marrow-derive mesenchymal stem cells (C, n=5) were treated with vehicle or 100 ng/ml Wnt-3a for 12 h, mRNA levels determined by real time PCR showed ADAMTS-4 was inhibited by Wnt-3a. (D) Human chondrocytes were pretreated with vehicle or DKK1 for 30 min then exposed to 100 ng/ml of Wnt-3a for 12 h, the RT-qPCR showed the Wnt-induced inhibition of ADAMTS-4 was prevented by DKK1 (n=3). (E) si-CTNNB1 was transfected into chondrocytes for 48 h, RT-qPCR revealed the expression of ADAMTS-4 was higher comparing to the si-control group (n=3). (F) Silencing of β -catenin rescued the Wnt-3a-induced down regulation of ADAMTS-4 (n=3). Data are expressed as mean \pm S.D. of separate experiments. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

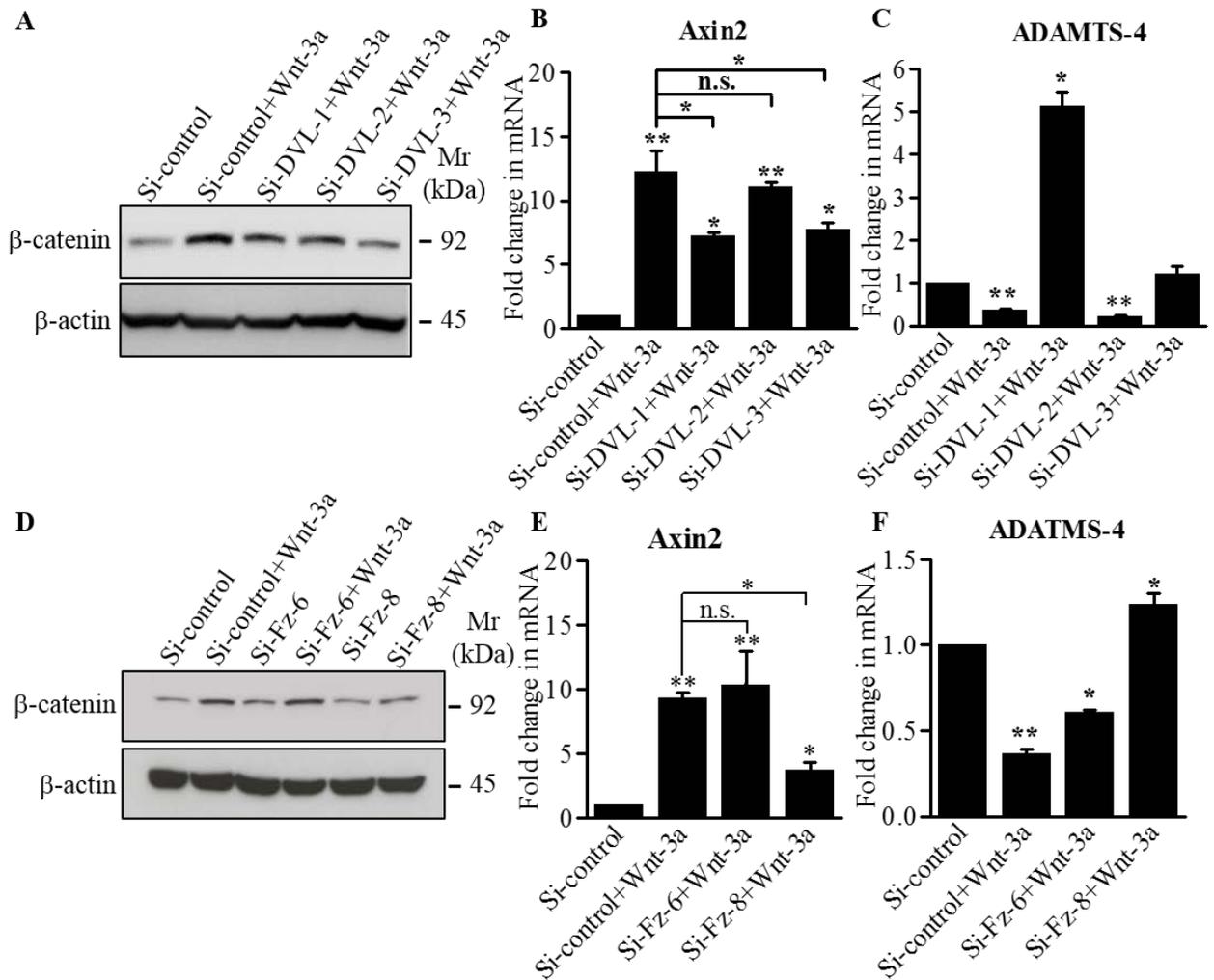


Figure 2. DVL-1/3 and Fz-8 mediate Wnt canonical pathway and down-regulation of ADAMTS-4

