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WNT MODULATORS IN DIFFERENTIATING AND ARTICULAR CHONDROCYTES

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List of Abbreviations

AA	Ascorbic acid
AB	Alcian blue
AC	articular cartilage
Acan	Aggrecan
ADAMT	A Disintregrin and Metalloproteinase Domain with Thrombospondin Motif
AER	apical ectodermal ridge
ANOVA	analysis of variance
ANP32a	Acid (leucin-rich) nuclear phosphoprotein 32 family, member A
APC	Adenomatous polyposis coli
AR	Alizarin red
ATM	ataxia-telangiectasia mutated
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
Ca2+	Calcium
CAM	constitutively activated mutant
CamKII	Calcium/calmodulin-dependent kinase II
cDNA	(complementary) Deoxyribonucleic Acid
CI	confidence intervals
ChIP	Chromatin-immunoprecipitation
CK1α	Caseine kinase 1α
CLK2	CDC-like kinase 2
Col10a1	collagen type 10a1
Col2a1	collagen type 2a1
CS	chondroitin sulfate
Ct	comparative threshold
DAB	3,3'Diaminobenzidine
DISH	Double fluorescent in situ hybridization
DKK	Dickkopf
DMEM	Dulbecco's modified Eagle's medium
DMMB	di-methylmethylene blue
DMOARD	disease modifying anti-osteoarthritis drug
DOT1L	disruptor of telomeric silencing 1-like
DS	dermatan sulfate
DYRK1A	dual-specificity tyrosine phosphorylation-regulated kinase 1A
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EtOH	Ethanol
EXT1	Exostosin
EZH2	enhancer of zeste homologue 2
FBS	Fetal Bovine Serum
FRZB	Frizzled-related protein
Fz	Frizzled receptor
FZD	frizzled

GAG	Glycosaminoglycan
GDF	Growth differentiation factor
GSK3	Glycogen Synthase Kinase 3
GWAS	genome wide association study
H3K79	histone H3 lysine 79
hACs	human articular chondrocytes
HBP1	HMG-box transcription factor 1
HIF1 α	Hypoxia-inducible factors 1α
HH	hedgehog
HME	Hereditary multiple exostoses
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1
HRP	Horseradish peroxidase
HSPG	and heparan sulfate-proteoglycan
Ihh	Indian Hedgehog
IL	Interleukin
INHAT	inhibitor of histone acetyltransferase
ITS	Insulin, Transferrin, Selenite
JNK	c-Jun N-terminal kinase
KO	knock out
KD	knowckdown
KS	keratan sulfate
LCAR	low-complexity acidic region
LDL	low density lipoprotein
LEF	Lymphoid enhancer-binding factor
LGRs	Leucine-rich repeat-containing G-protein coupled receptors
LRP	Low-density lipoprotein receptor-related protein
MMP	matrix metalloproteinase
mRNA	Messenger Ribonucleic Acid
NAC	N-acetyl-cysteine
$NaClO_3$	sodium chlorate
NFAT	Nuclear factor of activated T-cells
NSAIDs	non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
ORF	open reading frame
PBS	phosphate buffered saline
PCP	planar cell polarity
PI3K	phosphatidylinositide 3-kinases
PIC	protease inhibitor cocktail
PKC	Protein kinase C
PP2A	phosphatase 2A
QQ	quantile-quantile
Rock	Rho-associated protein kinase
Rspo	R-spondin
RT-PCR	reverse transcription polymerase chain reaction

RT-qPCR	quantitative real-time polymerase chain reaction
RUNX2	Runt-related transcription factor 2
S29	ribosomal protein S29
SD	Standard Deviation
SFRP	Secreted frizzled-related protein
sGAG	sulfated glycosaminoglycans
SIRT1	Sirtuin-1
SNP	Single nucleotide polymorphism
SOX9	Sex determining region Y – box 9
TBS (-T)	Tris-buffered Saline (with Tween)
TCF	T-cell factor
TCF/LEF	T-cell factor/lymphoid enhancer factor
WG	Wingless
WIF	Wnt-inhibitory-factor
WISP	Wnt-1 induced secreted protein
WLS	Wnt ligand secretion mediator
WNT	Wingless integration site
WT	wild type
Y2H	yeast two-hybrid

Chapter 1 General introduction

The vertebrate skeleton is a fascinating organ that fulfills many different functions [1]. It provides support to the body, protection to the inner organs and the blood-forming bone marrow, acts as an endocrine regulator of many physiological processes, is a critical storage of calcium and, not in the least, allows movement. For the latter function, the different bones are articulating in the joints, the connections between bony levers. Most joints, in particular those in the peripheral skeleton, are synovial joints. The ends of the different bones are capped with a thin layer of articular cartilage, a softer, water-rich tissue essential for smooth movement. Between the cartilages of the bones, the synovial cavity is found, lined by the synovium, a thin connective tissue that produces the synovial fluid, thus contributing to the lubrication of the joint [2].

Given the essential roles of the skeleton in the vertebrate body, it is no surprise that skeletal malformations and diseases can have an enormous impact on the individual [3]. Thus, understanding of the molecular and cellular mechanisms that are involved in skeletal development, growth, homeostasis and disease is of uttermost importance. Current evidence suggests that molecular signaling pathways with key roles in the normal development of bone and cartilage, are also important in postnatal life and disease. Among these, a number of growth factors and morphogen molecular cascades have gathered extensive attention. Prominently among these features the Wnt signaling pathway [4]. Fine tuning of this molecular pathway is key to skeletal health and therefore an area of intensive research. Over the last couple of years, the first therapeutic strategies for skeletal and joint disease that target the Wnt signaling cascade, have emerged [5]. Still, many aspects of this biological system and its regulation remain unknown. This thesis will focus on novel regulators of Wnt signaling and how these affect skeletal development and joint health.

1.1 Cartilage

Cartilage is an extra-cellular matrix-rich connective tissue, softer than bone but still resistant to mechanical forces, and a structural component of the joints, but also of the ears, nose, tendon and ligament insertions, trachea, bronchi and intervertebral discs. Cartilage tissue can be subdivided into three different groups by its histological characteristics and

matrix composition: hyaline cartilage, elastic cartilage and fibrous cartilage [6]. **Elastic cartilage** is for instance found in the ears and the matrix is rich in elastin fibers. **Fibrous cartilage** is typically found in the entheses, insertions sites of tendons and ligaments into the bone. Here the matrix is rich in type 1 collagen fibers. In contrast, **hyaline cartilage** features a matrix that is rich in type 2 collagen fibers and proteoglycans (Fig. 1). Hyaline cartilage is the most broadly present cartilage in the body and can be further classified into two functionally distinct tissues: permanent cartilage as found on the joint surfaces and transient cartilage, an essential template during skeletal development. The articular cartilage does not ossify and is required for smooth movement throughout life, while the transient cartilage is critical for skeletal patterning and growth. The transient nature of this cartilage which is also referred as temporary cartilage is reflected in its eventual replacement by bone in the process of endochondral ossification.

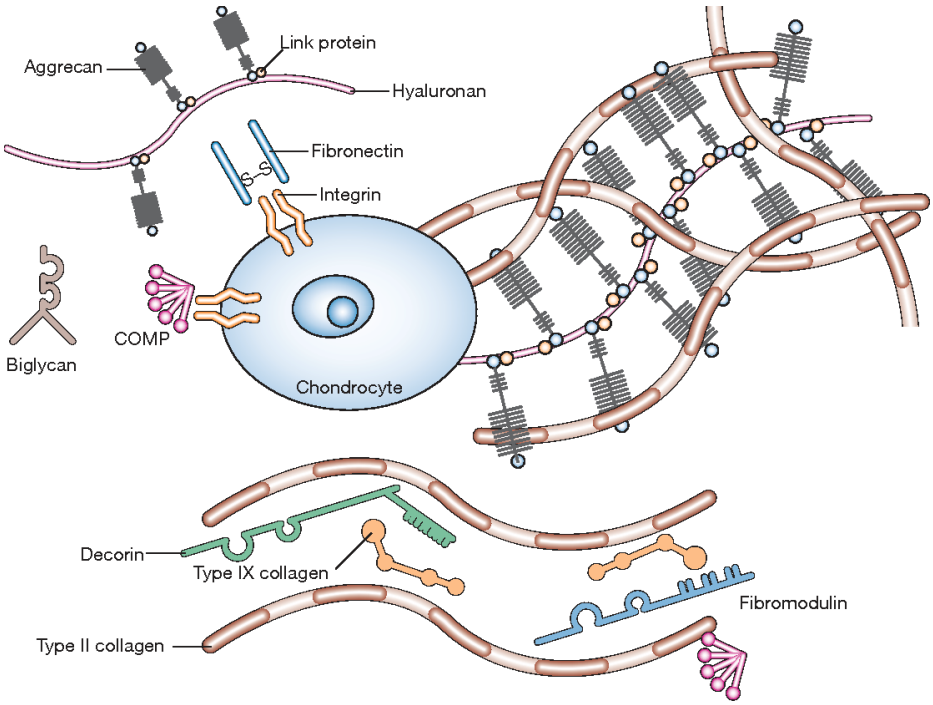


Fig 1. Extracellular matrix (ECM) of the cartilage.–Three classes of proteins exist in articular cartilage: collagens (mostly type 2 collagen); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils is responsible for the compressive and tensile strength of the tissue, which resists load *in vivo*. Reused with permission from [7].

1.2. Endochondral bone formation and skeletal growth

The majority of the bones in our body are formed by endochondral ossification [8]. The basic principle of endochondral ossification is defined by the differentiation of skeletal progenitor cells into chondrocytes. The resulting cartilage serves as a template that will ultimately be replaced by bone. This two-step process contrasts with intramembranous or direct ossification in which skeletal progenitor cells directly differentiate into bone cells. The latter contributes mostly to the bones of the skull and to the cortex of long bones, whereas endochondral ossification forms the nucleus of most axial and appendicular skeletal elements and is also key to the natural healing of bone fractures.

The endochondral bone formation process has been extensively studied and many steps are well defined [8]. It starts with the condensation of skeletal progenitor cells at the prospective site for the skeletal element to be formed. This is best studied in the context of limb development. The cell condensation – thereby forming a so-called "anlagen" – is taking place proximal to the apico-ectodermal ridge that represents the front of the limb outgrowth. Intercellular contacts within the condensation appear to trigger proliferation and differentiation of the skeletal progenitor cells that are committing to the chondrocyte lineage, characterized by the presence of transcription factor sex determining region Y – box 9 (SOX9). The differentiating cells start to produce a typical cartilage matrix, in particular rich in type 2 collagen. The early commitment of the skeletal progenitor cells towards chondrogenic differentiation appears to be dependent on a reduction in Wnt signaling. Active Wnt signaling is present at the apico-ectodermal ridge and decreases more proximal from this site, thereby facilitating chondrogenic differentiation. Chondrogenic differentiation is directly stimulated by bone morphogenetic proteins (BMP), another growth factor cascade.

Subsequently, the cells align for longitudinal growth with cells distant from the proliferative center further differentiating towards hypertrophic chondrocytes. These cells produce a different matrix, rich in type 10 collagen. The coordinated differentiation of cells and linked growth of the skeletal template is dependent on a complex feedback loop

involving hedgehog and parathyroid-hormone like protein signaling. The Wnt signaling cascade also stimulates the differentiation towards hypertrophy [8].

The matrix produced by the hypertrophic chondrocytes is calcified and the terminally differentiated cells produce vascular endothelial growth factor (VEGF) and matrix metalloproteinase 13 (MMP13). The former will attract vessel- and bone progenitor cells, the latter will breakdown the cartilage matrix, ultimately resulting in the progressive replacement of the cartilage template by bone tissue. Recent evidence also suggests that hypertrophic chondrocytes can transdifferentiate towards osteoblasts [9]. Further strength to the bone is conveyed by the direct ossification of the bone collar originating from periosteal progenitor cells.

The original area of bone development is referred to as the primary ossification center [1]. During skeletal development secondary ossification centers form in the epiphyses. Primary and secondary ossification centers evolve into growth plates that are further executing the endochondral differentiation process. The growth plate, or epiphyseal plate, is a thin growing tissue located at the end of the long bones in children and adolescents. Four distinct zones can be defined: the resting zone, proliferating zone, prehypertrophic zone and the hypertrophic zone. The resting zone is composed of round chondrocytes distributed singly or in pairs and may function as a reservoir of chondrocyte progenitors which is capable of forming new proliferative chondrocytes. The proliferating zone is characterized with dividing and flattening cells which are arrayed in columns parallel to the long axis of the bone. The high proliferating rate leads to the stacks of chondrocytes. As cells develop into prehypertrophic and then hypertrophic cells, the chondrocytes continue their columnar arrangement but will cease proliferating. Chondrocytes in the upper region increase in size, while the lower region induces matrix mineralization. The calcified zone of growth plate is invaded by vessels and bone cells as in the developmental process described above and eventually in a remodeling process the cartilage is replaced by bone. The growth plate is the only region that allows longitudinal growth of long bones postnatally until the end of adolescence.

Joints between the bones are formed within the same time-frame [2]. The joint interzone marks the location of the future joint. Cells within the joint interzone do not commit to endochondral bone formation but will differentiate into the connective tissue cells of the joint such as ligaments, synovium and menisci. The joint cavity is formed by apoptosis of interzone cells. The precise origin of the articular chondrocytes is still debated with both interzone cells as well as secondary ossification zone cells likely contributing to the multilayered structure described below (Fig. 2).

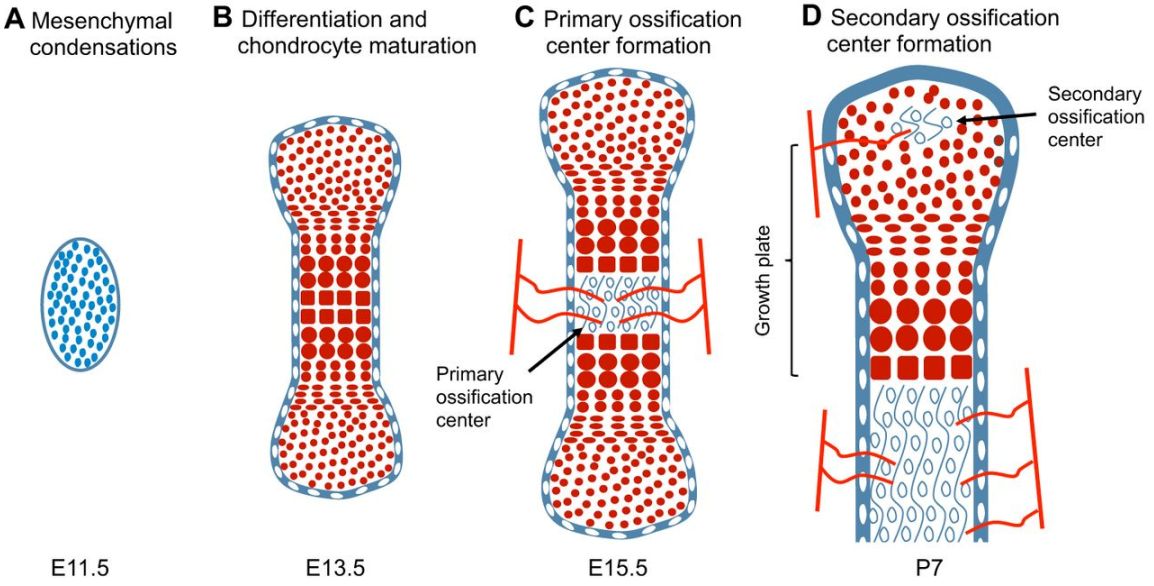


Fig 2. The stages of endochondral bone formation in the developing mouse hindlimb. (A) Schematic representation of mesenchymal cells (blue) that begin to form condensations at E11.5 in the hindlimb buds. (B) By E13.5, mesenchymal cells differentiate into chondrocytes (red cells), and the process of chondrocyte maturation and hypertrophy initiates. The cartilage anlage is surrounded by a layer of perichondrium (white cells). (C) By ~E15.5, vascularization (represented by red lines) takes place in the center of the cartilage anlage, resulting in replacement of chondrocytes with endochondral bone (open circles) in the primary ossification center. (D) The secondary ossification center (SOC) forms postnatally, at ~P7, and also becomes vascularized. The timing of SOC formation varies slightly between different bones. The schematics of the developing bones are approximately drawn to scale. Reused with permission from [10].

1.3 The anatomy and histology of the normal joint

The synovial joint is an organ characterized by a fluid-filled joint cavity, which facilitates the articulation between two or more skeletal elements, to allow and limit the movement

of the skeletal elements. As mentioned above, it contains different tissues, such as articular cartilage, bone, ligaments, synovium, and is enclosed by the fibrous capsule (Fig. 3).

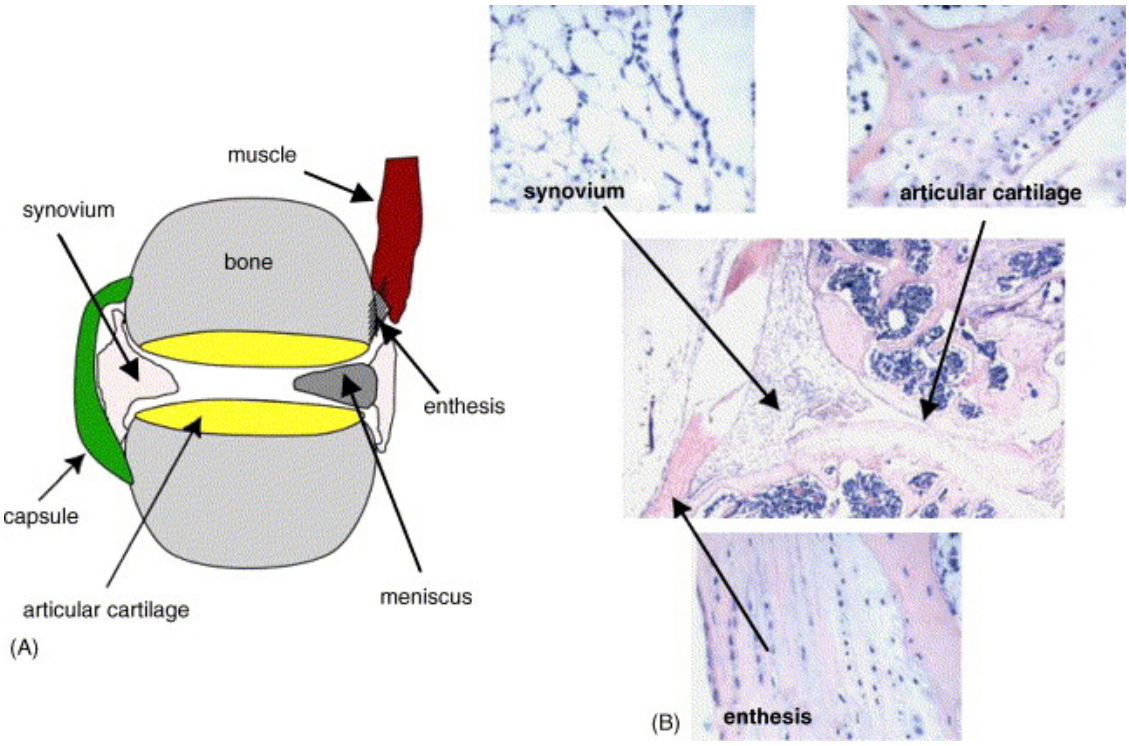


Fig 3. The anatomy of the synovial joint. (A) Schematic drawing of a synovial joint. The articular surfaces are connected by the synovium that consists of a thin pseudo-epitheloid lining layer and a loose connective tissue sublining zone. In some joints a fibrocartilaginous meniscus is found. The joint is encapsulated. The enthesis is an anatomic region referring to the insertion of tendons and ligaments in the underlying bone. **(B)** Microscopic image of a knee joint in the mouse. Images of the synovium, the articular cartilage and the enthesis are further enlarged. Reused with permission from [11].

The joint **capsule** separates the joint from the neighboring tissues and confines it. The outer layer of joint cavity is a tough membrane of collagen fibers and is continuous with the periosteum of the bones. The inner joint cavity is lined by the loose connective tissue of the **synovium**. It produces the **synovial fluid** that delivers nutrients and oxygen to the joint. Moreover, it can also produce lubricin and hyaluronic acid which can lower the friction to allow smooth joint movement and prevent pinching of the synovial membrane. **Menisci** are crescent-shaped fibrocartilaginous tissues which present in some joints like medial and lateral tibiofemoral joint surfaces that help to distribute and attenuate joint loading and to reduce friction. **Ligaments and tendons** can further stabilize the joint and restrict undesired joint motion. The articular bone ends are covered by a thin layer of hyaline or

articular **cartilage** caps. It is responsible for resistance of impact upon compressive loading and sustaining shearing forces and ultimately to allow almost frictionless movement [1].

The **articular cartilage** is an avascular, aneural connective tissue and its characteristic properties are related to the unique combination of only around 5% cells, the articular chondrocytes, and a large mass of extracellular matrix (ECM). The chondrocytes play an essential role in the maintenance of the joint homeostasis throughout life by producing the matrix molecules. Chondrocytes can also produce matrix degrading enzymes and control growth factor production to synthesize and remodel the ECM, for instance as joint loading is changing over time. However, articular chondrocytes are in a strongly quiescent state and only show limited repair capacity upon cartilage damage [7]

The ECM of the articular cartilage is very well hydrated and water-rich. It consists of two important families of macromolecules that define its critical mechano-physical properties: the collagen fibers and the proteoglycans [6,12]. The collagens are the structure-defining molecules whereas the proteoglycans define the function of the tissue. Damage to the collagen network is therefore often irreversible whereas proteoglycans may be more easily replaced. Type 2 collagen is the major ECM collagen in the articular cartilage (90-95%), but other forms (type 1, 4, 5, 6, 9 and 11 collagen) contribute to the maintenance of the collagen fiber network and together all collagen fibers give strength and structure to the cartilage [13]. The proteoglycan family is characterized by a core protein covalently attaching a variable number of glycosaminoglycan (GAG) side chains. The GAGs are commonly sulfated and can mainly be classified as chondroitin sulfate/dermatan sulfate (CS/DS), keratan sulfate (KS), and heparan sulfate (HS)/heparin. The largest in size and most abundant in weight cartilage proteoglycan, aggrecan, possesses over 100 CS and KS chains. Being a negatively charged molecule, aggrecan can attract polarized water molecules. Shifts in these water molecules provide cartilage its capacity to translate the loads imposed upon the joint by movement. Other proteoglycans that can be found in cartilage are cell surface syndecans and glypican, the small leucine-rich proteoglycans decorin, biglycan, fibromodulin, lumican and epiphycan and the basement membrane proteoglycan, perlecan [14].

Four distinct layers can be identified within the human articular cartilage: a superficial, transitional, deep, and calcified layer, which can be distinguished from each other by the configuration of the ECM and the shape of the articular chondrocytes [3]. The very thin superficial zone of articular cartilage is composed of flat chondrocytes, water and parallel, highly organized collagen fibers with very limited concentration of proteoglycans. The transitional zone of articular cartilage is the largest zone that makes up 50% of the total cartilage. Chondrocytes appear here to be more randomly distributed while collagen fibers are thicker and start to be arranged into radial bundles. The deep zone features columnar spherical chondrocytes in a proteoglycans-rich and lowest water concentration network. It carries the largest collagen fibers with a more organized structure. The zone of calcified cartilage is the deepest zone that separates cartilage tissue from subchondral bone. In this layer the matrix becomes calcified. The tidemark is a histological feature that separates the calcified articular cartilage from the non-calcified layers.

2. Osteoarthritis

Osteoarthritis is the most common chronic joint disease that is affecting over 250 million people worldwide [15]. Over 80% of those patients report limitations with joint movement and over 25% are not capable of doing normal activities of daily life. Therefore, osteoarthritis is rightfully considered a serious disease with a very high socio-economic impact and a high burden on individuals. As aging and obesity feature prominently among risk factors for the development of disease and its progression, the impact of osteoarthritis on society is likely to further increase in the near future.

