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Osteoarthritis and Cartilage

ANP32A represses Wnt signaling across tissues thereby protecting against osteoarthritis and heart disease

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Objectives: To investigate how ANP32A, previously linked to the antioxidant response, regulates Wnt signaling as unraveled by transcriptome analysis of *Anp32a*-deficient mouse articular cartilage, and its implications for osteoarthritis (OA) and diseases beyond the joint.
Methods: Anp32a knockdown chondrogenic ATDC5 cells were cultured in micromasses. Wnt target genes, differentiation markers and matrix deposition were quantified. Wnt target genes were determined in articular cartilage from *Anp32a*-deficient mice and primary human articular chondrocytes upon *ANP32A* silencing, using qPCR, luciferase assays and immunohistochemistry. Co-immunoprecipitation, immunofluorescence and chromatin-immunoprecipitation quantitative PCR probed the molecular mechanism via which ANP32A regulates Wnt signaling. *Anp32a*-deficient mice were subjected to the destabilization of the medial meniscus (DMM) OA model and treated with a Wnt inhibitor and an antioxidant. Severity of OA was assessed by cartilage damage and osteophyte formation. Human Protein Atlas data analysis identified additional organs where ANP32A may regulate Wnt signaling. Wnt target genes were determined in heart and hippocampus from *Anp32a*-deficient mice, and cardiac hypertrophy and fibrosis quantified.
Results: Anp32a loss triggered Wnt signaling hyper-activation in articular cartilage. Mechanistically, ANP32A inhibited target gene expression via histone acetylation masking. Wnt antagonist treatment reduced OA severity in *Anp32a*-deficient mice by preventing osteophyte formation but not cartilage degradation, contrasting with antioxidant treatment. Dual therapy ameliorated more OA features than individual treatments. *Anp32a*-deficient mice also showed Wnt hyper-activation in the heart, potentially explaining the cardiac hypertrophy phenotype found.
Conclusions: ANP32A is a novel translationally relevant repressor of Wnt signaling impacting osteoarthritis and cardiac disease.

Introduction

Tissue homeostasis requires orchestrated activation and restriction of gene expression programs. How specific genes are switched on and off at the correct time and in the right place is a central question in biology. Furthermore, understanding how gene regulatory processes might be perturbed by disease or by factors such as genetic variants is key to establish the rationale for the

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development of effective targeted therapies and personalized medicine.

We reported that acidic leucine rich nuclear phosphoprotein-32A (*ANP32A*) protein switches on transcription of the ataxia telangiectasia mutated serine threonine kinase (*ATM*) gene to tightly control a central regulatory network that prevents oxidative processes in cartilage, cerebellum and bone[1]. In cartilage, absence of ANP32A leads to severe osteoarthritis and oxidative stress[1]. Earlier, genetic variants in *ANP32A* were associated with this disease[2]. Current treatments for osteoarthritis are limited to symptom relief and in advanced cases joint replacement surgery may be the only option. Thus, developing an effective therapy that arrests or reverses disease progression is urgently needed. As osteoarthritis is a disease of the whole joint affecting different tissues, distinct pathways with varying downstream pathological effects are likely involved and effective treatment may require combination therapies.

ANP32A is a pleiotropic protein, influencing several phenotypic traits. Pleiotropic proteins are central in protein-protein interaction networks and may control multiple biological pathways[3]. Key unanswered questions are whether ANP32A has a role in multiple molecular mechanisms of homeostasis and disease, in particular in the joint, and whether there are yet undiscovered phenotypic traits critically influenced by ANP32A.