Primary chondrocytes were transfected with siRNA specific to DVL-1, DVL-2, DVL-3 or siRNA control for 48 h then treated with Wnt-3a (100 ng/ml) or vehicle for 12 h and the protein level of β -catenin was determined by Western blotting, β -actin was used as loading control (A, n=3), expression of *Axin2* (B, n=3) and *ADAMTS-4* (C, n=3) was determined by RT-qPCR. After the 48 h transfection with si-control, siFz-6 or siFz-8, chondrocytes were treated with 100ng/ml Wnt-3a for 12 h. The expression of β -catenin was detected by Western blotting (D, n=3) while expression of *Axin2* (E, n=3) and *ADAMTS-4* (F, n=3) was measured by RT-qPCR. One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (n.s., statistically not significant; *, p < 0.05; **, p < 0.01).

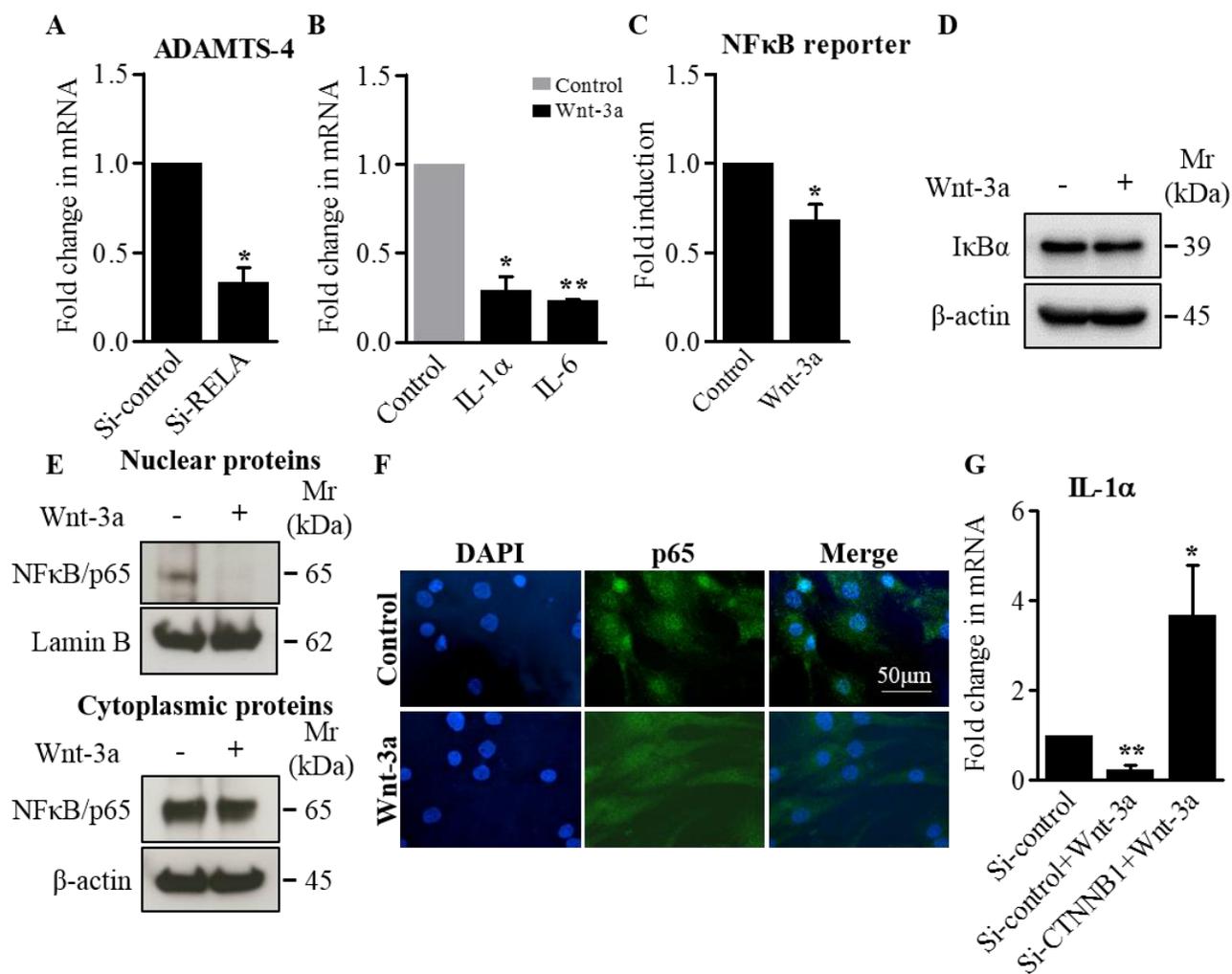


Figure 3. Wnt-3a inhibited the expression of ADAMTS-4 via NF- κ B-dependent pathways