Currently, there is no cure for the disease and there are only limited treatment options. Standard approaches include a combination of physiotherapy and exercise programs combined with painkillers and anti-inflammatory drugs [16]. Local treatments of disease flares by intra-articular steroid injections is part of common practice. Strong painkillers have been associated with severe side-effects including increased mortality and rapidly progressive osteoarthritis in some patients [17,18]. Currently, there are no so-called structure or disease modifying treatments available and in huge groups of patients, in particular those with large joint involvement, prosthesis or other types of joint surgeries

are the only viable solution. Prosthesis surgeries are a form of joint replacement rather than joint restoration approaches and the life-time of the implants may become too limited for the increased life expectancy of many patients, thus leading to potential re-do surgery in very elderly patients.

The onset and course of osteoarthritis are not easily explained in a simple model. In fact, osteoarthritis is best considered as the outcome of a range of interactions between contributing processes and factors depending on the characteristics of the individual patient. Risk factors include among others obesity, metabolic disorders, injury, altered anatomy and biomechanics of the joint and inflammation [16]. With many different mechanisms contributing to the initiation as well as to the progression of disease, it is not surprising that the development of specific targeted therapies is a major challenge in the long process from bench to bedside, and that the clinical development of any such strategies will need to focus on well-defined subpopulations in order to establish quality data on an eventual effect of the intervention.

Osteoarthritis has been considered to be initiated by changes at articular cartilage which result in secondary changes in the underlying subchondral bone and other tissues, but recent observations have indicated that the early changes may involve the entire joint or that any of these tissues may be the site of disease initiation. Indeed, failure in one of the joint components, not limited to cartilage damage and loss, but also the subchondral bone remodeling, the formation of osteophytes, the development of bone marrow lesions, inflammatory changes in the synovium, damage to the joint capsule, ligaments and periarticular muscles, and meniscal tears and extrusion, can lead to the progressive failure of the whole joints.

2.1 Articular cartilage

The articular chondrocyte has a vital role in mediating the normal turnover of cartilage matrix components to maintain the homeostasis, by synthetizing and degrading ECM components. As indicated above, these cells are in a relatively quiescent state and have limited repair potential.

Osteoarthritis can result from failure of the chondrocytes to maintain the balance between anabolic and catabolic processes. Triggers like excessive joint loading, trauma or inflammation can stimulate the chondrocyte to increase proliferation and ECM synthesis, however paralleled by increased production of cytokines and tissue degrading enzymes such as interleukin 1 (IL-1), matrix metalloproteinases and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes.

Synovial macrophages can also release pro-inflammatory cytokines, like tumor necrosis factor (TNF), IL-1 and IL-6 after exposure to molecules released from the damaged cartilage. Those cytokines can then further activate chondrocytes to release tissue breakdown enzymes and decrease type II collagen production, accompanying by increased chondrocyte apoptosis thus triggering a destructive chain reaction.

Another important factor is the progressive loss of the molecular phenotypic characteristics of the articular chondrocytes. In the process of osteoarthritis, part of the articular chondrocytes re-enter a differentiation-like program and achieve molecular characteristics of hypertrophic chondrocytes, producing matrix molecules like type 10 collagen, growth factors like VEGF and tissue degrading enzymes like MMP13. Together this results in a change in matrix composition with suboptimal biomechanical properties and will contribute to the progressive cartilage decay [3].

All these changes in the articular cartilage will therefore progressively result in cartilage softening, fibrillation of the superficial layers, fissuring into the deeper layers, with diminished cartilage thickness. Ultimately the degenerative cartilage is lost and ultimately the subchondral bone exposed.

2.2 Subchondral bone

The subchondral bone consists of the subchondral bone plate and the underlying trabecular bone and bone marrow space, a site that features a close biomechanical and biochemical relationship with the overlying articular cartilage. In the pathogenesis of osteoarthritis, subchondral bone is commonly reported to play a vital role and has been attracting increasing interest. In patients with osteoarthritis, the subchondral bone shows altered bone remodeling processes that can lead to a number of structural changes like sclerosis and

thickening of the subchondral bone plate, altered trabecular bone structures, the development of subchondral bone cysts and bone marrow edema-like lesions that can be visualized by magnetic resonance imaging [19]. Although the bone may appear sclerotic on conventional X-radiations, it may be of inferior quality characterized by reduced mineralization due to the intensity of tissue turnover with limited time for the mineralization process.

2.3 Synovium

In patients with osteoarthritis, the synovial membrane can display signs of hyperplasia with an increase of the synovial lining cell layer thickness accompanied by focal infiltration of the sublining layer with lymphocytes and monocytes. Other features include increased, vascularity likely through neoangiogenesis and fibrosis in long-standing disease. It is believed that the proteolytic degradation products from the cartilage matrix and other factors like microcrystals contribute to the development of synovitis. As suggested above, this is likely the basis of a deleterious chain reaction.

2.4 Meniscus

The menisci play an important protective role for the joint through shock absorption and load distribution, especially in the knee [20]. It is common to see meniscal degeneration in patients with osteoarthritis. Those menisci are typically torn, fissured, fragmented, macerated or completely destroyed. The changes usually initiate at the inner rim and further spread to the surface over time. A normal meniscus contains around 70% water and the dry weight is comprised of collagen (60%-70%), non-collagenous proteins such as elastin (8%-13%) and proteoglycans (1%). Peri-meniscal synovitis and meniscal calcification contribute to these intra-meniscal changes and result in meniscal degeneration and reduced meniscal tensile strength [21]. The affected menisci are not able to cope with loading and force transmission and this further contributes to degenerative damage.

To conclude, osteoarthritis is a multifactorial disease affecting the whole joint, with a complex pathogenesis in which all tissues of the joint get involved at some point in time. Better understanding of these complicated processes are required to develop specific therapies. One molecular pathway of interest in this context is the Wnt signaling pathway.

3. The Wnt signaling pathway

The molecular name Wnt is derived from the terms Wingless, the *Drosophila* melanogaster segment-polarity gene, and Int, the vertebrate homologue. The Int molecules, including Int1, were first identified as oncogenes in mouse mammary tumors. In 1987, investigators sequenced wingless in Drosophila and found it was the homolog of Int1 [22]. Thus, the int/Wingless family became the Wnt family and Int1 became Wnt1. The Wnt ligand family consists of a large group of lipid-modified secreted proteins which are cysteine-rich and highly hydrophobic [23]. These molecules are highly conserved during evolution and among organisms. Wnts are produced as precursor proteins that contain a short N-terminal signal sequence for secretion followed by 22-24 cysteines and a mature segment that varies in length from approximately 350 to 400 amino acids [24]. In mammals, there are 19 different Wnt homologs whose expression is spatially and temporally regulated during development [25].

Wnts can modulate biological processes from early embryonic development to organogenesis, to growth and postnatal homeostasis, and are involved in a number of diseases [26]. Mutational analysis has shown that Wnts have diverse roles during development, in governing cell fate, polarity, differentiation, migration and death. Postnatally, Wnts also have a role in tissue and organ homeostasis [27]. Mutations in components of the Wnt signaling pathway or their modulators are associated with a wide spectrum of diseases, such as cancer, aortic valve calcification and several bone related phenotypes [25]. Beyond mutations and syndromes, diverse roles for Wnt signaling in a variety of diseases have been suggested [28].

Wnt proteins act as ligands for the Wnt signaling pathway and lead to the activation of two main different pathway branches: the so-called canonical and non-canonical Wnt signaling pathways (Fig. 4).

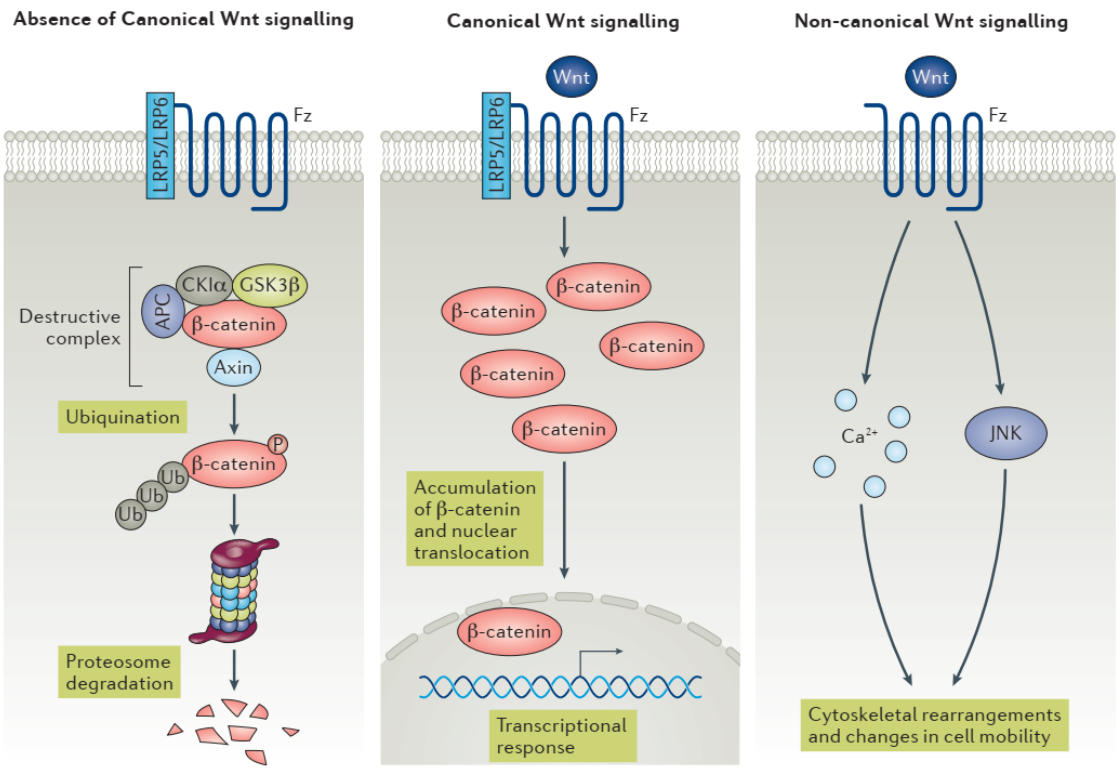


Figure 4. Schematic diagram of Wnt signaling cascades. **1) Canonical Wnt signaling pathway.** Upon the stimulation of Wnt ligands, lipoprotein related protein 5/6 (LRP5/6) co-receptors and frizzled receptor (Fz) complex deactivate the β-catenin destruction complex and allow β-catenin free from ubiqutinylation and proteasomal degradation. The accumulated β-catenin translocates to the nucleus and binds with transcriptional factors, ultimately actives canonical Wnt signaling pathway. **2) Non-canonical Wnt signaling pathway**. With the presence of specific Wnt ligands, changes with intracellular calcium or mitogen-activated kinases like c-Jun N-terminal kinase (JNK) can cause cytoskeletal remodeling and adjustments in cell movement. Reused with permission from [31].

3.1 Canonical Wnt signaling

The canonical Wnt pathway is also called β-catenin-dependent pathway [4]. In the absence of Wnt ligands, β-catenin is phosphorylated through a destruction complex consisting of Axin, tumor suppressor protein adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β) and casein kinase 1α (CK-1α). CK-1α phosphorylates β-catenin on the Ser45 residue, then β-catenin is phosphorylated on Thr41, Ser37, and Ser33 residues by GSK-3β, which subsequently leads to ubiquitination and degradation of the molecules [29].

When the canonical Wnt signaling is activated by binding of Wnt ligands to their Frizzled receptor (FZD) and low density lipoprotein (LDL) receptor-related protein (LRP) 5/6 co-receptor, the FZD receptor recruits the cytoplasmic phosphoprotein dishevelled (DSH) and requires the kinase GSK-3β, CK-1α and Axin, leads to disassembly or tethering of the β-catenin destruction complex. Therefore, non-phosphorylated β-catenin accumulates in the cytoplasm and then translocates to the nucleus where it can bind with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to drive downstream gene expression [4].

3.2 Non-canonical pathway

The non-canonical Wnt pathways are also referred to as β-catenin independent pathways, and include the planar cell polarity (PCP) pathway and the Wnt/calcium (Ca^{2+}) pathway [24]. The PCP pathway signals are initiated by binding of Wnt ligand to the Frizzled receptor and lead to the recruitment of DSH. The complex can activate Rho-associated protein kinase (Rock), c-jun N-terminal kinase (JNK) and profilin signaling leading to a rearrangement of the actin cytoskeleton ultimately changing cell polarization. The Wnt/Ca^{2+} pathway shares a number of components with the PCP pathway but also has clear differences and is therefore classified as a separate branch. This pathway was discovered with the finding that some Wnts and Fz receptors could stimulate intracellular Ca^{2+} release from the endoplasmic reticulum [30]. The release and accumulation of intracellular Ca^{2+} then activates several Ca^{2+} sensitive enzymes such as protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamKII).

3.3 Modulation of Wnt signaling

When we focus on the canonical Wnt signaling pathway, several groups of modulators can further regulate pathway activation at the ligand, receptor, intracellular and nuclear levels. **(1) Secreted Wnt modulators**. Some of those secreted molecules can directly bind with Wnt ligands and include the secreted frizzled related proteins (sFRPs) and Wnt inhibitor factors (WIFs). Others modulators like the DKK family members or sclerostin can competitively bind to the Wnt ligand co-receptors, LRP, to reduce Wnt ligand–receptor complex binding [28]. Norrin and R-spondin family members (RSPO) can directly bind with the FZD receptors and provide a co-stimulatory signal [32]. The extracellular enzymes

Tiki and Notum act as a protease and a carboxylesterase to cleave the N terminus of Wnts or remove their palmitoleate moiety, both resulting in Wnt inactivation [33-35]. **(2) Transcription factor complexes**. When the canonical Wnt signaling pathway is activated, accumulated β-catenin translocates to the nucleus and regulate downstream gene expression by binding to transcription factors from the TCF/LEF family to form transcriptional complexes that can then bind with DNA [31]. There are four identified members in TCF/LEF family. TCF1, 3, 4 and LEF1, and TCF4 is the most abundantly expressed in human cartilage. Evidence showed that Hypoxia-inducible factors 1α (HIF1 α) can modulate Wnt signaling via TCF4 by lowering its transcriptional activity and displacing it from MMP13 gene [36]. However, the understanding of the complex regulation of transcription by TCFs and LEFs is still limited. (3) **Epigenetic regulation of Wnt signaling**. Epigenetic mechanisms such as DNA methylation, covalent histone modifications like methylation and acetylation, and noncoding regulatory RNA regulation, are key regulators of biological processes. DOT1-like histone H3K79 methyltransferase (DOT1L), specifically methylates histone H3 lysine 79 (H3K79) and functions as a positive mediator of gene transcription and DNA repair and as a regulator of the cell cycle [37]. DOT1L has been reported as a key regulator of Wnt signaling in cartilage by direct binding and inhibition of sirtuin-1 (SIRT1) [38]. Another study showed that enhancer of zeste homologue 2 (EZH2) activity increases active canonical Wnt signaling by enhancing H3K27 methylation on the promoter of the gene encoding sFRP1 a negative modulator of Wnt as described above [39]. **(4) Other signaling pathway regulations**. Interactions with many other signaling components can also change the activity of Wnst, such as GDF5 signaling, Hedgehog signaling, IFT80 signaling and some pro-inflammatory cytokine signaling (TNF and IL-1) [31].

4. Role of Wnt in skeletal development and in joint disease

4.1 Wnt signaling in chondrogenesis

Chondrogenic differentiation is tightly controlled by a complex network of signaling cascades and the Wnt signaling pathway has emerged as a critical regulator of this process. The complex regulation may depend on the context of the cell or stage of differentiation. Evidence proving the involvement of Wnt signaling in cartilage development was initially

discovered by studies looking for expression patterns of Wnts and a Wnt antagonist, frizzled related proteins (Frzb/Frp3/Sfrp3), in the cartilage of chick developing limb model [40]. Further studies on expression patterns of other Wnt ligands, receptors and antagonists have been used to confirm the direct participation of Wnt in developing cartilage [40]. Functional studies revealed that inactivation of β-catenin can shift the cell fate of the mesenchymal progenitor cells from becoming osteoblasts towards chondrocytes [41]. However, as indicated above, Wnts may inhibit early chondrogenic differentiation but act as stimulators for the terminal differentiation towards hypertrophic chondrocytes. This is also important postnatally in a disease context. In a Col2a1-ICAT-transgenic mouse study which showed inactivation of β-catenin in adult cartilage, increased chondrocyte apoptosis and articular cartilage destruction was observed [42], whereas articular chondrocyte hypertrophic differentiation and the development of an OA-like phenotype were found in adult mice with chondrocyte-specific activation of β-catenin signaling [43].

SOX9 is a pivotal transcription factor in both developing and adult cartilage and required for chondrogenesis [44]. It secures chondrocyte lineage commitment, promotes cell survival, and transcriptionally activates the genes for many cartilage-specific structural components and regulatory factors. Studies have showed that interactions between SOX9 and β-catenin can regulate chondrocyte hypertrophy. Either overexpression of SOX9 or inactivation of β-catenin in chondrocytes of mouse embryos lead to a similar dwarfism phenotype characterized by decreased proliferation, delayed hypertrophic and endochondral bone formation [45]

Wnt antagonists, FRZB and DKK1, can limit hypertrophic differentiation and endochondral ossification [46]. Additionally, our group found that FRZB increases expression of chondrogenic markers in a cell differentiation model [47] while other research teams described that overexpression of FRZB-1 caused dwarfism, joint fusion, disorganized growth plates, a delayed hypertrophic maturation and inhibition of matrix mineralization [48]. More evidence also demonstrated that SFRP1 is a critical negative regulator of Wnt signaling for the normal progression of chondrocyte differentiation [49]. In general, activation of canonical Wnt cascades inhibits the early stages of chondrogenesis but stimulate chondrocyte hypertrophy.

4.2 Wnt signaling in osteoarthritis

A potential role for Wnt signaling in osteoarthritis emerged based on a series of different discoveries. First, in their search for genetic factors potentially associated with osteoarthritis, investigators found evidence that polymorphisms in the *SFRP3* gene, that encodes FRZB, were associated with hip osteoarthritis [50]. Functional analyses suggested that the identified variants affected the Wnt antagonizing properties of FRZB. Interestingly, other investigators document that expression levels of *SFRP3* were rapidly downregulated upon *in vitro* cartilage injury [51]. Our research group had developed *Sfrp3* knockout mice to study the role of this molecule in skeletal development [52]. However, primary analyses of this novel mouse strain did not demonstrate obvious skeletal abnormalities. However, when studying these animals postnatally we demonstrated that the Sfrp3 knockout mice showed more severe osteoarthritis in different induced models, that FRZB appeared to interact directly with cartilage matrix destructive enzymes such as MMP 3, and that its absence also affected cortical bone thickness. The data from this mouse model were thereby the first evidence that increased activation of Wnt signaling in the joint contributes to the development of osteoarthritis.

As mentioned above key role for β-catenin in the maintenance of cartilage and joint health was subsequently confirmed by two studies with opposing strategies [42,43]. First, cartilage specific inactivation of β-catenin was demonstrated to lead to articular cartilage destruction mainly by cell death, whereas overexpression of a constitutively active form also resulted in osteoarthritis now characterized by increased cartilage cell hypertrophy. Together these studies provided clear evidence that Wnt signaling must be tightly regulated within the joint in order to maintain homeostasis and prevent joint disease such as osteoarthritis.

Since these original discoveries, a large body of evidence has been assembled that supports the view that controlling Wnt signaling is an essential mechanism in joint homeostasis [31]. From the deleterious perspective, strong activation of the canonical Wnt signaling pathway affects the molecular characteristics of the healthy articular chondrocyte, stimulates their further differentiation towards hypertrophic cells associated with the production of a

calcified and therefore biomechanically inferior extracellular matrix, and also appears to contribute towards increased subchondral bone remodeling and osteophyte formation, two factors that are also associated with osteoarthritis development. In contrast, in the superficial layer of the articular cartilage, Wnt16 production stimulates the synthesis and secretion of lubricin, a lubricant molecule that optimizes near zero-like friction between the opposing cartilage surfaces in the moving joint (Fig. 5) [53].

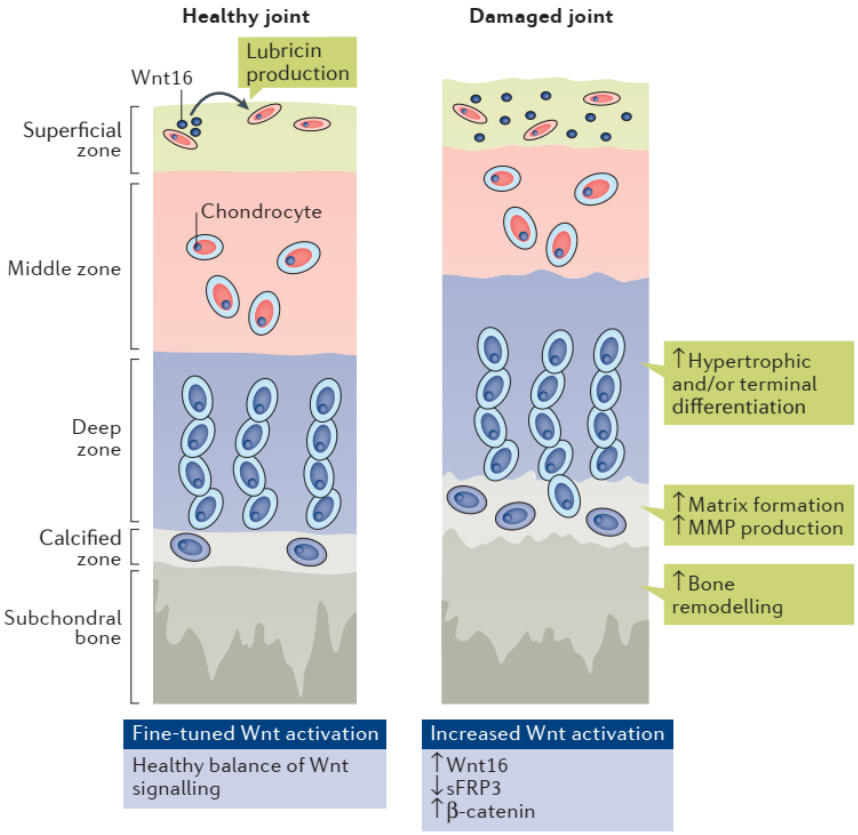


Figure 5. Cartilage homeostasis is tightly regulated by Wnt activity. Under normal circumstances, the healthy cartilage is divided into four different zones and lubricin, which is synthesized and secreted by superficial zone chondrocyte progenitor cells, could maintain the nearly frictionless motion of joints and protect chondrocytes. While hyperactive Wnt signaling pathway is triggered, the cartilage could undergo pathological changes such as increases of terminal differentiation and chondrocyte hypertrophy, higher expression of tissue destructive enzymes and consequently subchondral bone remodeling. Reused with permission from [31].

In a drug library screening, different Wnt modulators were identified of which lorecivivint was selected for studies on osteoarthritis [54-56]. Of note, the vivint suffix definition has been attributed to the novel class of Wnt pathway inhibitors. Preclinical development highlighted the potential of lorecivivint to be used in osteoarthritis. *In vitro* experiments demonstrated that the addition of lorecivivint to differentiation cultures of human mesenchymal progenitor cells towards cartilage increased chondrogenesis as demonstrated by increased extracellular matrix accumulation, including proteoglycans and collagens. Further cartilage protection could be conferred by the inhibitory effects of lorecivivint on the expression of tissue destructive enzymes from the MMP family. Of note, in cell culture the compound also demonstrated anti-inflammatory effects by reducing the expression of cytokines such as IL-1b, TNF and IL-6 [56].

These promising results prompted the use of lorecivivint in *in vivo* models of osteoarthritis. Remarkably, intra-articular injection resulted in sustained high levels of the molecule over several months, mostly in cartilage and to a lesser extent in bone. Systemic levels of the drug were extremely low. Intra-articular injection also provided clear protection against osteoarthritis development in rats, thus paving the way for human trials [55].