Here, we identify that ANP32A adjusts the transcriptional response of Wnt signaling, a central network in tissue homeostasis and disease with a key role in osteoarthritis[4–6]. We unravel that ANP32A performs this regulatory role via histone acetylation masking. From a translational perspective, we evaluate disease-modifying effects of targeting Wnt hyper-activation in a model of osteoarthritis in *Anp32a*-deficient mice, and benefits of a combination with antioxidant treatment in this setting. Additionally, we explore whether ANP32A regulates Wnt signaling in tissues beyond cartilage. Our findings unveil that ANP32A is a regulatory molecule of Wnt signaling across multiple tissues, with links to osteoarthritis, and with potential roles in cardiac hypertrophy and Alzheimer's disease.

Materials and methods

Additional information is found in supplementary data

Study design

Our aim was to determine how ANP32A protects against osteoarthritis by regulating pathways beyond oxidative stress and whether such mechanisms also affect other tissues and organs. Human cartilage and genetically engineered mice were used in *ex vivo* and *in vivo* studies using unchallenged ageing mice and a model of joint disease, combined with *in vitro* assays. Mouse models are reported following the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) (Supplementary Table 1).

Patient materials

Human articular chondrocytes were isolated from hips of patients undergoing hip replacement surgery after informed consent. The University Hospitals Leuven Ethics Committee and Biobank Committee (Leuven, Belgium) approved the study (S56271).

Mice

Anp32a$^{-/-}$ (*Anp32a*^{tm1Hzo}) mice were a gift from Dr. P. Opal (Northwestern University Medical School, Chicago, USA)[7] and backcrossed onto the C57Bl/6J background. In the experiments reported, mice were between the 11th and 21st generation of backcrossing. Wild-type C57Bl/6J, purchased from Janvier (Le Genest St Isle, France), were used as controls. All studies were approved by the Ethics Committee for Animal Research (P114-2008, P198-2012, P159-2016; KU Leuven, Belgium) (License LA1210189). Genotypes of animals were confirmed by polymerase chain reaction (PCR)[1].

Statistics

Data analysis and graphical presentation were performed with R-Studio (version 1.1.463) and GraphPad Prism version 8. Power analysis was performed with G*Power (version 3.1.9.4). Data are presented as mean and SD or as individual data points, representing the mean of technical replicates as indicated in figure legends. Raw data are available in Supplementary File 1. R code is available in Supplementary File 2. Gene expression data and image quantifications were log-transformed for statistical analysis. All tests performed were two-tailed. Further information is found in Supplementary methods.

Results

Articular cartilage transcriptome analysis suggests dysregulation of Wnt signaling in Anp32a-deficient mice

To investigate which central networks are potentially regulated by ANP32A in joint biology, we sought to identify signaling cascades driving differences in gene expression in articular cartilage from *Anp32a*$^{-/-}$ mice (mice with global deletion of ANP32A) compared to wild-type mice. We took advantage of our earlier microarray transcriptome study (geonr: GSE108036)[1], and applied the Upstream Regulator Analysis approach from Ingenuity Pathway Analysis software. Strikingly, pathway analysis of identified genes classified as “transcriptional regulators” indicated that Wnt signaling drives gene expression changes resulting from loss of ANP32A [Fig. 1(A)]. Our earlier observations[1] were also confirmed as P53 signaling, directly linked to ATM, appeared as main enriched pathway mediating loss of ANP32A's effects on gene expression [Fig. 1(A)].