(A) Knockout of p65 by si-RELA inhibited basal mRNA expression of ADAMTS-4. (B) After 12 h treatment of 100 ng/ml Wnt-3a, IL-1 α and IL6 mRNA levels was lower comparing to the vehicle. (C) Human chondrocytes were transfected with NF κ B reporter construct or empty vector together with pRL-TK and treated with Wnt-3a (100 ng/ml) or vehicle for 24 h and then luciferase activity was measured in cell extracts. Luciferase activities were normalized to pRL-TK vector activity and were expressed relative to the basal activity of empty vector. (D) The degradation of I κ B α was not altered by the stimulation of Wnt-3a for 12 h. (E) After 12 h treatment of 100ng/ml Wnt-3a, chondrocytes nuclear proteins and cytoplasmic proteins were separated to detect NF κ B P65. Western blot showed Wnt-3a inhibited the basal translocation of NF κ B P65 into nuclear. (F) NF κ B P65 was stained with immunofluorescence after stimulation of Wnt-3a for 12 h in human chondrocytes. (G) The blockage of Wnt canonical pathway by si-CTNNB1 well abolished the inhibition of IL-1 α by Wnt-3a. One representative blot or immunofluorescence of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

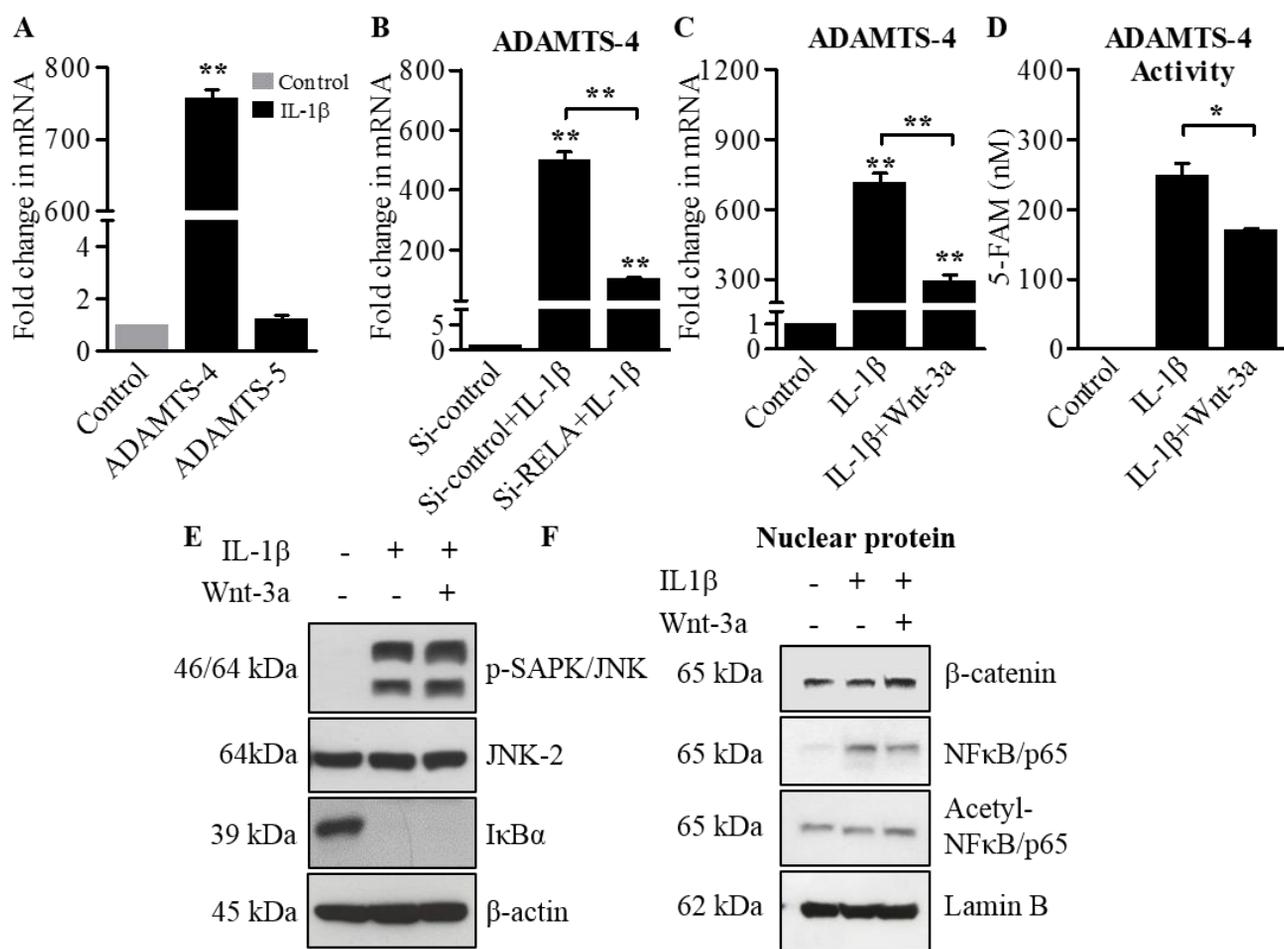
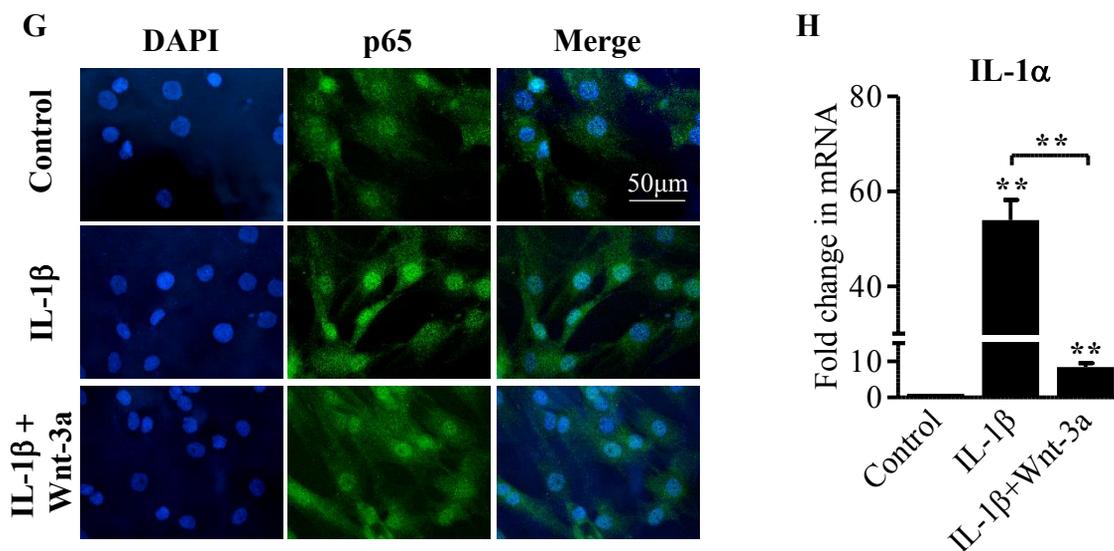