Further *in vitro* work identified a unique mechanism of action for lorecivivint [56]. The compound works as a kinase inhibitor and was demonstrated to mainly affect the activity of CLK enzymes, in particular CLK2 in nanomolar concentrations, and DYRK enzymes, in particular DYRK1A, acting in nanomolar concentrations. These molecules had not yet been clearly associated with Wnt signaling, thereby requiring further investigations. CLK2 plays an important role in the steps from DNA transcription towards protein translation. Indeed the messenger RNA that is the result of gene transcription, requires further processing by splicing, for example to remove introns. Inhibition of CLK2 affects this process and results in abnormal transcripts with dominant negative effects. TCF7, a key transcription factor in the Wnt pathway features prominently in this list and the abnormally spliced variants appear to have a dominant negative effect. Experimental data suggest that the effects on Wnt signaling transcription factors reduce the levels of Wnt target genes and simultaneously increase the levels of markers associated with the healthy chondrocyte

profile such as aggrecan, collagen type 2 and DOT1L. Moreover, negative modulation of CLK2 activity also appears to inhibit NFκB signaling, a strong pro-inflammatory cascade.

Kinase inhibitors rarely have total specificity. This also applies to lorecivivint. Interestingly, in addition to inhibition of CLK2. Additional inhibition of DYRK1A offers an interesting perspective on the mechanism of action [56]. DYRK1A is associated with anti-inflammatory effects as it affects the JAK/STAT signaling pathway. Moreover, DYRK1A also affects activity of transcription factor FOXO1 and of epigenetic modulator SIRT1. Inhibition of SIRT1 in a context of Wnt hyperactivation may contribute to cartilage homeostasis. Positive regulation of FOXO1 stimulates cartilage homeostasis and lubricin production.

These preclinical data have been used to support the use of lorecivivint for the treatment of knee osteoarthritis in human clinical trials [54]. Based on a phase 1 study with an excellent safety profile, phase II trials have recently been finished suggesting effectiveness on both signs and symptoms as well as structural damage in patients with knee osteoarthritis, in particular those without widespread pain. Currently phase III trials are ongoing. Current observations match with the preclinical data suggesting both symptomatic and structural effects.

The complexity of the signaling pathway and its involvement in a wide range of biological processes appears to be a daunting perspective for the development of therapies that have an impact on a disease such as osteoarthritis. In addition, therapies for an aging-associated disease that is slowly progressing may require sustained administration of drugs over a long time-period in a population of patients that will likely also show an increase in co-morbidities over time. Hence, safety of treatments is an important consideration. We propose that there are at least two ways to overcome these challenges, either by searching for tissue-specific mechanisms of action or by developing highly tissue-specific targeting approaches for drug delivery.

Currently, multiple approaches can be considered (Fig. 6). Inhibition of porcupine will shut down Wnt secretion. Antibodies or molecular mimics could be used to interfere with ligand-receptor interactions. Such strategies may also be developed towards co-agonists

such as the R-spondin family, molecules that amplify canonical signaling pathway activation. Drugs can stabilize the destruction complex thereby stimulating beta-catenin breakdown. Finally, intranuclear effects on gene transcription may confer the tissue specificity desired for.

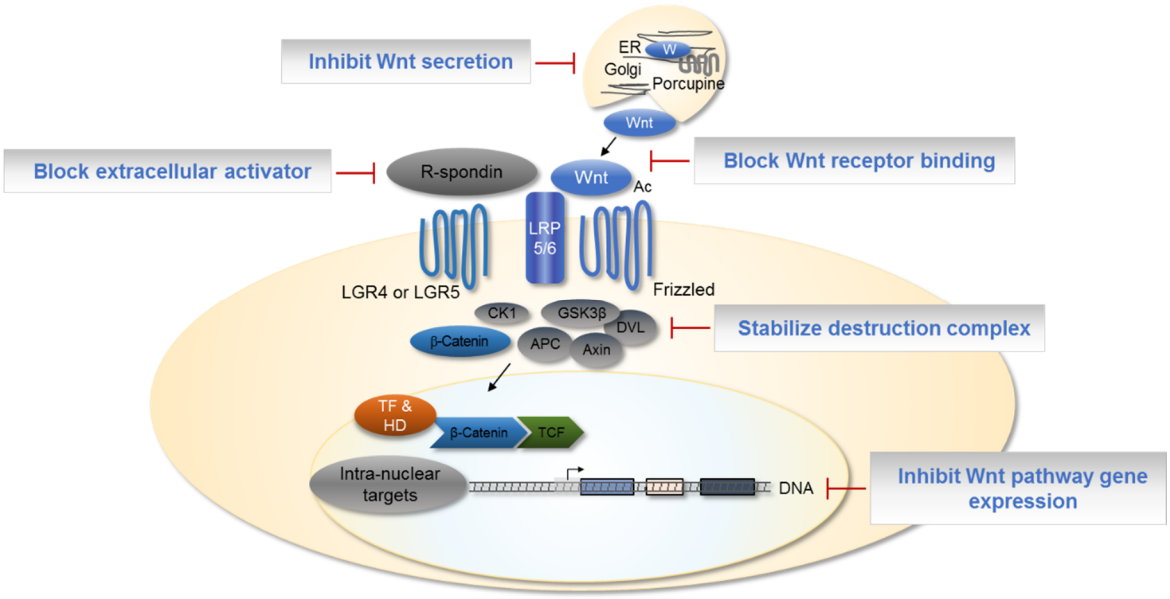


Figure 6. Potential therapeutic targets associated with activation of the Wnt signaling pathway. Inhibition of porcupine will stop Wnt ligand secretion. Wnt receptors, Wnt co-receptors and accessory signals such as R-spondin-LGR4/5 interactions can be inhibited. The destruction complex can be stabilized to increased beta-catenin degradation. Transcription complex formation can be disturbed by chemicals.

5. Wnt, heparan sulphate proteoglycans (HSPGs) and exostosin-1 (EXT1)

Wnts typically act as morphogens by establishing concentration gradients relying mostly on autocrine and paracrine signaling. The lipid tail associated with the Wnt proteins has a strong impact on their solubility and spread throughout the tissues and body. However, to achieve more long-distance gradient formation, several mechanisms have been proposed that mediate intercellular Wnt transport, including (1) modifications to the lipid tail that makes the ligand more soluble, (2) transfer of ligands by cell to cell contact, (3) shielding of the Wnts inside micelles-like aggregates or by lipid-binding proteins, (4) secretion on membranous particles acting as cargo, (5) direct transfer from cell to cell by a lateral diffusion mechanism (Fig. 7) [57].

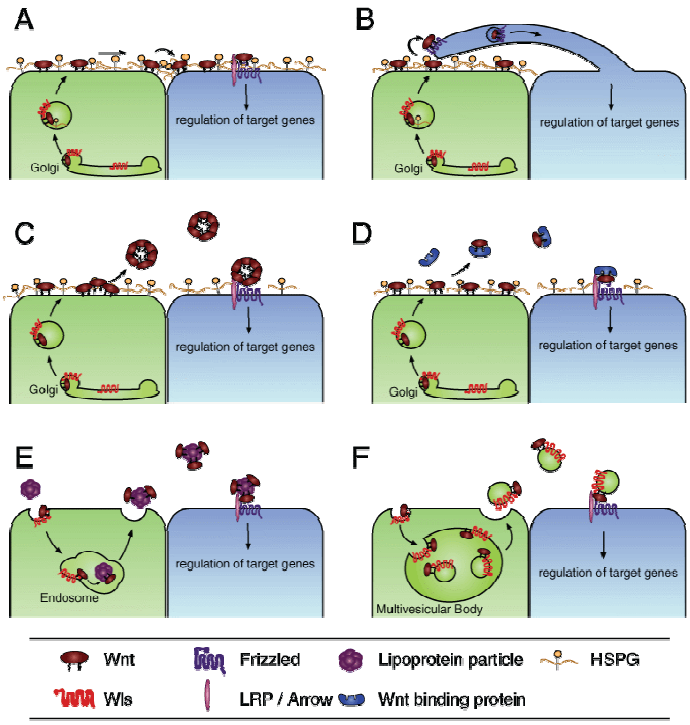


Figure 7. Models for Wnt gradient formation. A) Lateral diffusion aided by HSPG. B) Wnt transfer at sites of cell–cell contact. Receiving cells grow cytonemes to contact producing cells. C) Wnt proteins form soluble micelles. D) Wnts are bound and solubilized by specific lipid-binding proteins. E) Wnts are secreted on lipoprotein particles. F) Release of exosomes containing Wnt ligand secretion mediator (Wls) and presenting Wnts on their surface. Note that these models could act in parallel and their use might depend on the tissue and developmental context. Reused with permission from **[57].**

In this context, increasing evidence supports the view that heparan sulfate (HS)-carrying proteoglycans (HSPGs) that are present at the cell surface and in the extracellular matrix

play a critical role for morphogen gradient formation. Not limited to Wnts, HSPGs also regulate many other growth factors and morphogens, including many that play a role in skeletal development [58]. HSPGs are composed of a core protein with several attached chains of HS glycosaminoglycans (GAG) and can be classified into several families based on their core protein. According to their location, they can be further divided into groups as membrane HSPGs, including syndecans and glypicans, the secreted ECM HSPGs (agrin, perlecan, type 18 collagen), and the secretory vesicle proteoglycan, serglycin. (Table 1).

Heparan sulfate proteoglycans	
Cell surface	Transmembrane syndecans
	Glycosylphosphatidylinositol-anchored glypicans
Extracelullar matrix	Agrin
	Perlecan
	Type XVIII collagen
Intracellular vesicles	Serglycin

Table 1. Classification of HSPGs according to their location. Reused with permission from [59].

Previous research in *Drosophila* has shown that HSPGs can stabilize Wnts ligands to enhance Wnt diffusion [60]. For example, overexpression of the Drosophila glypican gene, *dallylike (dlp),* leads to an accumulation of extracellular Wingless. On the contrary, another cell study showed that compromised biosynthesis of GAGs can dramatically decrease the Wingless (Wg) accumulation [61]. Moreover, mutation of HSPGs-biosynthesis gene, *sugarless (sgl)*, impairs surface expression of Wingless, suggesting that proteoglycans contribute to maintain the local concentration of Wg molecules for their receptors. HSPGs are also suggested to limit Wingless diffusion and thereby facilitate the binding of Wingless to its receptor [62]. Other research in the *Zebrafish* and *Xenopus* developmental models confirm that HSPGs play a role in modulating Wnt signaling and have an impact on the concentration gradients formed [63]. In addition, HSPGs may also influence Wnt morphogen gradient formation by acting as receptors for extracellular vesicles in the internalization of Wnt-bearing lipoproteins [64]. In chick development, glypican-4 may

also allow the formation of long-range gradients of Wnt morphogen by mobilizing the source cells, neural crest cells, to improve the delivery process instead of Wnt ligand mobilization [65]. RSPO2 and RSPO3 can potentiate WNT/β-catenin signaling in the absence of leucine-rich repeat-containing G-protein coupled receptors (LGRs) but require HSPGs [66].

Many studies on HSPGs have highlighted the importance of their HS chains. HS chains are long linear polysaccharides synthesized in the Golgi apparatus and contain repeating D-glucuronic acid and N-acetyl-D-glucosamine residues. The processes of HSPG biosynthesis involve distinct glycosyltransferases and modification enzymes depends on the glycosyltransferases of the exostosin family (EXTs). Within the Golgi-associated EXT1/EXT2 protein complexes, EXT1 protein has a major catalytic function in HS polymerization, while EXT2, though required, may have more structural or supportive roles [67]. Studies have shown that the glycosyltransferase activity associated with EXT1 is specific and indispensable for HS assembly [68]. Loss-of-function mutations in EXT1 lead to the human bone disorder multiple hereditary exostoses (HME), in which multiple benign cartilaginous tumors called exostoses form next to growth plate of the skeletal elements [67]. Further research highlighted the physiological importance of EXTs and HS in skeletal development. In this thesis, we will investigate the role of Ext1 in the regulation of Wnt signaling in the process of chondrogenesis

6. ANP32A, key molecule in osteoarthritis and potential regulator of Wnt signaling

The acidic (leucine-rich) nuclear phosphoprotein 32 kDa (ANP32) family is composed of small, evolutionarily conserved proteins characterized by an N-terminal leucine-rich repeat (LRR) domain, a central domain and a C-terminal unstructured low-complexity acidic region (LCAR). They are approximately 250 amino acids in length. In mammals, the ANP32 family has three members, ANP32A, ANP32B, and ANP32E and have multiple functions including chromatin modification and remodeling, apoptotic caspase modulation, transcription regulation and regulation of intracellular transport [69].

A GWAS study in women with knee OA described a modest association between the disease and a variant located in chromosome 15 that mapped to the *ANP32A* gene which suggests that genetic variation at ANP32A could be involved in susceptibility to OA [70]. A follow-up study demonstrated that ANP32A is abundantly present in healthy and OA cartilage, also in growth plate chondrocyte and cartilage cells in osteophyte, and identified three SNPs in the *ANP32A* gene that are significantly associated with hip OA [71].

Our lab recently reported that ANP32A is down-regulated in human and mouse OA cartilage compared to healthy cartilage, and that ANP32A protects against OA by promoting the expression of antioxidant enzyme ATM, thereby reducing oxidative stress [72]. Antioxidant treatment can limit cartilage damage in Anp32a-deficient mice in different induced models. However, we found that this rescue is incomplete, thus the ANP32A/ATM axis likely does not explain the full role of ANP32A in OA.

Interestingly, a protein-protein interaction network screening using automated yeast two-hybrid (Y2H) interaction mating suggests interactions of ANP32A with a key component of Wnt signaling, AXIN1 [73]. Another study showed that ANP32A can specifically bind to the catalytic C subunit of phosphatase 2A (PP2A) and regulate its activity. PP2A has a dual and opposing role in β-catenin regulation as positive and negative regulator of Wnt signaling [74,75]. However, direct evidence to demonstrate a role for ANP32A in modulating Wnt activity is currently not available. To address this issue, we will study if Wnt signaling acts as central cascade under the regulation of ANP32A, beyond Atm/oxidative stress axis, in cartilage biology and osteoarthritis.

Chapter 2 Objectives

General objective:

Wnt signaling plays a critical role in joint development and homeostasis, and its dysregulation is associated with the development of joint disorders like OA. Based on previous evidence, EXT1 and ANP32A are hypothesized to play a role in these processes by modulating Wnt activity in cartilage. We therefore aim to further elucidate the role of EXT1 and ANP32A in cartilage development and disease by studying their regulatory effects on Wnt signaling.

Specific objectives:

1. **Investigating the regulation of Wnt signaling at the extracellular level in cartilage**. We aimed to define the impact of EXT1 and HS content in determining the strength of Wnt signaling activation during chondrogenesis. To this end, we interfered genetically with HS-synthetizing enzyme Ext1 in ATDC5 cells cultured as micromasses, a well established in vitro model of chondrogenic differentiation.

2. **Investigating intracellular regulation of Wnt signaling by ANP32A in cartilage.** We aimed to investigate whether the protein ANP32A, genetically and functionally linked to OA, has a regulatory role on Wnt signaling in cartilage. ANP32A gene expression was modified in ATDC5 cells and in human articular chondrocyte.

Chapter 3 Exostosin-1 enhances canonical Wnt signaling activity during chondrogenic differentiation.

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Abstract

Objective: Exostosin-1 (*Ext1*) encodes a glycosyltransferase required for heparan sulfate (HS) chain elongation in HS-proteoglycan biosynthesis. HS chains serve as binding partners for signaling proteins, affecting their distribution and activity. The Wnt/β-catenin pathway emerged as critical regulator of chondrogenesis. Yet, how EXT1 and HS affect Wnt/β-catenin signaling during chondrogenesis remains unexplored.

Methods: Ext1 was stably knocked-down or overexpressed in ATDC5 chondrogenic cells cultured as micromasses. HS content was determined using ELISA. Chondrogenic markers *Sox9, Col2a1, Aggrecan*, and Wnt direct target gene *Axin2* were measured by RT-qPCR. Proteoglycan content was evaluated by Alcian blue and DMMB assay, canonical Wnt signaling activation by β-catenin Western blot and TOP/FOP assay. ATDC5 cells and human articular chondrocytes were treated with WNT activators CHIR99021 and recombinant WNT3A.

Results: Ext1 knock-down reduced HS, and increased chondrogenic markers and proteoglycan accumulation. *Ext1* knock-down reduced active Wnt/β-catenin signaling. Conversely, *Ext1* overexpressing cells, with higher HS content, showed decreased chondrogenic differentiation and enhanced Wnt/β-catenin signaling. Wnt/β-catenin signaling activation led to a down-regulation of *Ext1* expression in ATDC5 cells and in human articular chondrocytes.

Conclusions: EXT1 affects chondrogenic differentiation of precursor cells, in part via changes in the activity of Wnt/β-catenin signaling. Wnt/β-catenin signaling controls *Ext1* expression, suggesting a regulatory loop between EXT1 and Wnt/β-catenin signaling during chondrogenesis.

Keywords: EXT1, Heparan sulfate proteoglycans, Wnt/β-catenin signaling, Chondrogenesis, Cartilage

The majority of bones in our body are formed by endochondral ossification. This process is initiated by condensation of mesenchymal progenitor cells, which then differentiate into cartilage-forming chondrocytes to generate a template of the future bone. Chondrocytes in these templates undergo a differentiation program from proliferating into mature chondrocytes, which eventually become hypertrophic and are ultimately replaced by bone [76]. Chondrogenic differentiation is tightly controlled by a complex network of signaling cascades. The Wnt/β-catenin pathway (also known as canonical Wnt signaling), composed of Wnt ligands, receptors, antagonists, and intracellular signaling molecules, has emerged as a critical regulator of skeletal cell differentiation processes [77,78].

The expression patterns and the modulation of Wnt pathway components in chick and mouse models of the developing limb provide strong evidence for the involvement of this cascade in chondrogenesis and endochondral bone formation [79-83]. In the early phase of skeletogenesis, expression of Wnts, such as WNT3A, in distal cell populations at the apical ectodermal ridge (AER) keeps the mesenchymal progenitor cells in a proliferative and undifferentiated state that is essential for limb outgrowth, preventing differentiation into chondrocytes. However, low Wnt signaling in cells distant from the Wnt source allows them to express the chondrogenic master transcription factor SOX9 and differentiate into chondrocytes [77]. Thus, the current model is that canonical Wnt signaling opposes a chondrogenic cell differentiation program in early chondroprogenitor cells. Mouse embryos expressing a constitutively active mutant form of β-catenin in developing cartilage under the control of collagen 2 promoter/enhancer, thereby increasing active Wnt signaling in cells that have acquired a chondrocyte identity, exhibit severe dwarfism and skeletal deformity. In these mice, synthesis of cartilage matrix molecules is strongly reduced and the growth plate structure fails to organize properly [45,82,84]. Thus, excessive activation of this signaling can disturb the normal endochondral bone formation process and lead to skeletal disorders. Therefore, Wnt/β-catenin signaling should be tightly controlled for normal cartilage development.

The Wnt/β-catenin pathway demonstrates an extraordinary degree of complexity and fine-tuning, and many aspects of its regulation remain uncharacterized [31,77]. Although the

interactions of Wnt and other signaling molecules during endochondral ossification have been intensively studied, mechanisms that control the extracellular distribution of Wnt molecules and subsequent signaling strength during chondrogenesis are less well understood. In this context, increasing evidence supports that heparan sulfate (HS)-carrying proteoglycans (HSPGs) that are present at the cell surface and in the extracellular matrix play a critical role in controlling the distribution and activity of secreted growth factors. Many growth factors and morphogens, including those that play a role in skeletal development, exhibit affinities for the HS chains of HSPGs [85,86]. HSPGs are composed of a core protein with several attached chains of HS glycosaminoglycans (GAG), and include syndecans, glypicans and perlecan [86]. The HS chains consist of repeating D-glucuronic acid and N-acetyl-D-glucosamine residues that are assembled into linear polysaccharides by glycosyltransferases of the exostosin family (EXTs) [85]. Within the Golgi-associated EXT1/EXT2 protein complexes, EXT1 protein has a major catalytic function in HS polymerization, while EXT2 –though required– may have structural or supportive roles [87]. The physiological importance of EXTs and HS in skeletal development is illustrated by the severe limb defects demonstrated in conditional *Ext1* deficient mice [88] and also by the human bone disorder Multiple Hereditary Exostoses, caused by mutations in EXT enzymes, with the most severe phenotypes associated with EXT1 mutations [87]. Of note, postnatal conditional ablation of β-catenin in cartilage induces ectopic periosteal chondroma-like cartilage formation in mice [89]. How EXT1 activity and associated HS levels may affect Wnt/β-catenin signaling activation during chondrogenesis remains unexplored.

In the present study, we aimed to define the impact of EXT1 and HS content in determining the strength of canonical Wnt signaling activation during chondrogenesis. To this end, we knocked-down or overexpressed HS-synthetizing enzyme EXT1 in ATDC5 cells cultured as micromasses. The ATDC5 cell line, derived from mouse teratocarcinoma cells and characterized as a chondrogenic cell line, exhibits a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation. Thus, it is a well-established *in vitro* model for skeletal development [90].

Materials

CHIR-99021 and XAV-939 were purchased from Sigma and Selleckchem, respectively. Sodium chlorate was obtained from Sigma, human recombinant WNT3A from R&D systems.

ATDC5 micromass cultures

ATDC5 cells were cultured in growth medium (1:1 Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 mix (Gibco)) containing 1% (vol/vol) antibiotic–antimycotic (Gibco), 5% fetal bovine serum (FBS) (Gibco), 10 µg/mL human transferrin (Sigma) and 30 nM sodium selenite (Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. High-density micromass cultures of ATDC5 cells were grown to study chondrogenic differentiation [91]. Cells were trypsinised, washed and resuspended at 2×10^7 cells/mL in a chondrogenic medium made of DMEM-F12 enriched by 1% (vol/vol) antibiotic–antimycotic, 5% FBS, 5 µg/mL human transferrin and 1× ITS (Insulin, Transferrin, Selenite) premix (resulting in 10 µg/mL insulin, 5 µg/mL human transferrin and 30 nM sodium selenite) (Life Technologies). One droplet (10 µL) was carefully placed in the centre of each well of a 24-well plate. Cells were allowed to adhere for 2 hours at 37°C, followed by addition of 500 µL chondrogenic medium. Micromasses and supernatants were collected at time points 1, 7 and 14 days for functional assays.

Generation of stably knocked-down ATDC5 cell line

Silencing of *Ext1* was performed using a GIPZ-shRNA construct directed against mouse *Ext1* (Dharmacon); and a non-interfering GIPZ vector (Dharmacon) was used as a control. ATDC5 cells were transfected using Lipofectamine LTX (Thermo Fisher) and after 24h, selection with 3 µg/ml puromycin (Thermo Fisher) was initiated and continued for 14 days. Three different antibiotic-resistant clonal colonies of each condition were isolated and grown independently. Knock-down efficiency was assessed by quantitative RT-PCR.

Generation of stably overexpressing ATDC5 cell line

ATDC5 cells were transfected with control pCMV6-entry vector, pCMV6-*Ext1* ORF clone (PS100001; RC200644; Origene) using Lipofectamine LTX reagent (Thermo Fisher). Selection was initiated 24h after transfection by supplementing the maintenance medium with 500 ng/ml Geneticin (Thermo Fisher). Clones were picked after 14 days of selection. Three different antibiotic-resistant clonal colonies of each condition were isolated and grown independently. Overexpression levels were assessed by quantitative RT-PCR. Antibiotic pressure was maintained for the whole duration of the experiments.

Human articular chondrocyte culture

Human articular chondrocytes were isolated from the hip of one non-OA patient (male, 68 years old) undergoing total hip replacement surgery, as previously described [38]. The University Hospitals Leuven Ethics Committee and Biobank Committee approved the study and specimens were taken with patients' written consent.

Quantitative RT-PCR

Total RNA was isolated using the NucleoSpin RNAII kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocol. Complementary DNA was synthesized from 0.5 to 1 µg of total RNA using the RevertAid H minus First Strand cDNA synthesis kit (Fermentas GmbH, Rockford, IL, USA). SYBR green analyses were performed as described previously using Maxima SYBRgreen qPCR master mix system (Fermentas) [38]. Gene expression levels were calculated following normalization to housekeeping gene *Hprt* mRNA levels using the comparative Ct (cycle threshold) method. Primers used for RT-qPCR analysis are listed in Supplementary Table 1.

Heparan sulfate ELISA

Supernatant, cell lysates and pellet rest fraction from *Ext1* knocked-down or overexpressing cells were collected at day 7 of micromass culture. The Heparan Sulfate Quantikine ELISA Kit (Amsbio) was used according to the manufacturer's instructions. Data were normalized to the total protein concentration measured by BCA protein assay (Pierce).