Figure 4. Wnt-3a counteracts IL-1 β -induced up-regulation of ADAMTS-4

(A) Human primary chondrocytes were treated with 10ng/ml IL-1 β for 12 h and mRNA levels of ADAMTS-4 and -5 were measured by RT-qPCR (n=3). (B) The blockage of NF κ B pathway by si-RELA partly prevented the IL-1 β induced augmentation of ADAMTS-4 (n=3). Treatment with 10 ng/ml IL-1 β alone or together with 100 ng/ml Wnt-3a was performed for 12 h in primary chondrocytes, then ADAMTS-4 mRNA level was determined by RT-qPCR (C, n=3), the activity of ADAMTS-4 in medium was measured using SensoLyte[®]520 Aggrecanase-1 Assay Kit (D, n=3). Chondrocytes were treated by 100 ng/ml Wnt-3a for 12 h then stimulated with vehicle or 10 ng/ml IL-1 β for 20 min, p-SAPK/JNK, JNK-2, I κ B α and β -actin in whole-cell extract (E, n=3) and β -catenin, NF κ B/P65, acetyl- NF κ B/P65 and Lamin B in nuclear extract (F, n=3) were detected by Western blot.



(G) Chondrocytes were treated with 100 ng/ml Wnt-3a or vehicle (control) for 12 h then simulated with 10 ng/ml IL-1 β , and NF κ B P65 was stained with immunofluorescence. (H) The RT-qPCR showed Wnt-3a reduced by about 5-fold the IL-1 β -induced up-regulation IL1 α . One representative western blot or immunofluorescence of three independent experiments is shown. PCR results were normalized as described previously. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

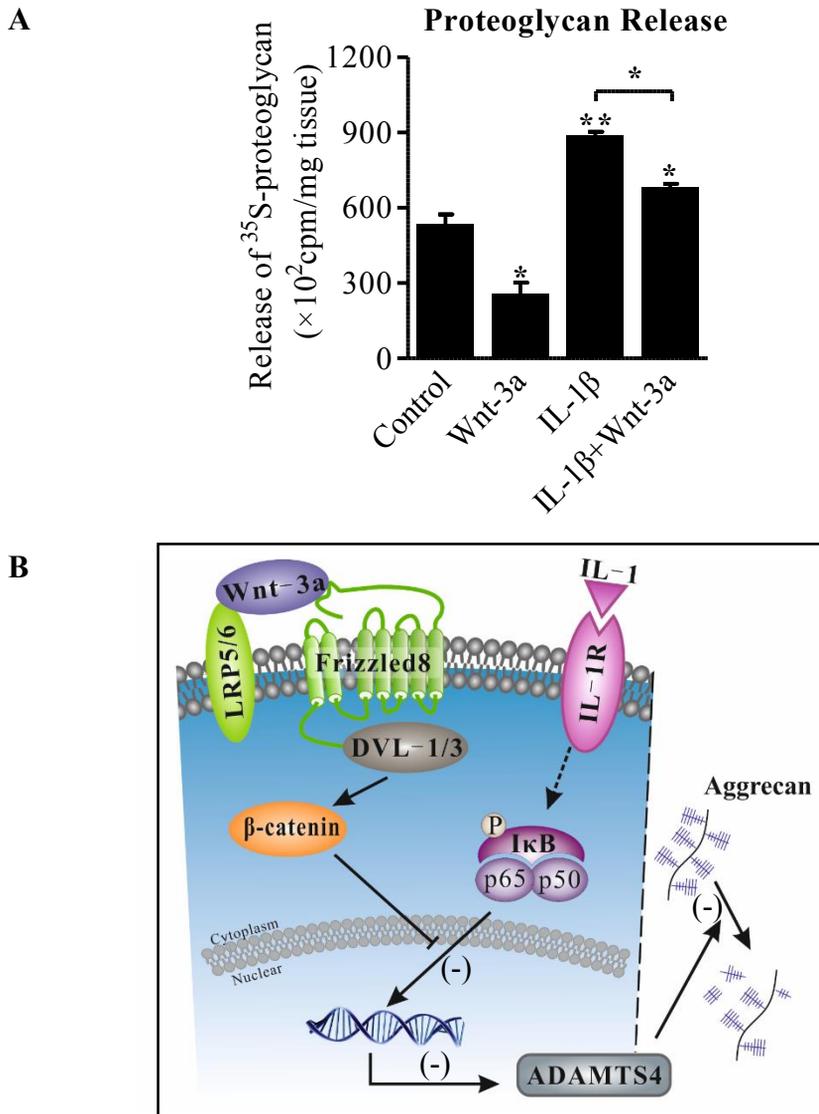


Figure 5. Wnt-3a counteracts IL-1β-induced cartilage degradation

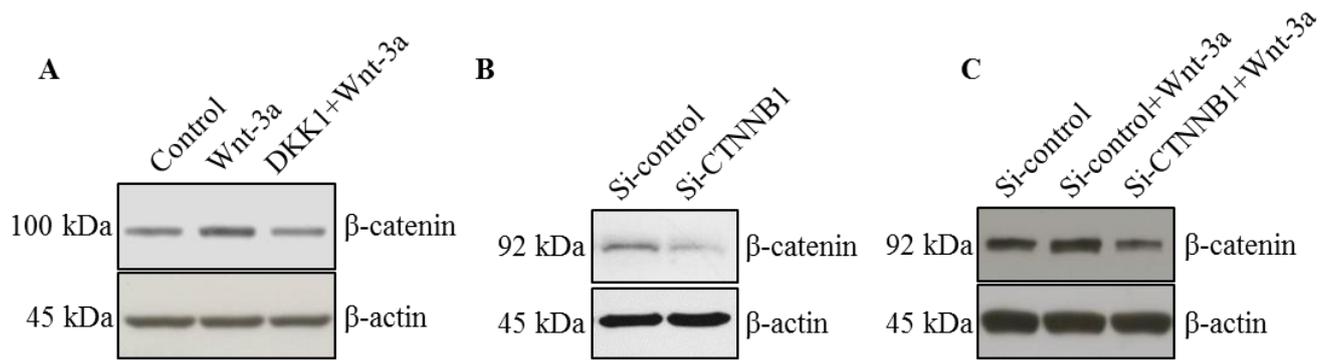
(A) The release of ³⁵S-Proteoglycan in the medium of the human cartilage explants treated with vehicle, Wnt-3a, IL-1β and both Wnt-3a and IL-1β for 48 h was quantified following CPC precipitation and radioactivity counting. results were normalized by the wet weight of cartilage (n=3). Data are expressed as mean ± S.D. Statistical analysis was performed with an unpaired Student's t-test (*, p < 0.05; **, p < 0.01). (B) Simplified schemes showing the interaction between Wnt canonical cascade and NFκB pathway.