Alcian blue staining

ATDC5 micromasses were washed with PBS and fixed with 95% ice-cold methanol for 30 min at 4°C. After rinsing three times with water, micromasses were stained with Alcian Blue (AB) (0.1% AB 8GX, Sigma), washed three times with water and air dried. Quantification of the staining was performed by dissolving the micromasses with 6M guanidine (Sigma) and measuring the absorbance at 595 nm with a spectrophotometer (BioTek Synergy).

Dimethylmethylene Blue (DMMB) assay

Biochemical analysis of DNA and glycosaminoglycan (GAG) amount was determined by DMMB assay, as previously described [91]. Harvested micromasses were digested at 56°C overnight with 0.1 M proteinase K in PBE buffer (1 mM EDTA, 10 mM Tris, pH 6.5). DNA amount was measured using the nanodrop (nanodrop 2000). Glycosaminoglycan amount was measured at 525 nm with a spectrophotometer (BioTek Synergy) using DMMB dye added to 50 µL of pellet digestion or undiluted supernatant and bovine chondroitin sulfate (Sigma) as a standard.

Luciferase Assay

Stably *Ext1* knocked-down or overexpressing ATDC5 cells (7500/well) were seeded in 96-well plates for 24h and transiently transfected with Super8X TOPFlash or Super8X FOPFlash (TOPFlash mutant control) (Addgene plasmids #12456 and #12457, respectively) for canonical Wnt signaling reporter using Lipofectamine LTX Reagent with PLUS Reagent (Life Technologies) according to the manufacturer's protocol. After 24h, cells were treated with 100 ng/ml recombinant WNT3A protein or vehicle and collected 24h later. Luciferase assay was performed using Promega Luciferase Assay system as previously described [38].

Western Blot Analysis

Cells were harvested and resuspended in RIPA buffer (Thermo Scientific) supplemented with 1 mM phenylmethanesulfonyl (Sigma-Aldrich), 5% protease inhibitor cocktail (Sigma-Aldrich), 2.3 mM Na_3VO_4 (Sigma-Aldrich) and 5 mM NaF (Merck Millipore,

Overijse, Belgium). Cell lysates were sonicated (two cycles of 7 s) and centrifuged at 18.000 g for 10 min, and supernatant containing proteins was collected. Protein concentration of the extracts was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Immunoblotting analyses were performed as previously described [38]. Antibodies against Actin (Sigma, A2066; dilution 1:4.000), active β-catenin (Merck Millipore, 05-665, clone 8E7; dilution 1:1.000) and total β-catenin (BD biosciences, 610154, clone 14; dilution 1:2.000), were used. Blotting signals were detected using the SuperSignalWest Femto Maximum Sensitivity Substrate system (Thermo Scientific).

Statistical analysis

Data are presented as mean and SD, representing the mean of technical replicates as indicated in figure legends. For the ATDC5 cells, experimental data reported in the manuscript are established from technical replicates of one representative colony. Replicates can therefore not be assumed to be strictly independent. The results obtained from other two colonies showed the same difference in a direction, but with different magnitude between the control and silencing or overexpression groups. In this hypothesis-finding exploratory study, no formal power analysis was performed. Statistical analyses were performed with GraphPad Prism software. Data distribution was evaluated on the basis of parameter characteristics, quantile-quantile (QQ) and residuals plots. T-tests or ANOVA tests were applied. Estimates of differences of means between groups (95% confidence intervals (CI)) are reported in the Results section. When different groups were compared by one-way ANOVA tests or when interaction was presented in two-way ANOVA tests, pair-wise t-tests were eventually performed applying a Bonferroni correction for multiple comparisons to control for type I errors in rejecting the null hypothesis as indicated in figure legends.

Results

Ext1 knock-down increases markers of chondrogenesis and proteoglycan deposition in ATDC5 cells

To study how EXT1 activity affects chondrogenic differentiation, we generated ATDC5 cells in which endogenous *Ext1* was knocked-down (KD). ATDC5 cells are mouse

chondrogenic precursor cells that differentiate and undergo chondrogenesis when cultured in micromasses. Following stable transfection, relative *Ext1* messenger RNA (mRNA) levels were determined in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (Gipz) and in cells with stable KD generated by shmiRNA against *Ext1* (shExt1), at baseline (day 1) and after 7 and 14 days in the differentiation culture. In the presented cell line, representative of at least 3 distinct colony-expanded clones (see [Fig. S1]), *Ext1* mRNA levels were on average 66% (95%CI: 23-89) down-regulated in shExt1 cells compared to Gipz cells [Fig. 1(A)]. This down-regulation was sustained during the differentiation process. We then assessed whether *Ext1* KD resulted in reduced HS biosynthesis. Effectively, the concentration of HS was decreased in shExt1 compared to Gipz cells (on average 16.69 ng/mg (95%CI: 4.6-28.77) in supernatant, 43.7 ng/ml (95%CI: -21.2-107.8) in cell lysate, and 34.01 ng/mg (95%CI: 4.09-63.92) in rest fraction) [Fig. 1(B)]. These results indicate that *Ext1* KD leads to reduced biosynthetic activity of HS proteoglycans during chondrogenesis.

Then, we measured mRNA expression of molecular markers of chondrogenic differentiation, in particular, *Sox9*, *Col2a1* and *Aggrecan*, by RT-qPCR. Gene expression of the master regulator of chondrogenic cell fate *Sox9* was enhanced in shExt1 cells, on average 0.8 difference between the means (95%CI: 0.14-1.476) [Fig. 1(C)] in the presented cell line, representative of at least 3 distinct colony-expanded clones. Similarly, expression of *Col2a1* and *Aggrecan* was up-regulated in shExt1 compared to control cells (10.46 difference between the means (95%CI: 5.9-15) and 712 differences between the means (95%CI: 561-862) [Fig. 1(C)]. Next, we evaluated the levels of total proteoglycans in the extracellular matrix of the micromasses, using Alcian blue staining. We observed an increase in total proteoglycans in shExt1 cells compared to controls (0.084 difference in absorbance (95%CI: 0.070-0.098) in the presented cell line, representative of at least 3 distinct colony-expanded clones) [Fig. 1(D)]. To validate these observations, we performed a DMMB assay. Again, we detected an enhancement in total proteoglycan content in shExt1 cells compared to controls, (3.199 difference between the means (95%CI: 1.037-5.36) and 10.77 difference between the means (95%CI: 7.98-13.56) for cell lysate and supernatant, compared to the lysate of control cells at day 1 in the presented cell line, representative of at least 3 distinct colony-expanded clones) [Fig. 1(E)]. Collectively, these

results indicate that *Ext1* deficiency and resulting HS reduction promotes chondrogenic differentiation.

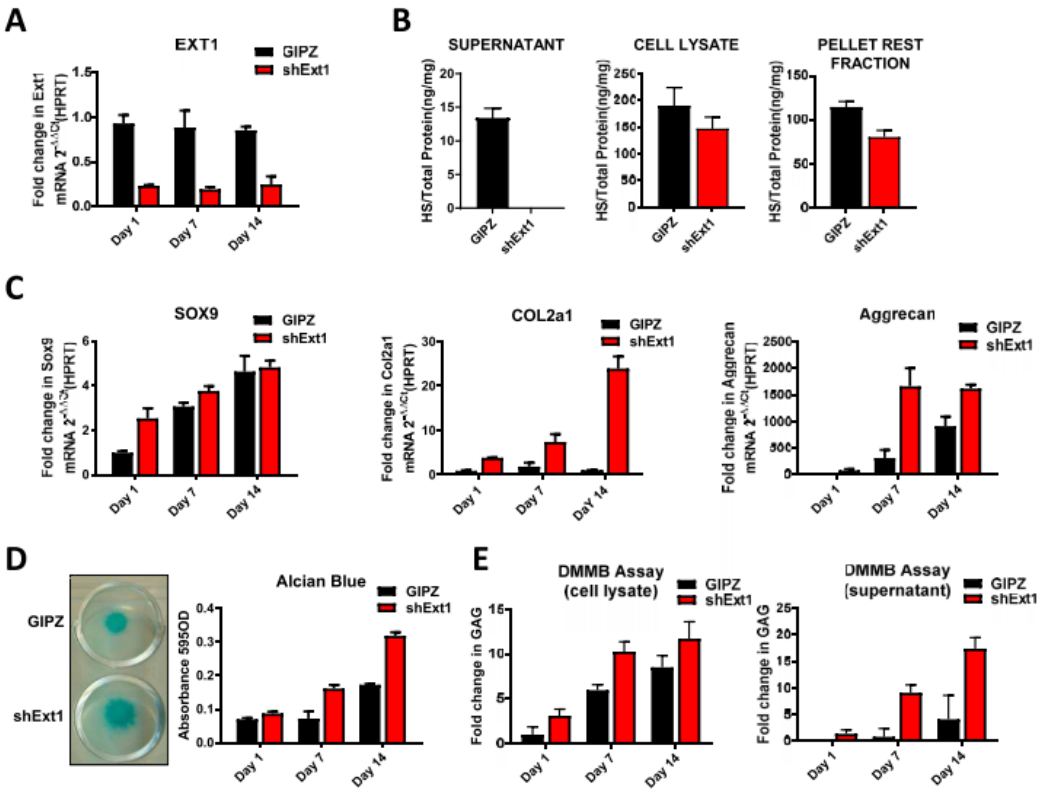


Fig. 1. *Ext1* knock-down enhances chondrogenic differentiation in ATDC5 cells. (A) Quantitative RT-PCR analysis in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (GIPZ) and in cells transfected with shmiRNA against *Ext1* (shExt1), showing successful knock-down (KD) of *Ext1* at day 1, 7 and 14 in shExt1 cells (p=0.015 in two-way ANOVA for silencing vs. control). (B) Heparan Sulfate (HS) ELISA assay showing reduced HS production in shExt1 cells in supernatant samples (p=0.019 by t-test), cell lysate (p=0.136 by t-test) and pellet rest fraction (p=0.039 by t-test). (C) *Ext1* KD increases mRNA expression of chondrogenic markers *Sox9* (p=0.028 in two-way ANOVA for silencing vs. control), *collagen 2a1* (*Col2a1*) (p=0.004 by two-way ANOVA for interaction between silencing and time) and *aggrecan* (p=0.014 by two-way ANOVA for interaction between silencing and time). (D) Alcian blue staining showing increased total proteoglycan deposition in shExt1 cells during chondrogenesis (p<0.0001 by two-way ANOVA for interaction between silencing and time). Left panel: representative image of staining at day 14 is shown. Right panel: quantification of staining by colorimetry. (E) Di-methylmethylene blue (DMMB) assay showing increased total glycosaminoglycan (GAG) content in shExt1cells, in both supernatants and cell lysates (p=0.015 and 0.0004 by two-way ANOVA for silencing vs. control, values are fold change compared to control day 1 cell lysates). Data shown are from one established cell line representative of at least three independent biological replicates, except for 1B (n=1 independent clone). Error bars indicate mean±SD of two (1A) or three (1B-E) technical replicates per condition.

Ext1 knock-down decreases canonical Wnt signaling activation during chondrogenesis

We then investigated the impact of *Ext1* loss-of-function on the activation of canonical Wnt signaling. In Wnt reporter-transfected cells, *Ext1* KD reduced luciferase activity in basal conditions (on average 0.23 unit decrease (95%CI: 0.10-0.36) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(A)]. In line with these observations, shExt1 cells showed lower active β-catenin protein levels than control cells during chondrogenic differentiation, assessed by Western Blot analysis [Fig. 2(B)]. Also, mRNA levels of direct Wnt target gene *Axin2* were decreased in shExt1 cells compared to controls (0.44 difference between means (95%CI: 0.14-0.75) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(C)]. Altogether, these findings indicate that, in basal conditions, canonical Wnt signaling activation is impaired in *Ext1* deficient cells during chondrogenesis.

Then, we aimed to evaluate how *Ext1* KD affects canonical Wnt signaling activation upon exogenous Wnt ligand stimulation. ShExt1 cells exhibited lower luciferase activity than control cells when the Wnt signaling pathway was activated by recombinant WNT3A protein (difference of the means 11.21 unit (95%CI: 10.04-12.38) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(D)]. Notably, in control cells, WNT3A stimulation led to a down-regulation in chondrogenic differentiation markers *Col2a1* (77.76 difference between means, 95%CI=17.21 to 138.3) and *Aggrecan* (71 difference between means, 95%CI: 16.6-128, both in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(E)], in line with previous reports [92,93]. However, in shExt1 cells, WNT3A treatment did not negatively affect the expression levels of chondrogenic markers. These data suggest that *Ext1* deficiency reduces canonical Wnt signaling activation and abolishes subsequent effects on chondrogenic differentiation.

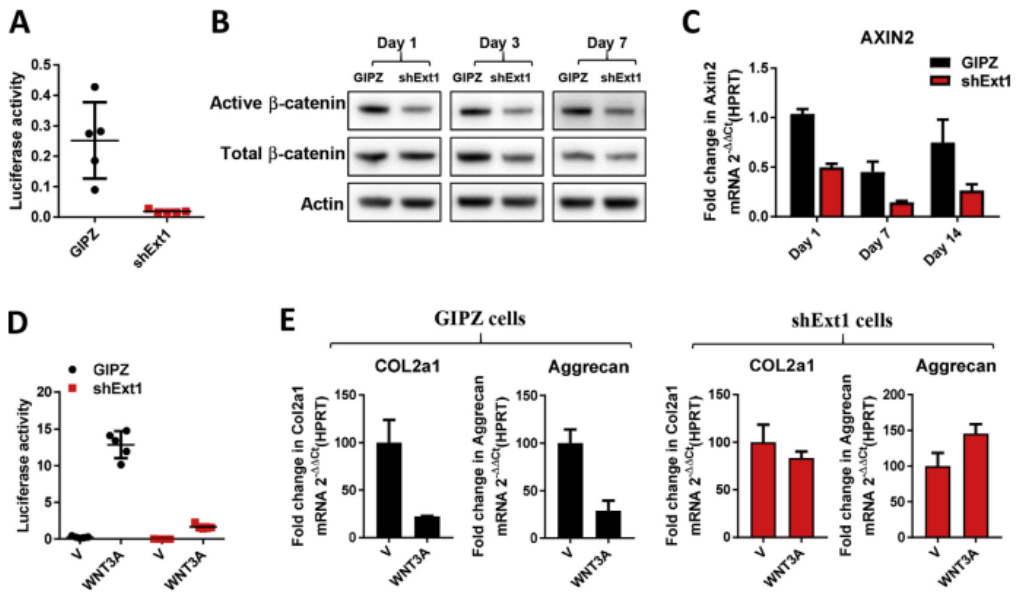


Fig. 2. *Ext1* knock-down limits canonical Wnt signaling activation in ATDC5 cells. (A) TOP/FOP luciferase reporter assay showing reduced Wnt/β-catenin activation in shExt1cells (p=0.0032 by t-test). (B) Western Blot analysis showing decreased active-β-catenin expression in shExt1cells at 3 different time points compared to control cells. Actin was set as loading control. (C) mRNA expression of Wnt target gene *Axin2* (p=0.0247 for silencing vs. control by two-way ANOVA). (D) TOP/FOP luciferase reporter assay in shExt1 cells showing less response than control cells upon exogenous Wnt signaling stimulation with recombinant WNT3A ($p<0.0001$ Bonferroni-corrected for 6 tests by two-way ANOVA). (E) Loss of chondrogenic markers *Col2a1* and *aggrecan* upon recombinant WNT3A treatment in control cells (left panel) is prevented in shExt1cells (right panel) (p=0.0154 and p=0.0164 Bonferroni-corrected for two tests by two-way ANOVA). Vehicle (V) expression was set as 100 %. Data shown are from one established cell line representative of at least three independent biological replicates except for 2E (n=1 cell line). Error bars indicate mean±SD of five (2A-2D), two (2C) and three (2E) technical replicates per experiment.

Ext1 overexpression reduces chondrogenic markers and proteoglycan deposition in ATDC5 cells

Next, we explored the effects of *Ext1* gain-of-function during chondrogenic differentiation. To this end, we established stable transfectants of ATDC5 cells where *Ext1* was overexpressed (*Ext1*+). Relative *Ext1* mRNA levels were determined in control cells expressing pCMV6-entry vector compared to *Ext1*+ cells after 1, 7 and 14 days of differentiation culture [Fig. 3(A)]. *Ext1* mRNA levels were increased in *Ext1*+ compared to control cells (difference between means 1.092 (95%CI: 0.2059-1.977) at day 1, 1.376 (95%CI: 0.9304-1.821) at day7, 4.005 (95%CI: 3.312-4.698) at day 14 in the presented cell

line, representative of 3 distinct colony-expanded clones). Then, we evaluated the impact of *Ext1* overexpression on HS content. As expected, we detected enhanced HS concentration in *Ext1*+ compared to control cells (114.6 ng/mg (95%CI: 76.9-152.2) and 133.9 ng/mg (95%CI: 103-164) for cell lysate and rest fraction) [Fig. 3(B)]. As opposed to shExt1, *Ext1*+ cells displayed a slight reduction in chondrogenic differentiation markers *Sox9* (0.09 difference between means (95%CI: 0.02-0.17) in the presented cell line, representative of 3 distinct colony-expanded clones) and *Col2a1* (4.9 difference between means (95%CI: 3.3-6.5) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 3(C)]. No changes were detected in *Aggrecan* expression in *Ext1*+ cells compared to controls in these conditions. Total proteoglycan content appeared to be reduced in *Ext1*+ cells, assessed by Alcian blue staining (on average 0.06 units (95%CI: 0-0.11)) [Fig. 3(D)] and by DMMB assay (0.32 difference between the means (95%CI: 0.20-0.43)) [Fig. 3(E)] in the presented cell line, representative of at 2 and 3 distinct colony-expanded clones, respectively. Interestingly, *Ext1*+ cells treated with sulfate synthesis inhibitor sodium chlorate ($NaClO_3$), which prevents effective HS biosynthesis [94-96], did not exhibit reduced total proteoglycan content [Fig. 3(F)]. Overall, these results indicate that excessive EXT1 function impairs chondrogenic differentiation.

Ext1 overexpression enhances canonical Wnt signaling activation during chondrogenesis

Then, we determined whether *Ext1* overexpression affects Wnt/β-catenin signaling activation. First, we evaluated luciferase activity in Wnt-reporter transfected cells, both in basal conditions and upon recombinant WNT3A stimulation. In non-stimulated conditions, we detected no differences in Wnt/β-catenin signaling activation between *Ext1*+ and control cells [Fig. 4(A)]. After WNT3A treatment, *Ext1*+ cells showed on average 14.18 units higher luciferase activity than controls (95%CI: 6.84-21.2) in the presented cell line, representative of 2 distinct colony-expanded clones. *Ext1*+ cells treated with $NaClO_3$ to block effective HS biosynthesis, did not show higher Wnt-reporter activity than control cells [Fig. 4(A)], indicating that differences in Wnt/β-catenin signaling activation rely on changes in HS content. Consistent with these findings, we detected higher protein levels of β-catenin in *Ext1*+ than control cells, assessed by Western Blot analysis [Fig. 4(B)].

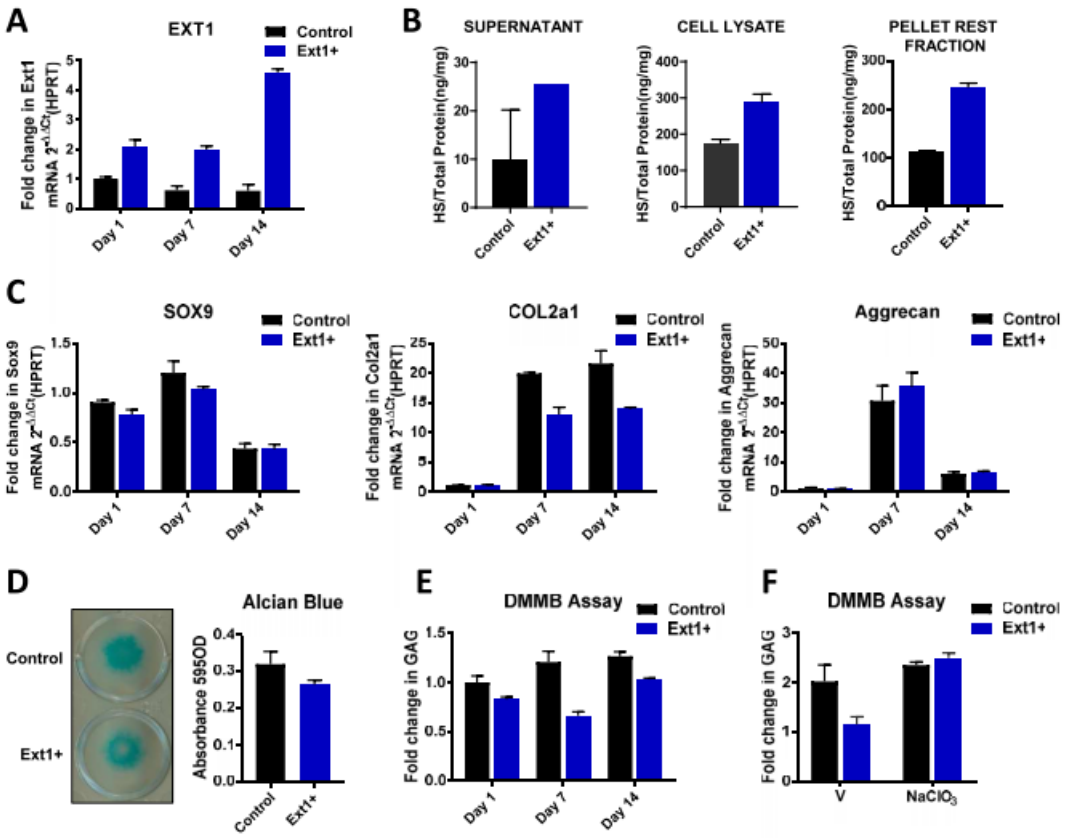


Fig. 3. *Ext1* overexpression reduces chondrogenic differentiation in ATDC5 cells. (A) Quantitative RT-PCR analysis of ATDC5 clones stably-transfected with control pCMV6-entry vector (controls) or with pCMV6-*Ext1* ORF (Ext1+) showing successful overexpression of *Ext1* in Ext1+ cells at day 1, 7 and 14 ($p<0.0001$ for interaction between overexpression and time by two-way ANOVA). (B) Heparan Sulfate ELISA assay showing HS production in Ext1+ cells and in controls (in cell lysate ($p=0.001$ by t-test) and in pellet rest fraction ($p=0.0027$ by t-test). (C) mRNA expression of chondrogenic markers *Sox9* ($p=0.0107$ for overexpression vs. control in two-way ANOVA), *collagen 2a1* (*Col2a1*) ($p=0.0150$ for interaction between overexpression and time in two-way ANOVA) and *aggrecan* ($p=0.32$ and 0.31 for overexpression vs. control and for interaction), in Ext1+ cells compared to controls. (D) Alcian blue staining showing reduced total proteoglycan deposition in Ext1+ cells during chondrogenesis ($p=0.059$ by t-test). Left panel: representative image of staining at day 14 is shown. Right panel: quantification of staining by colorimetry at day 14. (E) DMMB assay showing reduced total glycosaminoglycan content normalized to DNA content in Ext1+ cell lysates ($p<0.0001$ for interaction between overexpression and time in two-way ANOVA). (F) Treatment with sodium chlorate ($NaClO_3$), a selective chemical inhibitor of HS biosynthesis, prevents depletion of glycosaminoglycans in Ext1+ cells, assessed by DMMB assay of the cell lysates ($p=0.0012$ Bonferroni-correct for two tests in two-way ANOVA). Data shown are from one established cell line representative of at least three independent biological replicates, except for 3B (n=1 independent clone), 3D and 3F (n=2 independent clones). Error bars indicate mean±SD of three technical replicates per condition.

We also determined higher mRNA levels of Wnt direct target gene *Axin2* in *Ext1*+ cells than in controls (0.15 difference between the means (95%CI: 0.06-0.25) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 4(C)]. To investigate whether changes in Wnt/β-catenin signaling activation in *Ext1*+ cells contribute to impaired chondrogenesis, we treated these cells with Wnt antagonist XAV939 and determined total proteoglycan content as indicator of chondrogenic differentiation status [Fig. 4(D)]. XAV939 is a specific tankyrase inhibitor that stimulates beta-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex [97]. Wnt inhibition prevented the reduction in total proteoglycan content in *Ext1*+ cells. Thus, all these data demonstrate that EXT1 gain-of-function enhances the strength of canonical Wnt signaling and negatively affects chondrogenic differentiation.