Supporting Information

Wnt/ β -catenin signaling counteracts interleukine-1-induced ADAMTS4 expression and activity in human articular chondrocytes

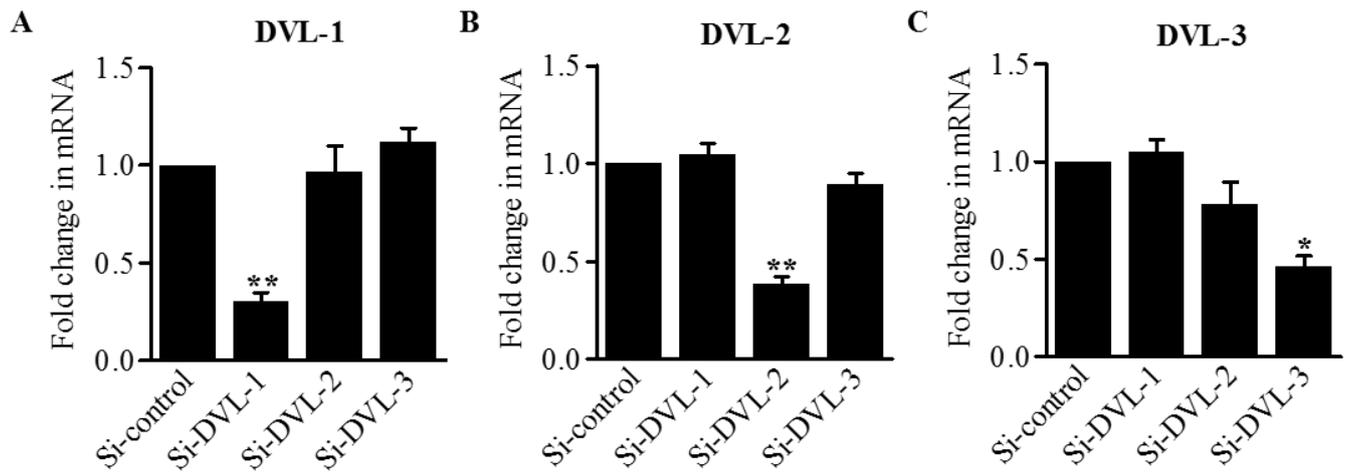
Zhe Xie, Khair Mostafa and Mohamed Ouzzine

Supplementary Figures 1-4



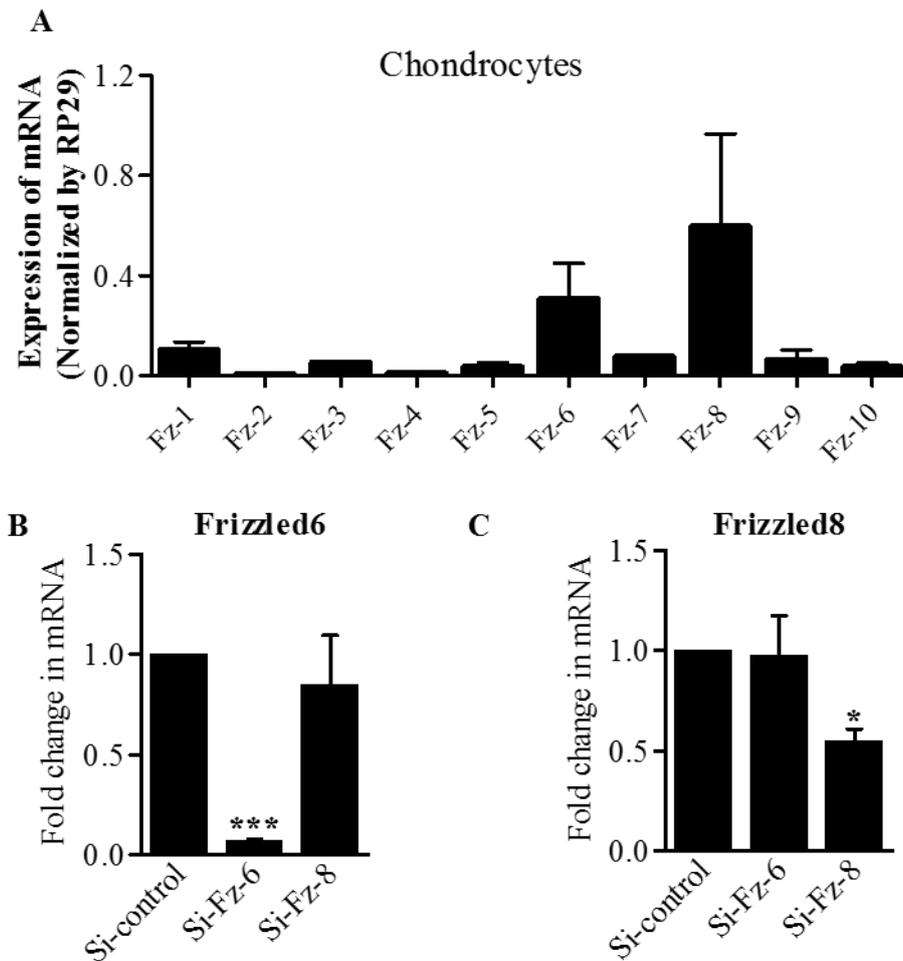
Supplementary figure 1. **The blockage of Wnt canonical pathway by pharmacology inhibitor and si-RNA.**