Canonical Wnt signaling regulates *Ext1* expression

Next, we investigated whether Wnt/β-catenin signaling reciprocally regulates *Ext1* expression. Pharmacological activation of Wnt/β-catenin signaling using CHIR-99021, a GSK3 inhibitor, resulted in a concentration-dependent down-regulation of *Ext1* expression in ATDC5 cells (difference between means 0.32 (95%CI:0.073-0.566) and 0.39 (95%CI:0.145-0.6387) for dosage 3 and 10 μM respectively) in the presented cell line, representative of 2 distinct colony-expanded clones [Fig. 4(E)]. Conversely, Wnt inhibitor XAV939 up-regulated *Ext1* expression (difference between means 0.25 (95%CI: 0.01-0.5)). These data, in combination with results reported above, suggest the existence of a regulatory loop between EXT1 and Wnt/β-catenin signaling during chondrogenesis [Fig. 4(F)].

Finally, we determined whether Wnt/β-catenin signaling also regulates EXT1 expression in human articular cartilage. Recombinant WNT3A treatment down-regulated *Ext1* mRNA expression, in a concentration-dependent manner, in human articular chondrocytes (difference between means 0.445 (95%CI: 0.09367-0.7978), 0.5080 (95%CI: 0.1143-0.9016), 0.6022 (95%CI: 0.2501 to 0.9543) for 10, 100, 1000 ng/ml WNT3A respectively [Fig. 4(G)]. These insights suggest that Wnt/β-catenin pathway also regulates Ext1 in human articular cartilage.

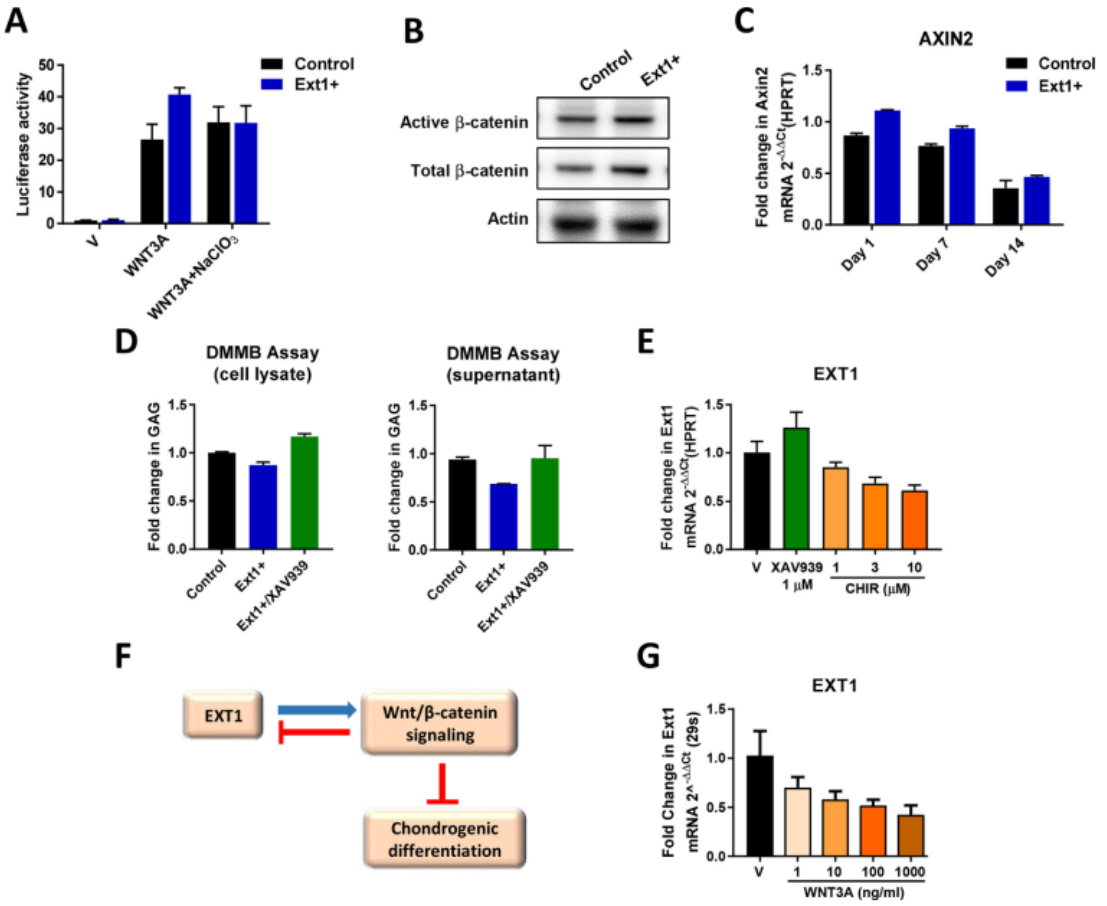


Fig. 4. *Ext1* overexpression enhances canonical Wnt signaling activation in ATDC5 cells. (A) TOP/FOP luciferase reporter assay in Ext1+ and control cells upon treatment with vehicle (V), recombinant WNT3A with or without $NaClO_3$ or $NaClO_3$ (p=0.0002 Bonferroni corrected for three tests by two-way ANOVA). (B) Western Blot analysis showing higher β-catenin protein expression in Ext1+ cells compared to control cells. Actin was set as loading control. (C) mRNA expression of Wnt target gene *Axin2* (p=0.011 for overexpression vs. control by two-way ANOVA) in Ext1+ cells during chondrogenic differentiation. (D) Treatment with Wnt inhibitor XAV939 restores glycosaminoglycan content in Ext1+ cells, assessed by DMMB assay (p=0.0015 and < 0.0001 Bonferroni-corrected for three tests in one-way ANOVA in cell lysate, p=0.075 in one-way ANOVA for supernatant). (E) mRNA expression of *Ext1* upon treatment with V, Wnt activator CHIR99021 (CHIR) and Wnt antagonist XAV939 (p=0.04, 0.0112 and 0.0028 for XAV939, CHIR 3 μM, CHIR 10 μM, Bonferroni-corrected for 4 tests by one-way ANOVA). (F) Model for the regulatory loop between EXT1 and Wnt/β-catenin signaling in chondrogenesis. (G) mRNA expression of *Ext1* in human articular chondrocytes upon treatment with vehicle (V) and recombinant WNT3A (1, 10, 100 and 1000 ng/ml) (p=0.075, 0.014, 0.012 and 0.002 Bonferroni-corrected for 4 tests by one-way ANOVA). Data shown are from one established cell line representative of 1 (4B), 2 (4A, 4D and 4F), 3 (4C) independent biological replicates and from one non-OA donor (4G). Error bars indicate mean±SD of three technical replicates per condition.

In this study, we report that EXT1, by modulating HS-proteoglycan content, affects the differentiation potential of ATDC5 chondroprogenitor cells, and these effects are in part attributable to modifications in the strength of Wnt/β-catenin pathway activation. Reciprocally, Wnt/β-catenin signaling regulates the expression of *Ext1*. Thus, our data suggest the existence of a regulatory loop between EXT1 and the Wnt/β-catenin signaling during chondrogenesis [Fig. 4(F)].

EXT1 and HS content affect chondrogenic differentiation potential of precursor cells

The importance of EXT1 and HS content in skeletal development and chondrogenic differentiation has been shown in several previous studies. In 2005, Hilton *et al*. analyzed full heterozygous *Ext1*-deficient mouse embryos and found enhanced chondrocyte proliferation and delayed hypertrophic differentiation in the mutant long bones, together with alterations in gene expression and protein distributions of molecules of the Indian Hedgehog pathway [98]. In another study, Matsumoto *et al*. ablated *Ext1* in limb bud mesenchyme using the *Prx1-Cre* driver [88]. These conditional *Ext1* mutant mice displayed severe limb skeletal defects, including shortened and malformed limb bones, oligodactyly, and fusion of joints. In developing limb buds of these mutant mice, chondrogenic differentiation of mesenchymal condensations was delayed and impaired. In this case, alterations in BMP signaling were reported. In another study, Pacifici and co-workers analyzed conditional *Ext1* ablation in perichondrium and lateral chondrocytes flanking the epiphyseal region of mouse embryo long bones [99]. In line with our results, they observed increased chondrogenic potential in these *Ext1*-deficient mice, and they found enhanced BMP signaling. In 2017, Inubushi *et al.* generated a model of multiple hereditary exostoses in which EXT1 was ablated in progenitor cells in the perichondrium, but not in chondrocytes in the growth plate [100]. Again, they reported enhanced chondrogenic differentiation with development of osteochondromas in the mutant mice, in this case associated to changes in BMP signaling.

EXT1 and HS content impact the strength of the Wnt/β-catenin pathway

We found that defects in *Ext1* and the resulting reduction in HS lead to reduced Wnt/β-catenin pathway activation, and the opposite was observed when *Ext1* was overexpressed. To our knowledge, mechanistic evidence linking EXT1 and HS to Wnt/β-catenin signaling during chondrogenesis was missing. It is well established that the canonical Wnt signaling pathway regulates chondrogenesis in precursor cells [101]. Consistent with our data, low Wnt signaling activation promotes a chondrogenic differentiation program, while high Wnt signaling reduces chondrocyte differentiation [101].

In agreement with our results, mutations in *tout-velu*, the Drosophila orthologue of *Ext1*, disrupt Wingless signaling [102]. Some studies in *Drosophila* also revealed connections between HSPGs and Wnt signaling. Genetic analyses of *Drosophila* mutants defective in the core proteins or in GAG biosynthesis indicated the requirement of HSPGs for Wnt signaling spreading [103]. Experiments showing that Wg (the best characterized *Drosophila* Wnt) can bind to heparin supports the idea that HSPGs regulate Wnt signaling possibly by maintaining the local concentration of Wnt proteins on the cell surface [104]. On the other hand, since Wg fails to move across a clone of cells defective in the synthesis of HSPGs, HSPGs also regulates the restrictive movement of Wnt in the extracellular space [103]. Here, we report that treatment with sodium chlorate that blocks N-sulfation of the GAG chains of HS, rescued the phenotype of *Ext1* overexpressing cells. In line with these findings, a *Drosophila* mutant defective in an enzyme required for N-sulfation of the GAG chains of HS exhibits impaired distribution and signaling of Wg [105]. Thus, HS modification by N-sulfation appears to be important in the regulation of Wnt activity during chondrogenesis.

Activation of Wnt signaling measured by a luciferase reporter assay without exogenous stimulation was very low in both *Ext1* overexpressing and control cells. Yet, a small increase was seen in total and active beta-catenin levels. This apparent discrepancy could be partially explained by the different techniques applied, the level of analysis (reporter gene expression vs. protein levels) but also because the overexpression model may have limited biological impact on endogenous Wnts as the amount of HSPGs may be sufficient to activate the cascade. This may not only apply to the reporter assay but also to other

results in the overexpression studies where effect sizes were small compared to the knock-down experiments.

The regulatory loop between EXT1/HS and Wnt/β-catenin signaling that our study identified during chondrogenic differentiation may also play a role in osteoarthritis (OA) as this loop also appears to be present in human articular chondrocytes, but additional studies are required to provide clear evidence on this assumption. EXT1 may contribute to hyper-activation of the Wnt signaling pathway, a deleterious event for cartilage homeostasis and important mechanism in the pathogenesis of OA [31].

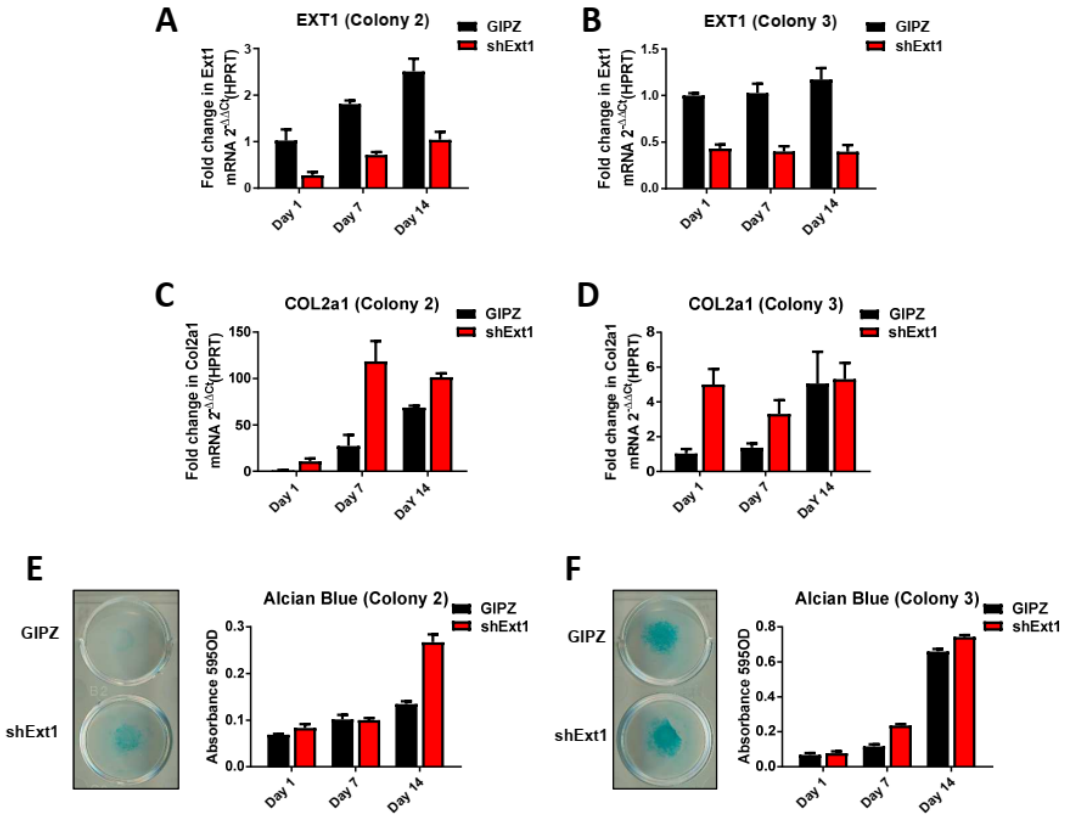
Our observations are currently restricted to an *in vitro* setting with several limitations. Overexpression and knock-down studies as presented here suffer from variation both within the same clone as in between cell lines. Hence, the biological relevance of the observed differences in the different *in vitro* experiments will need to be further assessed in more complex *ex* or *in vivo* models. Therefore, future studies are required to further demonstrate the presence of this regulatory mechanism in articular cartilage and its possible implications in OA.

Contributions

XW, SM and RJL were involved in study design, analysis of data and writing the manuscript. XW and FMFC were involved in data acquisition. All authors were involved in interpretation of data and revising the article and approved final content of the article.

Supplementary Table 1

Primer name	Sequence
mHPRT1 Fw	TGCTGACCTGCTGGATTACA
mHPRT1 Rv	TAGTCAGTTGCCCCCTGTAT
mExt1 Fw	AGCACAAGGATTCTCGCTGT
mExt1 Rv	CAAAGCCTCCAGGAATCTGA
mSox9 Fw	ATGAATCTCCTGGACCCCTT
mSox9 Rv	CTTCCTCGCTCTCCCTCTTC
mCol2a1 Fw	CTGCCAGTGGAAAATTAGGG
mCol2a1 Rv	TTCTCCCTTGTCACCACGAT
mAggrecan Fw	GCTGCAGTGATCTCAGAAGAAG
mAggrecan Rv	GATGGTGAGGGAAGACCCTA
mAxin2 Fw	GAGTAGCGCCGTGTTAGTGACT
mAxin2 Rv	CCAGGAAAGTCCGGAAGAGGTATG
h29S Fw	CCAGCAGCTCTACTGGAGTCA
h29S Rv	GCCTATGTCCTTCGCGTACT
hExt1 Fw	GCTCTTGTCTCGCCCTTTTGT
hExt1 Rv	TGGTGCAAGCCATTCCTACC



Suppl. Fig. 1. Independent *Ext1* knock-down colonies show similar increases in chondrogenic differentiation. (A, B) Quantitative RT-PCR analysis in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (GIPZ) and in cells transfected with shmiRNA against *Ext1* (shExt1), showing successful knock-down (KD) of *Ext1* at day 1, 7 and 14 in shExt1 cells (p=0.024 (A) and p<0.0001 (B) in two-way ANOVA for silencing vs. control). (C, D) *Ext1* KD increases mRNA expression of chondrogenic marker *collagen 2a1* (*Col2a1*) (p<0.001 (C) and p=0.032 (D) by two-way ANOVA for interaction between silencing and time). (E, F) Alcian blue staining showing increased total proteoglycan deposition in shExt1 cells during chondrogenesis (p<0.0001 (E, F) by two-way ANOVA for interaction between silencing and time). Left panel: representative image of staining at day 14 is shown. Right panel: quantification of staining by colorimetry. Data shown are from two different established cell lines (colony 2 and colony 3) that are independent from colony 1, which is shown as representative of the data in the main manuscript. Error bars indicate mean±SD of three technical replicates per condition.

Chapter 4 ANP32A Regulates Wnt Signaling in Articular Cartilage

Abstract

Objective: Previous work showed that ANP32A protects against cartilage damage by limiting oxidative stress through regulation of ATM expression. However, anti-oxidant treatment only partially rescues joint damage in *Anp32a KO* mice with induced osteoarthritis. A yeast two-hybrid screening suggested the presence of protein- protein interaction between ANP32A and Wnt signaling components. Therefore, we hypothesized that Wnt signaling may also play a role in the effect of ANP32A on cartilage homeostasis and disease.

Methods: Microarray data of articular cartilage from *Anp32a KO* mice were analysed to predict upstream regulators. *Anp32a* was stably knocked-down in ATDC5 chondro-progenitor cells cultured as micromasses, or silenced in human articular chondrocytes (hACs). Markers of chondrogenesis *Col2a1, Aggrecan*, *Col10a1* and Wnt target genes were measured by RT-qPCR. Proteoglycan content was evaluated by Alcian blue staining. Canonical Wnt signaling activation was checked by TOP/FOP assay. ATDC5 cells and human articular chondrocytes were treated with recombinant WNT3A or WNT inhibitor XAV939. Co-immunoprecipitation and immunofluorescence of hACs were performed to study protein interactions and subcellular location of ANP32A. TCF1 levels in cartilage samples from gene knockout mice were examined by immunohistochemistry.

Results: Transcriptomic analysis in *Anp32a KO* mouse cartilage suggested that ANP32A regulates the Wnt signaling. Anp32a deficiency in ATDC5 micromasses resulted in decreased chondrogenic markers and proteoglycan accumulation, and Wnt hyper-activation. In human and mouse articular cartilage, Anp32a deficiency also led to hyper-activation of Wnt signaling. Mechanistic studies demonstrated that Wnt signaling activation leads to ANP32A cytoplasmic switch in Wnt protein partners and nuclear translocation.

Conclusions: Collectively, this work reveals that ANP32A regulates two central networks in cartilage biology and osteoarthritis: the Wnt signaling and the Atm/oxidative stress axis.

Keywords: ANP32A, Wnt signaling, Chondrogenesis, Cartilage

Osteoarthritis (OA) is the most common chronic joint disease worldwide and has a tremendous societal burden [3]. It is characterized by cartilage degradation, bony changes and synovial inflammation, leading to joint space narrowing, pain, and loss of function. Current therapeutic approaches are limited and no disease-modifying OA drugs (DMOADs) have been approved yet.

The Wnt signaling pathway is highly conserved in different species and plays critical roles not only in skeletal and embryonic development, but also in cartilage homeostasis and the pathophysiology of OA [4]. Studies already showed that Wnt signaling affects mesenchymal progenitor cell proliferation, chondrocyte differentiation and function. It has also been generally accepted that cartilage degradation, as well as chondrocyte hypertrophy and joint inflammation are associated with excessive activation of canonical Wnt signaling [43]. Canonical Wnt signaling is also called the β-catenin-dependent Wnt signaling pathway [40]. For its activation, Wnt ligands form a complex with the cell-surface receptor Frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP) 5/6, thereby inhibiting the cytoplasmic β-catenin destruction complex. The β-catenin destruction complex consists of the Ser/Thr kinases glycogen synthase kinase 3 (GSK-3) and casein kinase 1 (CK1), the scaffolding protein Axin, the adenomatous polyposis coli (APC) protein, and the E3-ubiquitin ligase β-TrCP. Protein phosphatase 2A (PP2A) also associates with the complex. Inhibition of the destruction complex upon Wnt pathway activation allows β-catenin to accumulate and translocate to the nucleus to interact with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors in order to activate its target genes.

Our group reported that acidic leucine-rich nuclear phosphoprotein-32A (ANP32A) prevents oxidative stress in cartilage via promoting the expression of ataxia-telangiectasia mutated (ATM) gene [72]. Antioxidant treatment improves the outcome of a surgical model of OA in *Anp32a*-deficient mice, which indicates that ANP32A offers therapeutic opportunities for OA. However, antioxidant treatment only partially rescued the cartilage damage in the mouse model, which indicates the potential existence of a parallel mechanism involved in the effects of ANP32A on OA. Interestingly, a protein-protein

interaction network screening using automated yeast two-hybrid (Y2H) interaction mating suggested interactions of ANP32A with a key component of Wnt signaling, Axin1 [73]. Another study showed that ANP32A can specifically bind to the catalytic C subunit of PP2A and can regulate its activity [74]. All things considered, interactions with the canonical Wnt signaling pathway could therefore be the additional regulatory mechanism by which ANP32A affects OA development.

Materials and Methods

Patient materials

Human articular chondrocytes were isolated from the hips of patients undergoing total hip replacement surgery. The University Hospitals Leuven Ethics Committee and Biobank Committee approved the study (S56271) and specimens were taken with patients' written consent. Under Belgian Law and UZ Leuven's biobank policies, the hip joints are considered human biological residual material. Only age and sex of the patients are being shared between the surgeons and the investigators involved in this study. The material is fully anonymized without links to the medical file. Upon arrival of the specimen, these non-osteoarthritis hips were macroscopically evaluated to exclude signs of osteoarthritis.

Mice

Anp32a KO (*Anp32a*^{tm1Hzo}) mice were a kind gift from Dr. P. Opal (Northwestern University Medical School, Chicago, USA) [106] and were backcrossed onto the C57Bl/6J background. In the experiments reported here, mice were at least in the 11th generation of backcrossing. Wild-type *Anp32a*$^{+/+}$ littermates or wild-type C57Bl/6J, purchased from Janvier (Le Genest St Isle, France), were used as controls. Mice with normal immune status were housed in groups of 4-5 mice in Static micro-insulator cage with Macrolon filter with bedding material (composed of spruce particles of approximately 2.5 - 3.5 mm, type Lignocel® BK 8/15), under conventional laboratory conditions (14h light – 10h dark; 23+/-2°C), with standard mouse chow food (Sniff, Soest, Germany) and water provided *ad libitum*. All studies were performed with the approval from the Ethics Committee for Animal Research (P114-2008, P198-2012, P159-2016; KU Leuven, Belgium) (Licence number LA1210189).

Mouse genotyping

Genotypes of animals were confirmed by polymerase chain reaction (PCR). Genotyping primers were designed to distinguish *Anp32a*$^{+/+}$, *Anp32a*$^{+/-}$ and *Anp32a*$^{-/-}$ alleles: fw1 ACAGCAAAGCCTACTGGATA and rev1 ATGTTGGATAAGCACACCTC pair: 401bp (*Anp32a*$^{+}$ allele); fw1 ACAGCAAAGCCTACTGGATA and rev2 AGGAACCACAGGATGCTTCA pair: 601bp (*Anp32a*$^{-}$ allele). PCR reactions were performed as described [106]**.**

Histology of the mouse joint

Mice were sacrificed, and tibia and knees were fixed in 2% formaldehyde overnight at 4°C; decalcified for 3 weeks in 0,5M EDTA pH 7,5; and embedded in paraffin. Immunohistochemistry was performed on 5µm thick sections. Pictures were taken using a Visitron Systems microscope (Leica Microsystems GmbH) using Spot32 software.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded EDTA decalcified mouse knee sections. First, sections were treated with 3% H_2O_2/water for 10 minutes to inactivate endogenous peroxidase activity. Then, the sections were blocked in normal goat serum for 30 minutes and incubated overnight at 4°C with the primary antibodies against TCF1 (Abcam; ab96777). After incubation with the primary antibody, 1:100 peroxidase goat anti-rabbit IgG (Jackson Immunoresearch, Suffolk, UK) was applied for 30 minutes and peroxidase activity was determined using DAB (Dako, Carpinteria, CA, USA). Rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were used as negative controls. Quantification of the DAB immunohistochemical staining was performed with color deconvolution plugin (Jacqui Ross, Auckland University) in ImageJ Software (NIH Image, National Institutes of Health, Bethesda, MD, USA). Quantification was performed using two technical replicates for 3 different mice per condition, obtained in 3 different staining experiments.

Microarray hybridization and data acquisition

The articular cartilage and subchondral bone from the tibial plateau of the mouse knee joint of 8-week old male mice were carefully dissected in one piece and was homogenized in

TRIZOL (Invitrogen), using the Fastprep-24 tissue-homogenizer (MP Biomedicals, Solon, OH, USA). RNA was isolated using a manual phenol/chloroform extraction protocol. RNA concentration and purity were assessed with a NanoDrop Spectrophotometer (Nano Drop Technologies, Centreville, DE, USA) and integrity (RIN) was determined using RNA nanochips and the Agilent 2100 Bio-analyzer (Agilent Technologies, Diegem, Belgium). Only samples with acceptable quality and integrity (RIN above 7) were selected for the experiment.

Quantitative PCR

Total RNA was isolated using a manual phenol/chloroform extraction protocol or with the NucleoSpin RNA extraction kit (Macherey-Nagel, Filterservices) according to the manufacturer's instructions. cDNA was synthetized using 1 µg total RNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's recommendations. The relative expression level of transcripts was determined by real-time RT-PCR using cDNA as a template with Maxima SYBR Green qPCR Master Mix (Fermentas), following manufacturer's instructions and gene-specific primers on a Corbett Rotor-gene qPCR machine (Qiagen). Relative quantification was obtained with the ΔΔct method using Hprt or 29s as internal control, unless otherwise specified. Primers used in quantitative PCR are listed in supplementary Table 1. Results are reported as means of 3 technical replicates per individual or condition.

ATDC5 micromass cultures

ATDC5 cells were cultured in growth medium (1:1 Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 mix (Gibco)) containing 1% (vol/vol) antibiotic–antimycotic (Gibco), 5% fetal bovine serum (FBS) (Gibco), 10 µg/mL human transferrin (Sigma) and 30 nM sodium selenite (Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. High-density micromass cultures of ATDC5 cells were grown to study chondrogenic differentiation [91]. Cells were trypsinised, washed and resuspended at 2×10^7 cells/mL in a chondrogenic medium containing DMEM-F12 enriched by 1% (vol/vol) antibiotic–antimycotic, 5% FBS, 5 µg/mL human transferrin and 1× ITS (Insulin, Transferrin, Selenite) premix (resulting in 10 µg/mL insulin, 5 µg/mL human transferrin and 30 nM sodium selenite) (Life Technologies). One droplet (10 µL) was carefully placed

in the centre of each well of a 24-well plate. Cells were allowed to adhere for 2 hours at 37°C, followed by addition of 500 µL of chondrogenic medium. Micromasses and supernatants were collected at time points 1, 7 and 14 days for functional assays. For the rescue experiments, cells were treated with N-acetyl-cysteine (NAC) (Sigma) or XAV-939 (Selleckchem) at the indicated concentrations.

Generation of stably knocked-down ATDC5 cell line

Silencing of *Anp32a* was performed using a GIPZ-shRNA construct directed against mouse *Anp32a* (Dharmacon); and a non-interfering GIPZ vector (Dharmacon) was used as a control. ATDC5 cells were transfected using Lipofectamine LTX (Thermo Fisher) and after 24h, selection with 3 µg/ml puromycin (Thermo Fisher) was initiated and continued for 14 days. Three different antibiotic-resistant clonal colonies of each condition were isolated and grown independently. Knock-down efficiency was assessed by quantitative RT-PCR.

Micromass staining

ATDC5 micromasses were washed with PBS and fixed with 95% ice-cold methanol for 30 min at 4°C. After rinsing with water, micromasses were stained with either Alcian Blue (AB) (0.1% AB 8GX (Sigma)), Safranin O (Klinipath), Sirius red (0.1% Direct Red 80 (Sigma) in a saturated aqueous solution of picric acid) or Alizarin Red (AR) (1% AR (Sigma) in water pH 4.2), and washed with water to remove unbound staining and air dried.

Human Articular Chondrocyte Culture and transfection

Healthy articular chondrocytes were obtained from patients undergoing hip replacement for osteoporotic or malignancy-associated fractures. Cartilage was dissected from the joint explant surfaces and then rinsed with PBS. The tissue was cut into small pieces, using a sterile surgical blade. Cartilage explants were incubated with 2 mg/ml pronase solution (Roche) for 90 minutes at 37°C and digested overnight at 37°C in 1.5 mg/ml collagenase B solution (Roche) under continuous agitation. The preparation was filtered through a 70 µM strainer and cells were plated in culture flasks and cultured in a humidified atmosphere at 37°C, 5% CO_2. Culture medium consisted of DMEM/F12 (Gibco), 10% fetal bovine

serum (FBS) (Gibco), 1% (vol/vol) antibiotic/antimycotic (Gibco) and 1% L-glutamine (Gibco).

For small interfering RNA (siRNA) transfection, lipofectamin RNAiMAX (Invitrogen) was used as transfection reagent, together with siGENOME Anp32a siRNA (Dharmacon) or negative control siGENOME siRNA (Dharmacon), following the protocols provided by the manufacturer.

Luciferase assay

Primary human chondrocytes were plated into 96-well plates and after 24 h, cells were transfected with mGFP control plasmid (Origene PS100040) and Super8X TOPFlash or Super8X FOPFlash (TOPFlash mutant control) (Addgene plasmids #12456 and #12457, respectively) for canonical Wnt signaling reporter using Lipofectamine LTX Reagent with PLUS Reagent (Life Technologies), according to the manufacturer's protocol. After 24 h, cells were transfected with *Anp32a* or negative control siRNA, and 24 h later cells were treated with 100 ng/ml rWNT3A and collected 24 h later. Luciferase assay was performed using Promega Luciferase Assay system according to the manufacturer's protocol. Briefly, cells were lysed with 100 µl per well 1 X lysis buffer (E1531) and underwent two freeze-thaw cycles to ensure complete cell lysis. Afterwards, 20 µl of cell lysate was transferred to opaque 96-well plates, and 50 µl of Luciferase Assay Reagent was dispensed into the plate. Luciferase level was measured using Plate-Reading Luminoskan Ascent (Thermo Scientific).

Co-Immunoprecipitation

Co-Immunoprecipitation experiments were performed using the Pierce Co-Immunoprecipitation Kit (Thermo Scientific). Columns were conditioned following the manufacturer's recommendations. Antibody binding to the column was performed using 75 mg of either a mock antibody (donkey anti-goat IgG) as a control or ANP32A antibody (Novus Biologicals, NBP1-97576, clone RJ1). After antibody immobilization, the columns were washed, and 100 mg of the lysate's proteins were incubated overnight at 4°C under constant mixing. After three washings, retained proteins were eluted using 40 µl of Elution

Buffer (Thermo Fisher), and stored at -20°C. Protein complexes were then detected by Western blotting.

Immunofluorescence

Primary human chondrocytes were plated in 8-well glass chamber slides (Thermo) and treated with vehicle or recombinant WNT3A for 4 hours. After incubation, cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton-X-100. Rabbit polyclonal anti-ANP32A antibody (Abcam, ab5992, dilution 1:100) was added incubated overnight at 4°C. Alexa Flour 488-conjugated goat anti-rabbit secondary antibody (Abcam, ab150077, dilution 1:1000) was added and incubated for 1 hr at room temperature. Nuclei were stained with Hoechst 33342 (Thermo, dilution 1:10000). The stainings were imaged by wide-field fluorescence microscopy using an Olympus IX83 inverted microscope (Olympus) equipped with a DP73 camera. Fields were randomly selected, and five images were taken for each condition.

Cell lysis and western blotting

Cells were homogenized in IP Lysis/Wash buffer (Thermo Fisher) supplemented with 5% (vol/vol) Protease Mixture Inhibitor (Sigma) and 1mM phenylmethanesulfonyl (Sigma). After two homogenization cycles (7 s) with an ultrasonic cell disruptor (Microson; Misonix), total cell lysates were centrifuged 10 min at 18,000g, and supernatant containing proteins was collected. The protein concentration of the extracts was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Immunoblotting analyses were performed as described in previous studies [107]. Antibodies against Axin1 (Millipore, 05-1579, clone A5, dilution 1:1000), Active β-catenin (Cell signaling, 8814S, cloneD13A1, dilution 1:1000), Total β-catenin (BD Transduction Laboratories, 610154, clone 14/Beta-Catenin, dilution 1:2000), ANP32A (Abcam, ab189110; dilution 1:1000), total H3 (Abcam, ab1791; dilution 1:10000) and Actin (Sigma, A2066; dilution 1:4000) were used following manufacturer's instructions. The blotting signals were detected using the SuperSignalWest Femto Maximum Sensitivity Substrate system (Thermo Scientific).

Transcriptomic analysis of transcriptomics in *Anp32a KO* mouse cartilage suggests that ANP32A regulates Wnt signaling

To explore whether ANP32A regulates networks in cartilage biology beyond oxidative stress, we took advantage of our previous microarray study comparing gene expression profiles of messenger RNAs (mRNAs) in articular cartilage from *Anp32a* KO mice (mice with a global gene deletion of ANP32A) and wild-type mice [at 8 weeks of age] (geonr: GSE108036) [72]. We sought to identify which signaling cascades drive the gene expression changes in *Anp32a* KO mice. To this end, we performed further analysis using a novel approach, namely Upstream Regulator Analysis, from Ingenuity Pathway Analysis (IPA) software (cutoff $p<0.01$). Pathway analysis of identified regulators classified as "Transcriptional regulators", using PANTHER database, pointed out the p53 signaling pathway, directly linked to ATM, as main enriched pathway mediating ANP32A effects on gene expression programs(Fig.1), corroborating our earlier observations [72]. Strikingly, upstream regulator pathway analysis also suggested modulation of the Wnt signaling to explain the gene expression changes resulting from loss of ANP32A (Fig. 1).

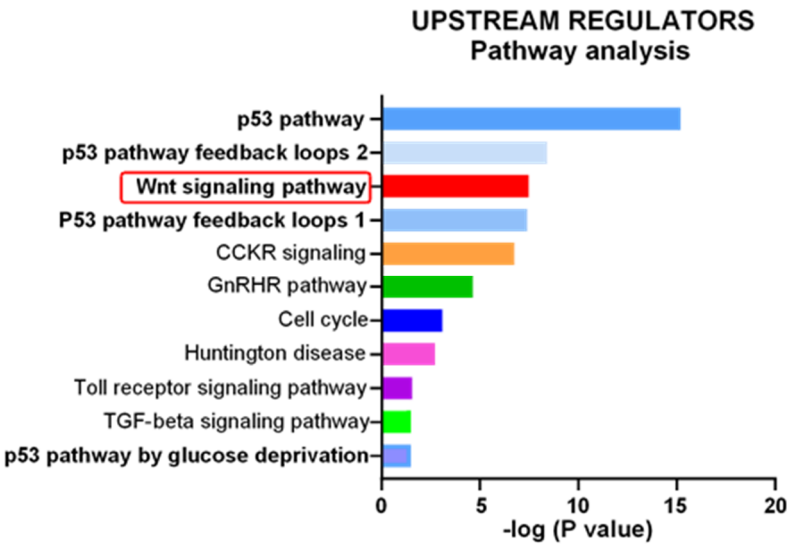


Fig.1 PANTHER pathway analysis of transcriptional upstream regulators identified in analysis of microarray data comparing articular cartilage of 8-week old male *Anp32a*-deficient to wild-type mice (n=4 per group) using Ingenuity Pathway analysis (IPA). ANP microarray data were uploaded in IPA, a cutoff (uncorrected p value ($p<0.01$)) was applied, and filters set for "Transcription regulators". All transcription regulators were listed in PANTHER and PANTHER pathway analysis was performed.

Anp32a deficiency impairs chondrogenic differentiation in ATDC5 micromasses

To interrogate whether ANP32A has a regulatory role on Wnt signaling in cartilage, we first used an *in vitro* model of cartilage differentiation in which Wnt signaling plays a key regulatory role. The ATDC5 cell line, characterized as a chondrogenic cell line, exhibits a multistep process of chondrogenic differentiation when they are seeded as micromasses [90]. In this model, mimicking the transcriptional events that occur in skeletal development, canonical Wnt signaling blocks the differentiation program when it is activated during the early differentiation phase [78,107]. We generated stable *Anp32a* knockdown (KD) ATDC5 cell lines, and micromass cultures were performed to induce a chondrogenic differentiation program. In *Anp32a* KD cells, relative *Anp32a* mRNA levels were effectively down-regulated at all time points studied, compared to control cells (Fig.2A). Then, we measured mRNA expression of early chondrogenic differentiation markers *collagen 2* (*Col2a1)* and *aggrecan (Acan),* and terminal differentiation marker *collagen 10* (*Col10a1)*, using qPCR. Expression of these genes was strongly down-regulated in *Anp32a* KD cells compared to controls (Fig. 2B).

To evaluate proteoglycan and collagen content of the micromasses, we performed Alcian blue, Safranin O and Picrosirius red staining. We observed a reduction in proteoglycans and collagen contents in *Anp32a* KD micromasses compared with controls (Fig. 2C). Mineralization of the micromasses, assessed by Alizarin red staining, was also reduced (Fig. 2C).

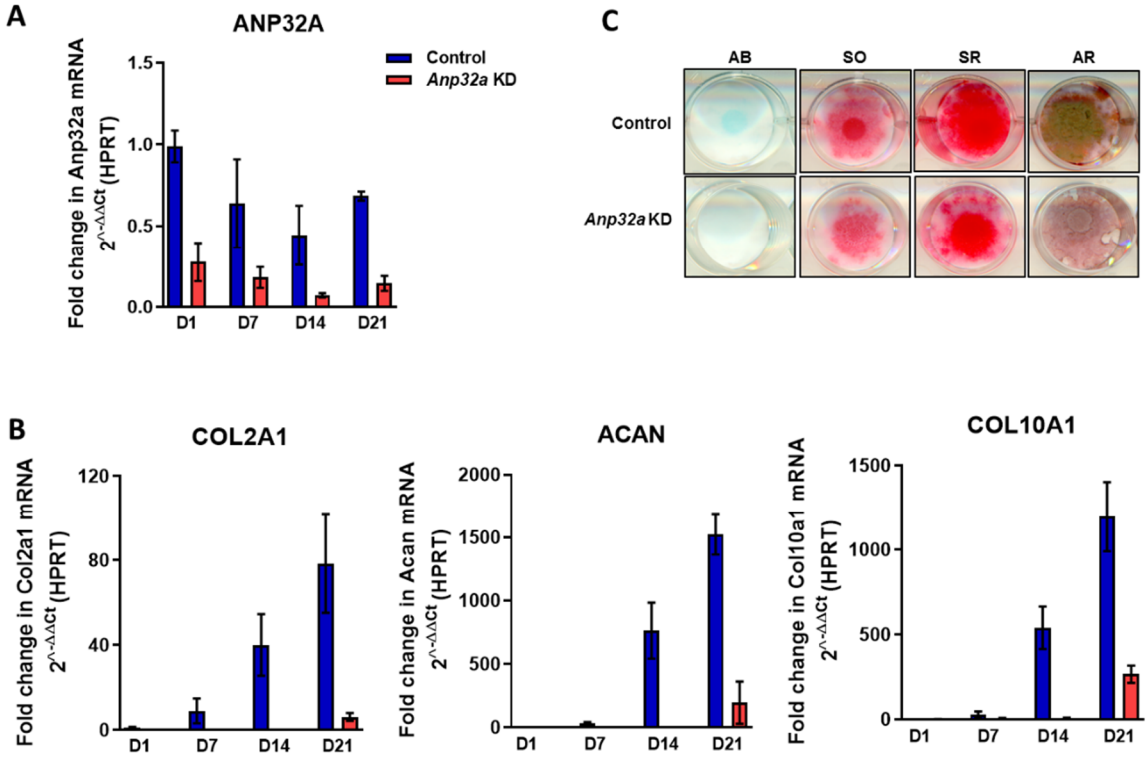


Fig. 2 (A) Real-time PCR analysis in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (GIPZ) and in cells transfected with shmiRNA against *Anp32a* (*Anp32a* KD), showing successful knock-down (KD) of *Anp32a* during the chondrogenic differentiation process ($p = 0.0006$ in two-way ANOVA for silencing vs. control). **(B)** Real-time PCR analysis of chondrogenic differentiation markers collagen 2 (COL2A1), aggrecan (ACAN) and collagen 10 (COL10A1) in control and *Anp32a* knockdown (KD) ATDC5 cells ($p=0.0005$ (COL2A1), $p<0.0001$ (ACAN, COL10A1) in 2-way ANOVA for interaction between silencing and time). **(C)** Alcian blue (AB), safranin O (SO), picrosirius red (SR) and alizarin red (AR) staining showing reduced proteoglycan deposition (AB, SO), collagen content (SR) and mineralization (AR) in Anp32a KD cells during chondrogenesis. Representative image of staining at day 21 is shown. Data shown are from one established cell line. Error bars indicate mean±SD of three technical replicates per condition.

Rescue effects of antioxidant treatment in *Anp32a* Knockdown ATDC5 micromasses.

Expression of *Atm* was only about 50% suppressed in *Anp32a* KD micromasses compared with controls (Fig. 3A). Treatment of *Anp32a* KD micromasses with antioxidant N-

acetylcysteine (NAC) had a low impact on the expression of chondrogenic markers and proteoglycan content (Fig. 3B).

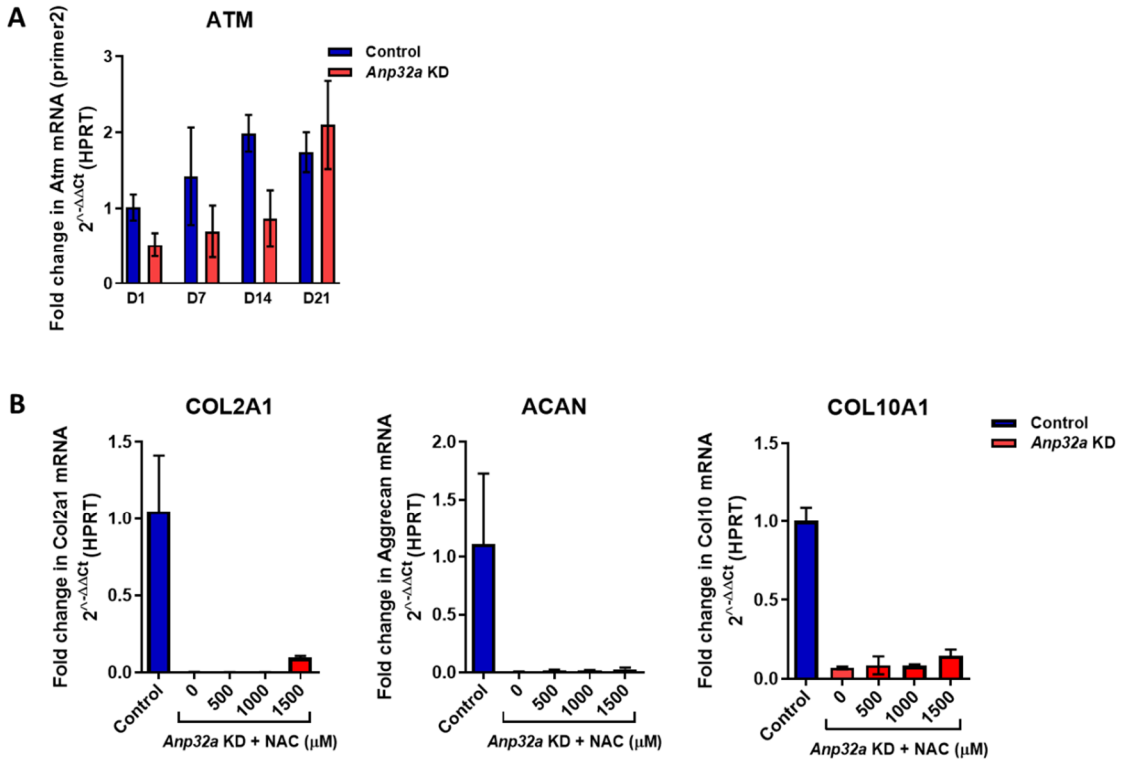


Fig. 3 (A) Real-time PCR analysis of *Atm* in control and *Anp32a* KD ATDC5 cells (p=0.0485 in 2-way ANOVA for interaction between silencing and time). **(B)** Real-time PCR analysis of chondrogenic differentiation markers at day 7 in control and *Anp32a* knockdown (KD) ATDC5 cells after treatment with antioxidant N-acetylcysteine (NAC) at the indicated concentrations. Error bars indicate mean±SD of three technical replicates per condition.

Collectively, these results point out the existence of mechanisms independent on Atm/oxidative stress that lead to impaired chondrogenic differentiation in *Anp32a*-deficient ATDC5 cells.

Anp32a deficiency leads to hyper-activation of Wnt signaling in ATDC5 micromasses

As suggested by the upstream regulator analysis, we investigated Wnt signaling activation in *Anp32a* KD micromasses. Gene expression levels of direct Wnt target genes were up-regulated in *Anp32a*-deficient cells compared to controls during the chondrogenic differentiation process (Fig. 4).

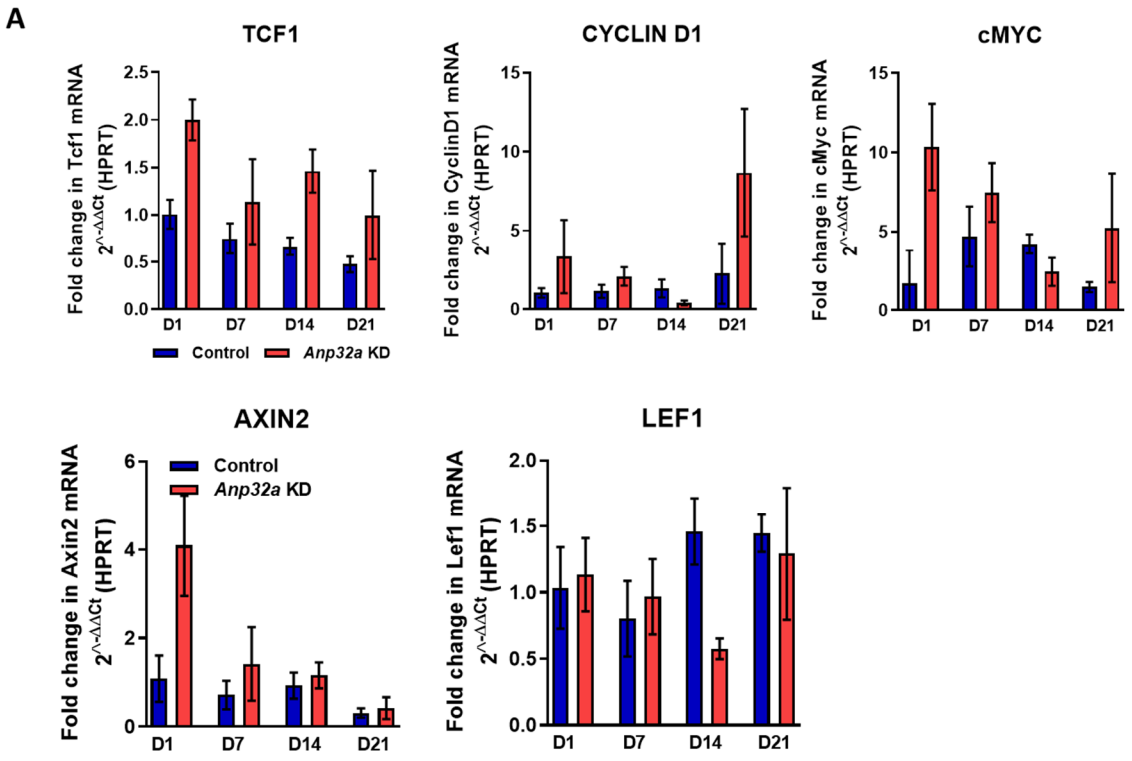


Fig 4. Real-time PCR analysis of Wnt direct target genes Tcf1, CyclinD1, cMyc, Axin2 and Lef1 in control and *Anp32a* KD cells (TCF1: interaction (p=0.1192), group (p=0.0136); CMYC: interaction (p=0.0015), group (p=0.0413); CYCLIN D1: interaction (p=0.0171), group (p=0.0670) in two-way ANOVA for silencing vs. control).