(A) Human chondrocytes were pretreated with vehicle or DKK1 for 30 minutes then exposed to 100ng/ml Wnt-3a for 12 hours, the Western blot showed the Wnt-induced accumulation of β -catenin was abolished by DKK1 (n=3), the β -actin was used as loading control. (E) si-CTNNB1 was transfected into chondrocytes for 48 hours, Western blot revealed the β -catenin level was lower comparing to the si-control group (n=3). (F) Silencing of β -catenin by si-CTNNB1 suppressed the Wnt-3a-induced augmentation of β -catenin (n=3). One representative western blot or immunofluorescence of three independent experiments is shown.



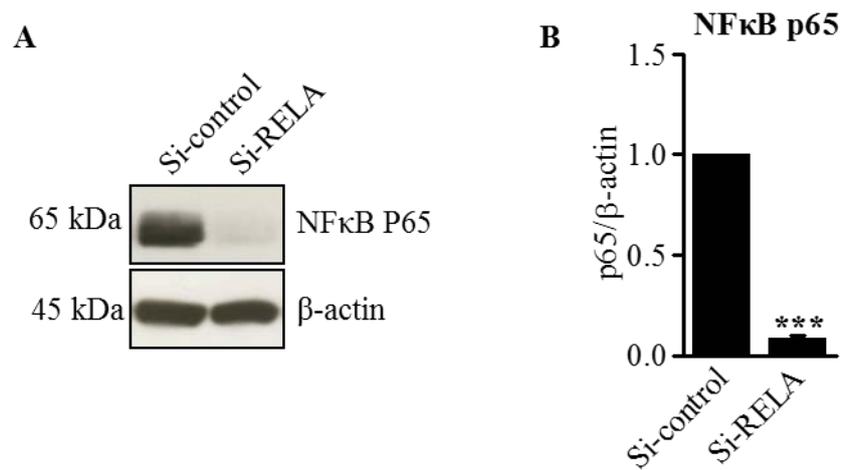
Supplementary figure 2. **siRNA efficiency on the expression of DVLs in human primary chondrocytes**

(A-C) Primary chondrocytes were transfected with siRNA specific to (A) DVL-1 (Si-DVL-1), (B) DVL-2 (Si-DVL-2), (C) DVL-3 (Si-DVL-3), or siRNA control (Si-control) for 48 h and the level of DVL-1, DVL-2 and DVL-3 was determined by RT-PCR (n=3). Knockdown of DVL-1, DVL-2 and DVL-3 did not affect other DVLs respectively (n=5). Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; **, $p < 0.01$).



Supplementary figure 3. **The relative expression of Fz receptors in human primary chondrocytes and the effects of si-Fz-6 and Fz-8.**

(A) Fz-6 and Fz-8 receptors are predominantly expressed in human primary chondrocytes (n=3). The relative expression of Fz receptors in human primary chondrocytes. Expression of different Fz receptors was evaluated by absolute Q-PCR. Specific DNA segment for each of the ten Fz receptors (standards) was amplified by RT-PCR, purified on agarose gel, quantified and used as template for Q-PCR. Absolute quantification was determined compared to standards that are run in parallel. (B and C) Human chondrocytes were transfected with si-control, si-Fz-6 or si-Fz-8 for 48 h, knockdown efficiency of si-Fz-6 (B, n=6) and si-Fz-8 (C, n=5) was determined by RT-PCR. Data are expressed as mean \pm S.D. of 3 separate experiments. Statistical analysis was performed with an unpaired Student's t-test (*, $p < 0.05$; ***, $p < 0.001$).