Rescue effects of Wnt inhibitor treatment in *Anp32a* Knockdown ATDC5 micromasses.

Blockade of Wnt signaling with XAV939, a specific tankyrase inhibitor that stimulates beta-catenin degradation [97], showed rescue effects on chondrogenic markers and proteoglycan content in *Anp32a* KD cells (Fig. 5A&B). These observations indicate that Wnt signaling contributes to the detrimental effects of loss of *Anp32a* in this cartilage differentiation model.

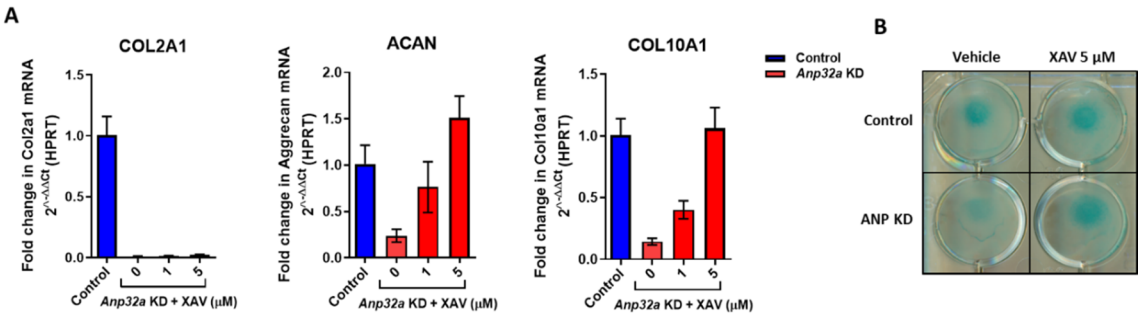


Fig 5. (A) Real-time PCR analysis of chondrogenic differentiation markers collagen 2 (COL2A1), aggrecan (ACAN) and collagen 10 (COL10A1) at day 7 in control and Anp32a knockdown (KD) ATDC5 cells after treatment with Wnt inhibitor XAV-939 (XAV) at the indicated concentrations ($P<0.0001$ (ACAN, COL10A1) Sidak corrected for 6 tests by one-way ANOVA). **(B)** Alcian blue staining at day 14 showing rescue in proteoglycan deposition in *Anp32a* KD cells after XAV treatment. Data shown are from one established cell line representative of at least three independent biological replicates. Error bars indicate mean±SD of three technical replicates per condition.

Anp32a deficiency leads to hyper-activation of Wnt signaling in mouse and human articular cartilage

We then investigated whether Wnt signaling is similarly dysregulated in articular cartilage of *Anp32a* KO mice, both at the gene expression and protein levels. We found that mRNA levels of Wnt direct target genes were up-regulated in *Anp32a* KO mice compared to wild-type littermates (Fig. 6A). Furthermore, protein levels of Wnt target gene TCF1 were also enhanced in articular cartilage from *Anp32a* KO mice, assessed by immunohistochemistry (Fig. 6B). Importantly, in human articular chondrocytes, expression of Wnt target genes was also up-regulated upon siRNA-mediated *Anp32a* silencing (Fig. 6C). In addition, *Anp32a*-silenced cells showed a stronger induction of the Wnt/β-catenin reporter TOP-FLASH than control cells, upon recombinant WNT3A stimulation (Fig. 6D). All these findings indicate that ANP32A negatively regulates the Wnt signaling in articular cartilage.

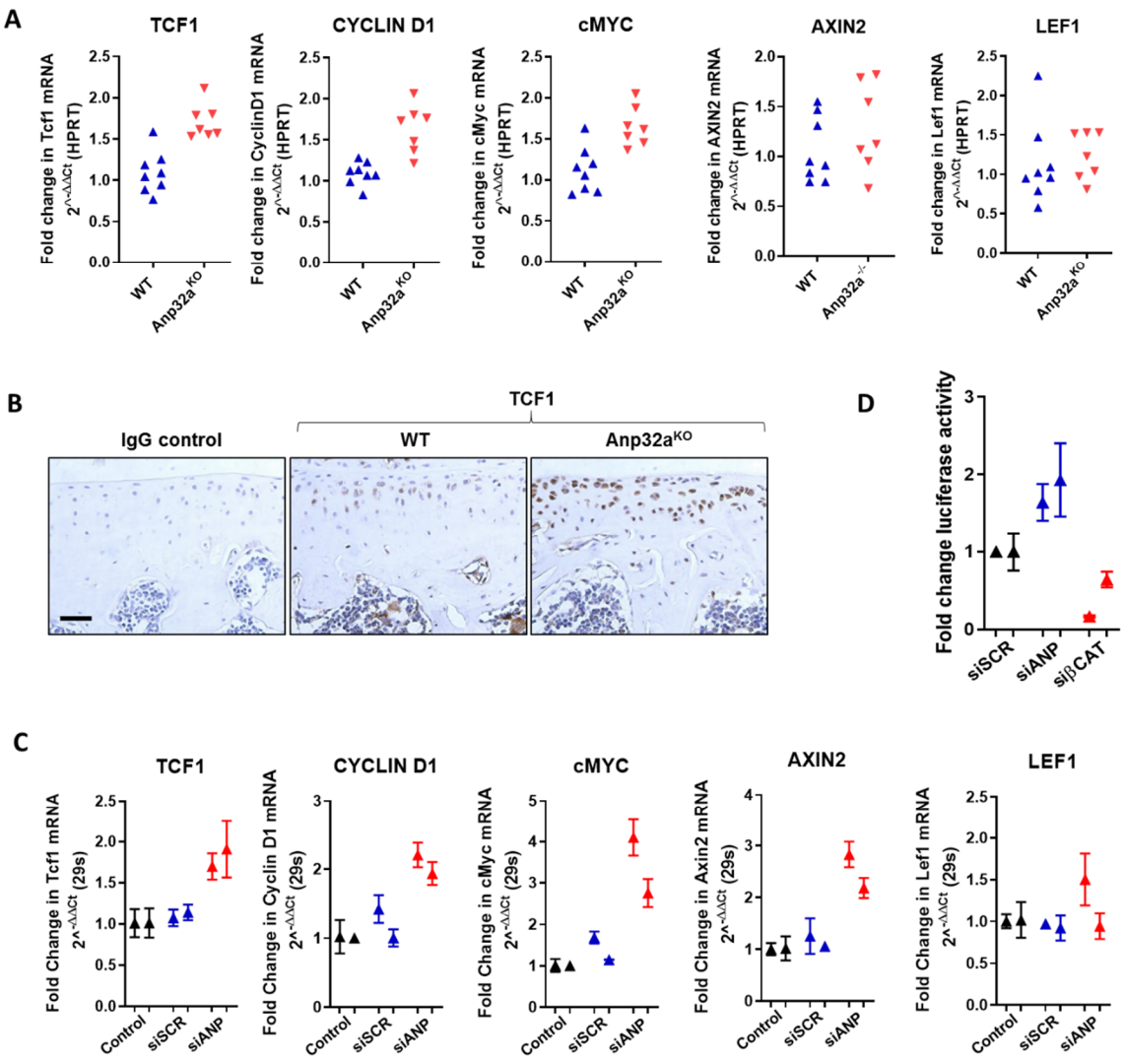


Fig. 6 (A) Real-time PCR analysis of Wnt target gene expression and **(B)** immunohistochemical staining for TCF1 protein expression (n=3) in articular cartilage from 8-week old male WT and *Anp32a*-deficient mice (p=0.0002 (TCF1), 0.0016 (cMyc) by t-test). Scale bar 50 µm. **(C)** Real-time PCR analysis of Wnt target gene expression in human articular chondrocytes transfected with Anp32a (siANP32A) or scrambled siRNA (siSCR) (n=2, error bars indicate mean ± SD of 3 technical replicates). **(D)** TOP/FOP reporter assay in human articular chondrocytes transfected with siANP32A, β-catenin siRNA (siβcat) or siSCR, and then treated with recombinant WNT3A. n=2 biologically independent experiments. P<0.001 by one-way ANOVA.

Wnt signaling leads to ANP32A cytoplasmic switch in Wnt protein partners and nuclear translocation

We next sought to investigate the molecular mechanism via which ANP32A negatively regulates Wnt signaling. The core of the Wnt pathway is the regulation of β-catenin protein

levels by a cytoplasmic destruction complex. In the absence of Wnt signaling, the destruction complex captures cytosolic β-catenin, leading to its phosphorylation by GSK3 and subsequent degradation by the proteasome. Wnt leads to functional inactivation of the destruction complex, resulting in β-catenin accumulation and nuclear entry, which regulates the transcription of Wnt target genes by binding to TCF/LEF transcription factors [22]. Interestingly, a high throughput yeast two-hybrid screening suggested interactions between ANP32A and key components of the Wnt signaling, in particular with Axin1 [73]. We thus investigated whether ANP32A interacts with Axin1 at the protein level in the articular chondrocyte. We carried out co-immunoprecipitations of endogenous ANP32A from extracts of cells treated with recombinant WNT3A ligand or vehicle control. This analysis revealed that ANP32A binds Axin1 in baseline conditions, and that this association is abolished when Wnt signaling is activated with WNT3A (Fig. 7A). These observations prompted us to investigate the functional relevance of the interaction of ANP32A with Axin1 for the cytoplasmic stabilization of β-catenin. Silencing of ANP32A did not affect β-catenin protein levels (active nor total) in human articular chondrocytes (Fig. 7B). These data suggest that ANP32A may regulate Wnt signaling downstream of β-catenin stabilization. Remarkably, the dissociation of ANP32A from Axin1 upon Wnt activation paralleled an enhanced association of ANP32A with β-catenin (Fig. 7A). Then, we investigated whether Wnt activation triggers nuclear translocation and accumulation of ANP32A, as it occurs for β-catenin. To address this question, we monitored the effect of recombinant WNT3A on the subcellular localization of endogenous ANP32A by immunofluorescence, in human articular chondrocytes. ANP32A was mostly cytoplasmic in untreated cells (Fig. 7C), in agreement with our previous data [72]. In contrast, ANP32A was also markedly localized in the nucleus after WNT3A treatment (Fig. 7C). We did not detect a protein-protein interaction between ANP32A and TCF/LEF factors using immunoprecipitation, suggesting that ANP32A regulates Wnt target gene transcription via TCF/LEF-independent mechanisms.

ANP32A is part of the inhibitor of histone acetyltransferase (INHAT) complex [108], which functions in transcriptional repression by binding to histones, preferentially histone 3, sterically inhibiting acetylation [109]. Hypoacetylation of histones is linked to condensed chromatin and transcriptional repression [110]. Wnt-induced ANP32A nuclear

accumulation in human articular chondrocytes (Fig. 7C) was paralleled by interaction of ANP32A with histone 3 (Fig. 7A), indicating that ANP32A may repress Wnt target gene expression via its inhibitory role on histone acetylation as part of the INHAT complex.

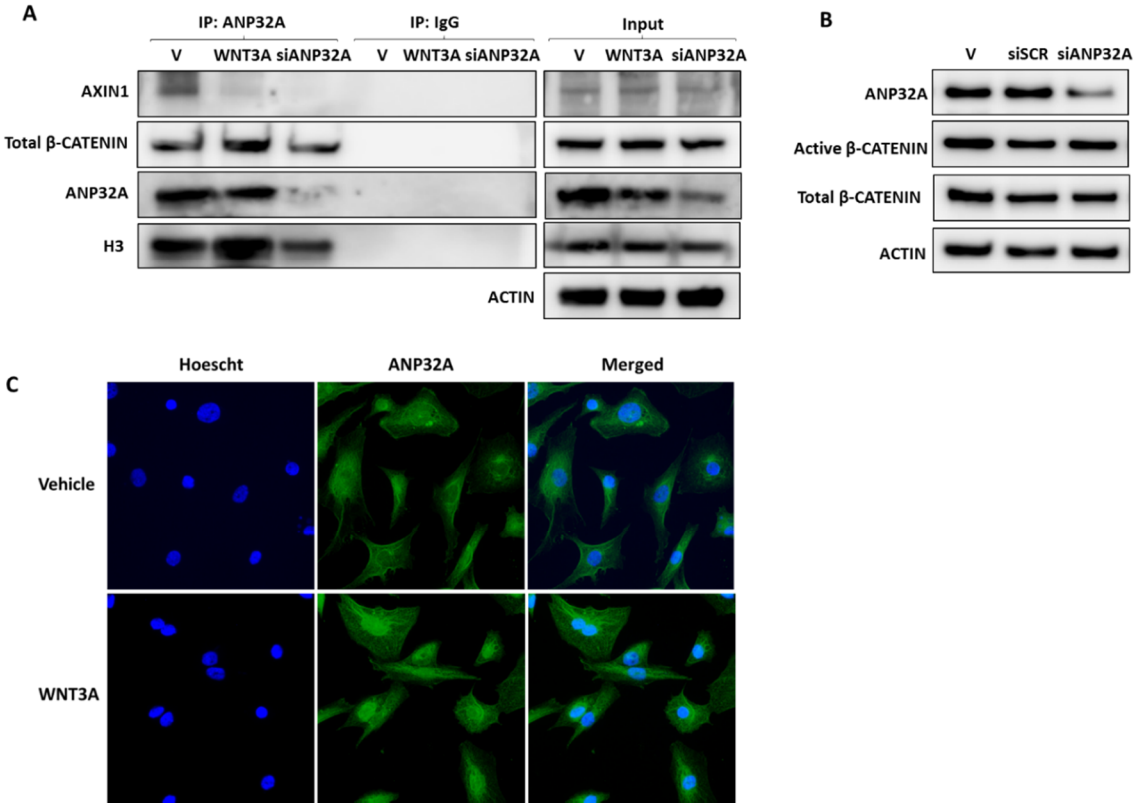


Fig.7 (A) Co-immunoprecipitation (Co-IP) using an anti-ANP32A antibody showing an association between ANP32A and Axin1 in untreated human articular chondrocytes that is abolished upon Wnt activation by recombinant WNT3A protein. Wnt activation increases the association of ANP32A with β-catenin and with histone 3. Silencing of ANP32A (siANP32A) is shown to assess the specificity of ANP32A antibody used in the immunoprecipitation. The image is representative of three independent experiments. **(B)** Immunoblot analysis of ANP32A, active β-catenin and total β-catenin protein expression (with actin as loading control) in human articular chondrocytes transfected with ANP32A (siANP32A) or scrambled siRNA (siSCR). **(C)** Immunofluorescence staining of ANP32A (green) and counterstaining of nuclei with Hoescht (blue) in human articular chondrocytes upon vehicle or recombinant WNT3A treatment (4h). Representative images are shown (n=2). Scale bar, 10 μm.

Discussion

Our findings reveal that ANP32A is a converging node regulating the transcriptional responses of two central cascades in cartilage biology: the Wnt signaling pathway and the

Atm/oxidative stress axis that was previously linked with ANP32A [72]. In this part, we show that ANP32A negatively regulates the transcriptional response triggered by Wnt signaling activation and preserves the homeostasis of articular cartilage. In basal conditions, ANP32A interacts with Axin1, one of the key components of the β-catenin destruction complex in the cytoplasm and it translocates to the nucleus upon Wnt activation upon binding with β-catenin. Hyperactivation of Wnt signaling in articular cartilage is associated with osteoarthritis. Hence, these novel finding of a link between ANP32A and Wnt signaling strengthens the importance of ANP32A in the endogenous protection against the development of OA.

We show that in the absence of ANP32A, Wnt signaling is hyperactivated in an *in vitro* model of cartilage differentiation, and in mouse and human articular cartilage. The Wnt signaling pathway has been extensively implicated in the pathogenesis of osteoarthritis [31,111]. A fine-tuned balance of Wnt signaling pathway is considered critical and indispensable for homeostasis of the articular cartilage and the synovial joint. Either excessive or lack of Wnt activity can lead to cartilage breakdown and OA in animal models. In humans, polymorphisms in genes involved in the Wnt signaling -particularly in the genes encoding extracellular inhibitor *FRZB* and epigenetic modulator *DOT1L*- are associated with an increased susceptibility to the development of OA [112,113]. In rodent models, loss of molecules that suppress Wnt signaling triggers osteoarthritis [38,52,53]. Furthermore, mechanical injury and inflammation are demonstrated to be potent inducers of Wnt in cartilage [51,114]. All this evidence justifies that Wnt signaling is increasingly recognized as a potential target for OA, and clinical trials with Wnt inhibitors are currently underway [5]. Further investigation of Wnt modulators and their underlying mechanisms could offer new translational opportunities to treat cartilage diseases such as OA.

Antioxidants have been successfully used to provide protection for cartilage in translationally relevant clinical settings [115-117]. Also, in our previous study, NAC treatment showed protective effects against OA in *Anp32a*-deficient mice [72]. Recently, a small-molecule Wnt pathway inhibitor which targets intranuclear kinases CDC-like kinase 2 (CLK2) and dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), has entered phase 3 clinical trials, based on earlier phase 2 trial results in

patients with knee OA as well as preclinically in models of chondrogenesis, and in an animal OA model [56,118]. However, combination of antioxidants and Wnt inhibitors for this disease has not been explored until now.

Wnt signaling is critical for joint health and many factors at different levels in the pathway contribute to the complexity of Wnt signaling regulation. For instance, there are many Wnt antagonists that can restrict the activity of Wnt pathway in articular cartilage [31]. Additionally, mutual crosstalk between β-catenin dependent and independent Wnt pathways and competitive antagonism between different Wnt ligands seem to be critical in limiting hyper-activated Wnt pathway [119]. Other factors contributing to Wnt modulation include switches in Wnt transcription factor complexes containing different TCFs and LEFs, epigenetic modulation, in particular by histone modifications, and reciprocal communications between the Wnt signaling pathway and other signaling pathways like Hedgehog signaling.

Here, our co-immunoprecipitation data demonstrated that endogenous ANP32A can bind with Axin1 at the protein level in primary human articular chondrocytes within the cytoplasm in baseline conditions, while Wnt hyper-activity induced by exogenous WNT3A abolishes this interaction. However, silencing Anp32a affected neither total nor active β-catenin expression at protein level which indicate that ANP32A regulate the activation of Wnt signaling at the downstream of β-catenin stabilization. Since an enhanced association between ANP32A and β-catenin was observed upon Wnt activation, we further investigated if ANP32A could translocate into the nucleus with β-catenin when Wnt is activated. This hypothesis was confirmed by immunofluorescence in human articular chondrocytes. A TCF/LEF-independent mechanism may be responsible for the effects of ANP32A on Wnt target genes as no direct protein-protein interactions were detected between ANP32A and TCF/LEF factors. Previous studies indicate that ANP32A's repressive function may be accomplished via its earlier-defined role as part of the INHAT complex, a multiprotein complex that sterically inhibits histone acetyltransferases by binding to histone tails [120-122]. This molecular mechanism contrasts with our previously reported findings for the role for ANP32A as a positive transcriptional regulator of the *Atm* gene and a previous report indicating that ANP32A enhances gene transcription of

interferon-stimulated genes [72,123]. Collectively, these observations indicate that ANP32A can either activate or repress relevant gene expression programs.

In conclusion, our study identifies that ANP32A controls the transcriptional response of Wnt signaling in cartilage, thereby suggesting ANP32A as a therapeutic target for osteoarthritis. This insight, together with our previous report discovering ANP32A as regulator of the antioxidant defense, points out ANP32A as a critical node regulating diverse key networks in the articular chondrocyte. Next step would be getting a deeper understanding on the transcriptional mechanisms by which ANP32A modulates Wnt signaling. As ANP32A expression is reduced in osteoarthritic cartilage [72], further research should focus on the factors that control ANP32A expression. In addition, if co-treatment with Wnt inhibitors and anti-oxidant drugs could have synergic effects on preventing cartilage loss in OA.

Supplementary

Table 1. Primers for qRT-PCR.

mHPRT1 Fw	TGCTGACCTGCTGGATTACA
mHPRT1 Rv	TAGTCAGTTGCCCCCTGTAT
mANP32A Fw	ACGCCCTCTGATGTGAAAGA
mANP32A Rv	CATCCGTGAGGCCTTCGATT
mATM Fw	TCTCCGATATGCCAGTCTTTTCA
mATM Rv	ATACAGACTCAAGGCTGCCC
mCol2a1 Fw	CTGCCAGTGGAAAATTAGGG
mCol2a1 Rv	TTCTCCCTTGTCACCACGAT
mAggrecan Fw	GCTGCAGTGATCTCAGAAGAAG
mAggrecan Rv	GATGGTGAGGGAAGACCCTA
mCOL10A1 Fw	CCTGGTTCATGGGATGTTTT
mCOL10A1 Rv	CAGGAATGCCTTGTTCTCCT
mTCF1 Fw	CCCCGATCTCTCTGGATTTT
mTCF1 Rv	AAGGGGACAGGGGGTAGAG
mCyclinD1 Fw	GCAAGCATGCACAGACCTTT
mCyclinD1 Rv	GCTTCAATCTGTTCCTGGCA
mCMYC Fw	CCTAGTGCTGCATGAGGAGAC
mCYMC Rv	GGTTTGCCTCTTCTCCACAGA
mAxin2 Fw	GAGTAGCGCCGTGTTAGTGACT
mAxin2 Rv	CCAGGAAAGTCCGGAAGAGGTATG
mLEF1 Fw	TCAGTCATCCCGAAGAGGAG
mLEF1 Rv	GGTAGGGCTCCTGAGAGGAC
h29S Fw	GGGTCACCAGCAGCTGTACT
h29S Rv	AAACACTGGCGGCACATATT
hTCF1 Fw	CACCCATCCTCAAAGAGCTG
hTCF1 Rv	ATGTTGTGCTGCTGCAGGTA
hCyclinD1 Fw	TGCTCCTGGTGAACAAGCTC
hCyclinD1 Rv	AGGACAGGAAGTTGTTGGGG
hCMYC Fw	TTTCGGGTAGTGGAAAACCA
hCYMC Rv	GCTCGAATTTCTTCCAGATATCCTC
hAxin2 Fw	GAGTAGCGCCGTGTTAGTGACT
hAxin2 Rv	CCAGGAAAGTCCGGAAGAGGTATG
hLEF1 Fw	CAGTCATCCCGAAGAGGAAG
hLEF1 Rv	AGGGCTCCTGAGAGGTTTGT

Chapter 5 General discussion

Osteoarthritis is the most common chronic joint disease worldwide affecting millions of people and a major cause of morbidity and disability with a huge socio-economic burden. Many efforts have been undertaken to provide an adequate answer to the different challenges presented by this disease. However, there are currently no marked-approved disease-modifying drugs available for OA. Therefore, further understanding of the disease mechanisms involved in the onset and progression of OA is an important research challenge to identify new potential therapeutic targets that can help drug design and development. The β-catenin-dependent Wnt pathway, also called canonical Wnt signaling pathway has been extensively implicated in the pathogenesis of osteoarthritis [31] and is currently considered as one of the key regulators in maintaining homeostasis of cartilage and chondrocyte development. Unbalanced Wnt activity, including either hyper- or hypo-activation, can lead to cartilage breakdown and osteoarthritis. This concept is based on different studies. Most evidence supports the view that hyperactivation of the pathway is associated with increased production of tissue destructive enzymes and with a phenotypic shift of the articular chondrocyte towards hypertrophy [38,43,52]. On the other hand, absence of beta-catentin mediated signaling also can lead to cell death and tissue damage. Hence, Wnt signaling pathway activity needs specific control in the joint. Hence, we focused in this PhD project on identifying modulators of this signaling pathway and elucidating their eventual mechanism of action.

In this PhD project, we studied two new Wnt modulators, EXT1 and ANP32A, and their potential implication as therapeutic targets involved in regulating Wnt activity in the pathophysiology of OA. In the first section, we discussed the influence of EXT1 on Wnt activity regulating chondrogenic differentiation in an in *vitro* model. Secondly, we described that the chondroprotective effects of ANP32A can be linked with modulation of the Wnt signaling pathway, together with the previous finding that ANP32A regulates oxidative stress via its effect on ATM.

In **chapter 3**, we discovered that EXT1, one of the key enzymes responsible for biosynthesis of HSPGs, can repress the differentiation capability of murine chondro-progenitor cells, and these effects are accompanied with modifications in the activity of canonical Wnt signaling pathway. Mutually, Wnt signaling also affects *Ext1* expression.

Collectively, there is a regulatory loop between EXT1 and the Wnt signaling during chondrogenic differentiation.

EXT1 encodes a glycosyltransferase required for HS chain elongation in the biosynthesis of HSPG and mutations in the *EXT1* gene have been associated with a human bone disorder called multiple hereditary exostoses [67,124]. Several previous studies have shown the important role of EXT1 and HS content in skeletal development and chondrogenic differentiation [98,125,126]. Most data obtained in studies of the heterozygous *Ext1*-deficient or tissue-specific/conditional *Ext1* ablation mice demonstrate enhanced chondrogenic differentiation via alterations in the Indian Hedgehog pathway or in bone morphogenetic protein signaling [125].

We demonstrated that *Ext1* deficiency increased chondrogenic differentiation in our cell model. Furthermore, overexpression of *Ext1* weakened the capability of those precursor cells to become chondrocytes. In addition our data suggest a mechanistic link between Ext1 and the canonical Wnt signaling pathway. Indeed, we found that *Ext1* deficiency and the associated reduction in HS resulted in decreased Wnt pathway activity, while *Ext1* overexpression lead to the opposite. Collectively, our findings are consistent with the established concept, low Wnt signaling activation promotes a chondrogenic differentiation program, while high Wnt signaling reduces chondrocyte differentiation.

Connections between EXT1/HSPGs and Wnt signaling were reported in other research models like *Drosophila* [61]. In agreement with our results, mutations of the *Drosophila* orthologue of *Ext1* or defective HSPGs formation can disrupt Wg signaling or spreading [102]. The experiments suggest a possible mechanism by which HSPGs can regulate Wnt levels in the extracellular space and Wnt signaling by maintaining the local concentration of Wnt proteins onto the cell surface. To confirm the existence of a similar mechanism in our experimental system, we used sodium chlorate, a compound can blocks N-sulfation of the GAG chains of HS, in the *Ext1* overexpressing cells to successfully rescue the impaired chondrogenesis. This finding is consistent with another study in a *Drosophila* mutant model which proved that N-sulfation of the GAG chains of HS is responsible for the distribution and signaling of Wg [127]. Therefore, modifications in the N-sulfation of HS chains could control for Wnt signaling pathway activity during chondrogenesis.

Of note, some of our results in the Ext1 overexpressing cell lines highlighted different activity levels of the Wnt pathways when comparing two different methods of assessment. The luciferase reporter assay showed a low Wnt activity level in Ext1+ and control cells in basal condition, but beta-catenin protein levels were slightly increased in Ext1 overexpressing cells. Beyond technical differences in the analyses and sensitivity to detect differences, these observations may be explained by the limited effect on endogenous Wnts from extra HSPGs by overexpression as the baseline levels were sufficient to active the cascade. As a result, most of knock-down experiments have a larger effect size than overexpression studies.

Lastly, our data in human articular chondrocytes suggested that the regulatory loop between EXT1/HS and Wnt signaling during chondrogenic differentiation may also be present in patients with osteoarthritis. In human primary cells, EXT1 could increase the activation of the Wnt signaling cascade. As we have described before, hyper-activation of the Wnt signaling pathway is a deleterious event for cartilage homeostasis and an important mechanism in the pathogenesis of osteoarthritis [31]. Full confirmation of our Ext1 hypothesis will require further experiments, in particular in *in vivo* models of disease.

Indeed, the absence of additional *in vivo* model data can be considered a remaining limitation towards the interpretation and translation of our work. We also encountered variation within either the same clone or cell lines. Therefore, multiple methods both *in vitro* and *in vivo* models should be used in the future for the assessment and the validation of the biological relevance of the observed differences in this thesis. Finally, future studies are needed to further substantiate our preliminary findings in the context of adult cartilage, and to confirm the presence and the mechanism of an EXT1/Wnt signaling regulatory loop in osteoarthritis.

We therefore aim, in the future, to study interactions between Ext1 and Wnt signaling in primary human articular chondrocytes, both in a healthy and an osteoarthritic context. Upon these observations, specific Ext1 loss- and/or gain-of-function in mouse genetic models, applied to a classical osteoarthritis model such as DMM, could provide translational evidence about the role of Ext1 in disease processes. These different types of experiments will also be useful to further confirm or elucidate the existence of the

regulatory loop between Ext1 and WNT signaling, which may represent an important mechanism in both health and disease.

In **chapter 4**, our findings revealed that ANP32A serves as a converging node regulating a protective network against OA by interaction with two different cascades: the previously reported regulation of Atm expression thereby acting on oxidative stress, and the hereby identified interaction with the Wnt signaling pathway (Fig. 1). The transcriptional response associated with activation of the canonical Wnt signaling pathway is negatively regulated by ANP32A in cartilage, and together with the inhibition of oxidative stress, ANP32A can enhance chondrogenesis and maintain articular cartilage homeostasis. ANP32A can bind with one of the key components of the β-catenin destructive complex, Axin1, within the cytoplasm and likely contributes to the sustenance of cartilage homeostasis. But when the Wnt cascade is specifically activated, ANP32A will translocate to the nucleus and bind with β-catenin repressing expression of Wnt target genes to prevent excessive Wnt activity.

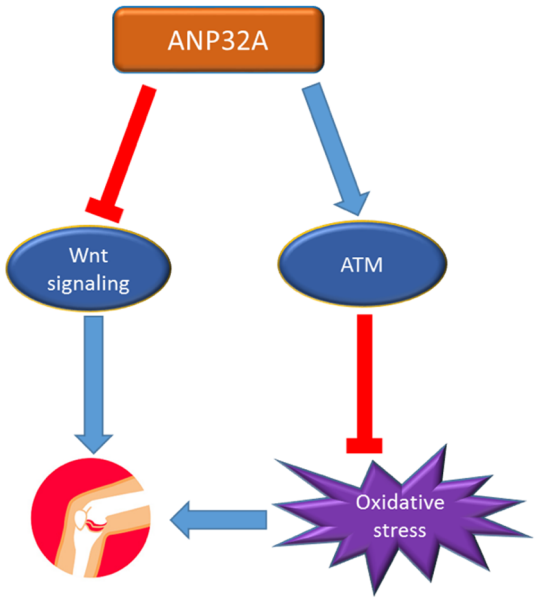


Fig. 1 ANP32A as a central hub of both oxidative stress and the Wnt signaling pathway.

We observed in earlier work from our research team that in the DMM-induced OA model when using *Anp32a*$^{-/-}$ mice, anti-oxidant treatment can rescue the cartilage loss but that this rescue was incomplete. Therefore, the connection with the Wnt signaling pathway triggered our interest. Interestingly, the interaction between ANP32A and Wnt signaling

pathway was also predicted by an advanced analysis from our earlier collected microarray data on articular cartilage from *Anp32a*$^{-/-}$ mice.

This prediction suggested us that ANP32A could be a central hub of two OA therapeutic targets, oxidative stress and the Wnt signaling pathway. As stated above, we earlier confirmed that antioxidants could prevent cartilage loss in *Anp32a* knock out mice [72]. Other research models also confirmed a cartilage protective role for antioxidants [115-117]. Similarly, the Wnt pathway is a promising therapeutic target for OA. Recently, investigators announced some success by intra-articular injecting a novel Wnt inhibitor for treating knee OA in a phase 2B study [118], and now this molecule is committed in phase 3 trials. Data on co-treatment of oxidative stress and Wnt signaling pathway in osteoarthritis model are still missing.

To validate our hypothesis, we further evaluated Wnt activity in chondrogenic cell lines, human articular chondrocytes, as well as in mouse and human articular cartilage, and confirmed that ANP32A deficiency leads to higher levels of Wnt signaling activation. To further confirm the interaction, we used a chemical Wnt inhibitor in our ANP32A deficient models to reduce the excessive Wnt activity and this approach could restore chondrogenesis, thus confirming the hypothesis. Of note, in the chondrogenesis model, silencing of Anp32a reduced Atm levels to about 50% but the earlier identified antioxidant effects were not confirmed in this setting when using NAC to rescue the observations. Therefore, our data show pathways different from oxidative stress to play a role in ANP32A-mediated chondrogenesis.

Subsequently, we aimed to figure out how ANP32A regulates the Wnt signaling pathway. The balance of Wnt activity in cartilage health is determined by many factors. For example, secreted Wnt modulators like sFRP3/DKK1/sclerostin, transcriptional factors like TCF4, epigenetic modulators like DOT1L and the miR-29 family members, and crosstalk with other signaling pathways (GDF5, Hedgehog, etc.) have been demonstrated to modulate the Wnt pathway [31]. Interestingly, a protein-protein interaction network screening by using automated yeast two-hybrid (Y2H) interaction mating revealed that ANP32A can bind with AXIN1, a component of the β-catenin destructive complex [73]. To confirm that this

interaction also occurs in chondrocytes, co-IP experiments were set-up to test protein-protein interactions in human articular chondrocytes and our data showed that in basal conditions, endogenous ANP32A can bind with AXIN1 in the cytoplasm, but that this binding was undone after triggering Wnt activity. Surprisingly, silencing ANP32A did not change total and active β-catenin protein levels suggesting that ANP32A does not affect the activity of the β-catenin destructive complex. We further evaluated whether the effects downstream of β-catenin stabilization are targeted by ANP32A. Based on the enhanced association between ANP32A and β-catenin after WNT3A stimulation, ANP32A is proposed to lose its binding to AXIN1 and regulate Wnt activity by nuclear translocation and association with β-catenin. In order to confirm this idea, we used immunofluorescence in human articular chondrocyte and the translocation into nuclear of ANP32A upon Wnt stimulation was demonstrated. Unlike the β-catenin protein which could bind with transcriptional regulator TCF/LEF, we did not find a direct interaction of ANP32A and TCF/LEF suggesting that ANP32A play its role in a TCF/LEF-independent mechanism. Hence, we concluded that ANP32A may translocate into the nucleus together with β-catenin and regulate the transcriptional response to Wnt signaling without binding to the TCF/LEF factors (Fig. 2).

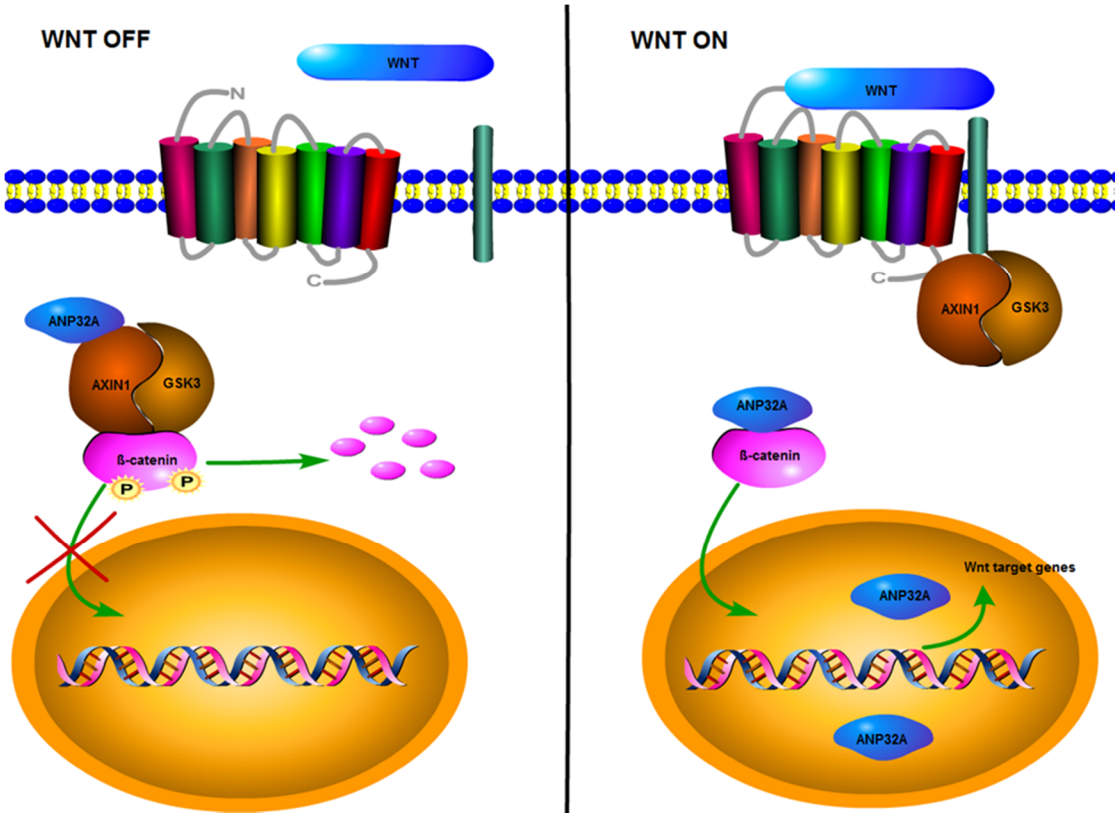


Fig. 2 Schematic diagram of the proposed regulatory role of ANP32A in the Wnt signaling pathway. In basal conditions, endogenous ANP32A binds with AXIN1 in the cytoplasm. Upon Wnt activation, ANP32A looses its binding with AXIN1 and translocates into the nucleus with β-catenin and regulatee the expression of Wnt target genes.

The repressive function of ANP32A on transcription has been intensively described because ANP32A plays a role in the INHAT complex, a multiprotein complex that sterically inhibits histone acetyltransferases by binding to histone tails [120-122]. But on the other hand, several studies also reported that ANP32A can be a positive regulator of gene expression, e.g. for the *Atm* gene and for interferon-stimulated genes [72,123]. Therefore, our future plan is to test if ANP32A could bind to Wnt target gene promotors via its function as inhibitor of acetyltransferases and inhibit histone acetylation to mask transcriptional expression of Wnt target genes in articular chondrocytes.

In addition to cartilage, ANP32A is ubiquitously expressed in all tissues of the body and biological roles have also been reported in other tissues. Particular attention has been given to the brain, where ANP32A is upregulated in patients and mouse models of Alzheimer's

[128-131]. Another study demonstrated memory impairments in mice that overexpressed ANP32A in the hippocampus [132]. Our group also demonstrated that ANP32A is essential to control oxidative stress via *Atm* expression in both brain and bone. On the other side, Wnt signaling also plays important roles in homeostasis and disease of many organs. For instance, low Wnt activity is linked with cognitive decline in Alzheimer's disease patients. To our knowledge, ANP32A has not been previously linked with Wnt signaling in brain disease [133,134]. Therefore, future research will evaluate if the biology of ANP32A could have therapeutic potential in other tissues via regulating Wnt signaling.

In conclusion, we described that EXT1 and ANP32A control the development and homeostasis of cartilage via Wnt signaling pathway, thereby suggesting these as therapeutic targets for osteoarthritis. The regulatory loop between EXT1/HS and Wnt signaling during chondrogenesis could offer insights for the treatment of OA, but additional studies will be required to provide further proof of concept. ANP32A act as a critical node regulating diverse key networks, including the Wnt signaling pathway and oxidative stress in the chondrocyte. Future research will focus on identifying the mechanisms by which ANP32A modulates Wnt signaling inside the nucleus and which factors control the expression of ANP32A. In vivo work by co-treatment of Wnt inhibitor and an anti-oxidative drug, for evaluating the synergistic effect on preventing cartilage loss, will be required to further assess the translational potential of our findings.

Summary

Cartilage is an important tissue in the skeleton. During skeletal development transient cartilage forms a template for the skeletal elements before it is replaced by bone. In the joints a non-transient form of cartilage, the articular cartilage, caps the ends of the bones and ensures very low friction between the bones to support mobility. Damage to this articular cartilage is a main feature of joint diseases. Osteoarthritis is the most common chronic joint disease, affecting millions of people world-wide and is characterized by progressive structural damage to the articular cartilage and the underlying subchondral bone, leading to pain and disability. Currently, no disease modifying treatments are available for patients with osteoarthritis, thereby defining an important unmet medical need.

The Wnt signaling pathway has been identified as a key player in cartilage development, homeostasis and disease. Hyperactivation of Wnt signaling inhibits early cartilage differentiation but stimulates the last stages of the developmental differentiation process. In the articular cartilage, low levels of active Wnt signaling appear important for cell survival, but hyperactivation of the pathway is associated with abnormal differentiation of the cells and an increase in the production of tissue-destructive enzymes thereby contributing to disease processes active in osteoarthritis. Hence, Wnt signaling is considered to be a therapeutic target for this disease. Therefore, understanding of the mechanisms that regulate this pathway in cartilage is important.

In this thesis, two regulatory mechanisms are investigated and demonstrated. First, activation of Wnt signaling is demonstrated to be dependent on the presence of heparin sulfate proteoglycans. *Exostosin-1* (*Ext1*) encodes a glycosyltransferase that is required for heparan sulfate (HS) chain elongation in proteoglycan biosynthesis. HS chains serve as binding partners for signaling proteins, affecting their distribution and activity. In knockdown experiments in a chondrocyte development model, HS levels were reduced and this positively impacted on chondrogenic differentiation and proteoglycan accumulation. *Ext1* knock-down reduced active Wnt signaling. Conversely, *Ext1* overexpressing cells, with higher HS content, showed decreased chondrogenic differentiation and enhanced Wnt. Wnt signaling activation led to a down-regulation of *Ext1* expression in chondrocytes.

EXT1 therefore affects chondrogenic differentiation of precursor cells, in part via changes in the activity of Wnt signaling. As Wnt signaling also controls *Ext1* expression, a regulatory loop between EXT1 and Wnt signaling during chondrogenesis is proposed.

Second, interactions between Wnt signaling and intracellular multifunctional molecule ANP32A were demonstrated. ANP32A was previously shown to protect against cartilage damage by limiting oxidative stress through regulation of key anti-oxidant regulator molecule ATM expression. However, anti-oxidant treatment only partially rescued joint damage in *Anp32a KO* mice with osteoarthritis. Analysis of global gene expression levels by microarray in the articular cartilage of *Anp32a KO* mouse cartilage suggested that ANP32A also can regulate Wnt signaling. Lack of Anp32a in a cartilage differentiation process resulted in inhibition of differentiation and lack of proteoglycan accumulation, associated with Wnt hyper-activation. In human and mouse articular cartilage, Anp32a deficiency was also linked to hyper-activation of Wnt signaling.

Therefore, this work reveals that EXT1 and ANP32A regulate the activity of Wnt signaling and can be considered potential targets to modulate this pathway in the treatment of osteoarthritis.

Samenvatting

Kraakbeen is een essentieel weefsel in het skelet. Tijdens de ontwikkeling van de bot elementen vormt kraakbeen een templaat dat progressief vervangen wordt door botweefsel. In de gewrichten vormt het gewrichtskraakbeen een niet-transiënte vorm van kraakbeen aan de botuiteinden dat zorgt voor beweging tussen de botten met lage wrijving. Kraakbeenschade is een belangrijk kenmerk van gewrichtsaandoeningen. Artrose of osteoartrose is de belangrijkste chronische gewrichtsziekte en treft miljoenen mensen wereldwijd. De ziekte wordt gekenmerkt door structuurschade aan het gewrichtskraakbeen en het subchondrale bot, hetgeen leidt tot pijn en functieverlies. Op dit moment zijn er geen structuur beïnvloedende behandelingen voorhanden voor patiënten met artrose en is het ontwikkelen hiervan een belangrijke medische noodzaak en uitdaging.

De Wnt signaalweg is een belangrijke factor tijdens kraakbeenontwikkeling, homeostase en ziekte. Hyperactivatie van Wnt signalisatie heeft een negatieve invloed op de vroege differentiatie van kraakbeencellen, maar stimuleert de laatste stappen in dit ontwikkelingsproces. In het gewrichtskraakbeen lijkt een laaggradige activering van de signaalweg belangrijk voor kraakbeenceloverleving, maar wordt hyperactivering geassocieerd met abnormale differentiatie en de productie van weefsel-destructieve enzymen. Dit draagt bij tot het ziekteproces bij patiënten met artrose. Daarom wordt de Wnt signaalweg beschouwd als een therapeutisch doelwit voor deze ziekte. Een verder begrip van de mechanismen die deze signaalweg regelen in het kraakbeen is daarom essentieel.

In dit proefschrift worden twee mechanismen die Wnt signalisatie kunnen reguleren aangetoond en onderzocht. Vooreerst wordt aangetoond dat de activering van Wnt signalisatie beïnvloed wordt door de aanwezigheid van heparansulfaat proteoglycanen. *Exostosin-1 (Ext1*) is een gen dat codeert voor een glycosyltransferase dat essentieel is voor de ketenverlenging van de heparansulfaten (HS) in de biosynthese van proteoglycanen. De HS ketens hebben een rol als partners in het binden van signaalmoleculen en beïnvloeden daardoor hun distributie en activiteit. In verlies van functie experiment in een kraakbeen differentiatiemodel, was de hoeveelheid HS verminderd en dit had een positieve impact of het differentiatieproces en de accumulatie van proteoglycanen. *Ext1* functieverlies reduceerde ook de activering van de Wnt signaalweg. In contrast hiermee blijkt dat in *Ext1*

overexpressie cellen, de hoeveelheid HS toeneemt en dat di teen negatief effect heeft op kraakbeendifferentiatie. Echter de activering van Wnt signalisatie is verhoogd. Bovendien blijkt dat Wnt signalisatie leidt tot een verminderde expressie van *Ext1* expressie in kraakbeencellen.

In een tweede deel worden de interacties tussen Wnt signalisatie en het multifunctionele intracellulaire molecule ANP32A aangetoond. Een rol voor ANP32A in kraakbeenbescherming werd eerder voorgesteld omdat ANP32A beschermt tegen oxidatieve stress in het kraakbeen via de expressie van antioxidant regulator ATM. Echter, antioxidant behandeling kon gewrichtsschade in *Anp32a* verlies van functie muizen met artrose slechts gedeeltelijk omkeren. Een analyse van het globale genexpressiepatroon in het kraakbeen van deze muizen suggereert dan ANP32A ook de Wnt signaalweg kan beïnvloeden. Een gebrek aan ANP32A tijdens het kraakbeen-differentiatie proces leidt tot inhibitie en een gebrek aan accumulatie van proteoglycanen, en kan worden geassocieerd met hyperactivatie van de Wnt signaalweg. In zowel muis als humaan kraakbeen wordt een gebrek aan ANP32A ook gelinkt aan deze hyperactivatie.

Dit werk toont daarom aan dat zowel EXT1 als ANP32A de activiteit van de Wnt signaalweg kunnen regelen en dat deze moleculen daarom kunnen beschouwd worden als potentiële therapiedoelwitten om Wnt signalen te moduleren in de behandeling van artrose.

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Scientific acknowledgements and personal contributions

All scientists that contributed to the different chapters presented in this thesis manuscript are listed in the respective author lists of the chapters.

Prof. Rik Lories and Prof. Silvia Monteagudo were responsible for the original concept of the work, the daily supervision and the final drafting and approval of the different chapters.

In chapter 3, all experiments were performed by Xiangdong Wang.

In chapter 4, all experiments were performed by PhD candidate Xiangdong Wang, with the exception of the upstream regulator analysis reported in Fig. 1 (performed by Silvia Monteagudo), the generation of the ANP32A KD ATDC5 cell line (generated by Tine Peeters) and the set-up, handling and immunohistochemistry analyses in the mouse experiments (performed by Frederique Cornelis).

Conflicts of interest

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Publications

***Wang Xiangdong**, Frederique M. Cornelis, Rik J. Lories, Silvia Monteagudo*. Exostosin-1 activity enhances the strength of Wnt signaling during chondrogenic differentiation. *Osteoarthritis and Cartilage*, 2019 Nov; 27(11):1702-1710.

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