Supplementary figure 4. **Knockdown efficiency of RELA in human primary chondrocytes.**

(A-B) Knockdown efficiency of RELA in human primary chondrocytes, as evaluated by Western blotting (A, n=3) and Q-PCR (B, n=3). One representative blot of three independent experiments is shown. Data are expressed as mean \pm S.D. Statistical analysis was performed with an unpaired Student's t-test (***, $p < 0.001$).

Résumé

La dérégulation de la signalisation Wnt est impliquée dans les anomalies du développement et dans la pathogenèse de nombreuses maladies, y compris l'arthrose. Au cours de ce travail, nous avons étudié l'effet de Wnt-3a sur l'expression de l'ADAMTS-4 dans les explants de cartilage et les chondrocytes primaires humains en culture. De façon inattendue, nous avons observé que Wnt-3a régule négativement l'expression de cette agrecanase et avons démontré que cette inhibition est médiée par Frizzled-8 via l'activation de la voie canonique Wnt/ β -caténine et inhibition de l'activité de NF κ B. En outre, nous avons montré que Wnt-3a est capable de s'opposer, dans une certaine mesure, à l'induction de l'expression de l'ADAMTS-4 par l'IL-1 β , indiquant que la voie Wnt/ β -caténine peut jouer un rôle protecteur dans l'arthrose. D'autre part, la signalisation non canonique de Wnt induit une perte de la stabilité phénotypique des chondrocytes articulaires qui représente un événement précoce dans l'arthrose, cependant les mécanismes impliqués restent à élucider. Au cours de ce travail de thèse, nous avons identifié la cascade Wnt/CaMKII/B-raf/ERK1/2 comme voie de signalisation non-canonique modulant le phénotype chondrocytaire et avons montré que le syndécan4 est un composant essentiel de cette voie. Nous avons démontré qu'en réponse à Wnt-3a, Frizzled 6 active la voie ERK1/2 en induisant la fixation de la kinase CaMKII α au syndécan4 et celle de B-Raf à DVL-2 conduisant à l'activation de B-Raf et à celle d'ERK1/2. Dans une boucle de rétrocontrôle, Wnt-3a inhibe l'expression du syndécan4 ce qui réduit l'activation et régule négativement la voie de signalisation. Ce travail révèle le rôle du syndécan4 dans la régulation du phénotype chondrocytaire et met en évidence de nouvelles cibles qui peuvent avoir un potentiel thérapeutique contre l'arthrose.

Mots clés: Wnt, syndécan4, chondrocyte, différenciation, ADAMTS-4, arthrose

Abstract

Dysregulation of Wnt signaling has been implicated in developmental defects and in the pathogenesis of many diseases, including osteoarthritis. Here, we studied the effect of Wnt-3a on ADAMTS-4 expression in human cartilage explants and primary chondrocytes and, unexpectedly, found that Wnt-3a negatively regulates the expression of this aggrecanase. We demonstrated that Wnt-3a inhibition of ADAMTS-4 expression is mediated by Frizzled-8 through activation of the canonical Wnt/ β -catenin pathway leading to inhibition of NF κ B activity and down-regulation of ADAMTS-4. Furthermore, we showed that Wnt-3a is able to counteract, to some extent, the induction of ADAMTS-4 by IL-1 β , therefore indicating that Wnt/ β -catenin pathway may play a protective role in osteoarthritis. On the other hand, Non-canonical Wnt signaling induces loss of phenotypic stability of articular chondrocytes which represents an early event in osteoarthritis, but the underlying mechanisms are poorly understood. In this thesis, we identify Wnt/CaMKII/B-raf/ERK1/2 cascade as non-canonical signaling pathway that modulates chondrocyte phenotype and revealed that syndecan4 is an essential component of this pathway. We show that in response to Wnt-3a, Fz-6 activates non-canonical signaling by triggering the docking of CaMKII α to syndecan4 and that of B-raf to DVL-2 leading to the activation of B-raf that transduces signals to MEK and ERK1/2 MAPK. In a feedback loop, non-canonical Wnt down-regulates the expression of syndecan4 to negatively regulate the signaling. Our finding uncovers a previously unanticipated role of syndecan4 as a regulator of chondrocyte differentiation. This study also provides new targets which may have therapeutic potential in osteoarthritis.

Key words: Wnt, syndecan4, chondrocyte, differentiation, ADAMTS-4, osteoarthritis