Anp32a deficiency impairs chondrogenic differentiation via Wnt hyper-activation

Combined genetic and experimental evidence strongly supports that a fine-tuned balance of Wnt activity is key for cartilage health, and excessive activation of Wnt signaling contributes to osteoarthritis[4,8]. To explore whether ANP32A has a regulatory effect on Wnt signaling in cartilage, we first used an *in vitro* cartilage differentiation model in which Wnt signaling plays a key role. The chondrogenic ATDC5 cell line exhibits a multistep differentiation process towards cartilage when seeded in micromasses[9]. In this model, Wnt signaling activation in the early phase blocks the differentiation program[10]. We generated stable *Anp32a* siRNA knockdown (KD) ATDC5 cell lines [Supplementary Fig. 1(A)] and found that gene expression levels of direct Wnt target genes were up-regulated in *Anp32a*-deficient cells compared to controls (Pillai = 0.964, $F_{2,3} = 40.34$, $P = 0.0068$ by MANOVA) [Fig. 1(B)]. Upon micromass differentiation culture, mRNA expression of early cartilage differentiation markers *collagen 2* (*Col2a1*) and *aggrecan (Acan),* and terminal differentiation marker *collagen 10* (*Col10a1*) were strongly down-regulated in *Anp32a* KD cells compared to controls [$F_{3,12} = 25.321$, $P < 0.0001$ (*Col2a1*), $F_{1.05,4.21} = 24.378$ $P = 0.007$ (*Acan*), $F_{3,12} = 42.775$ $P < 0.0001$ (*Col10a1*) by 2-way ANOVA] [Fig. 1(C)]. To evaluate proteoglycan and collagen content, we performed alcian blue, safranin O and picrosirius red staining. We observed a reduction in proteoglycans and collagen amounts in *Anp32a* KD micromasses compared with controls [Fig. 1(D)]. Mineralization, assessed by alizarin red staining, was also reduced [Fig. 1(D)]. Although expression of *Atm* was

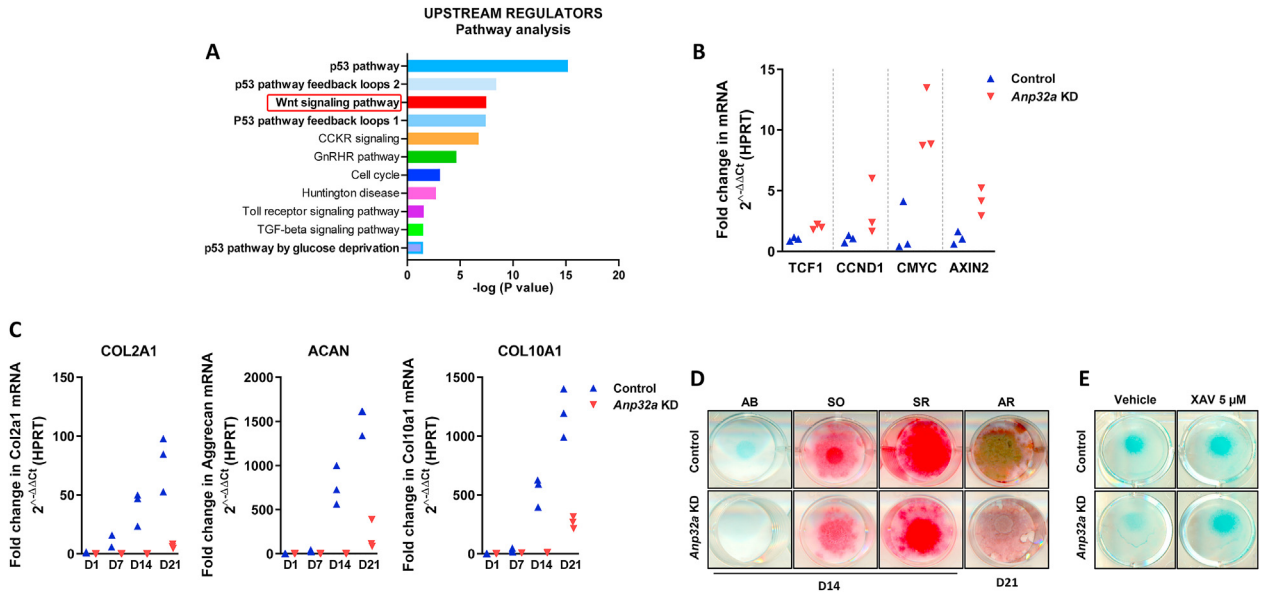

Fig. 1

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***Anp32a* deficiency impairs chondrogenic differentiation via Wnt hyper-activation.** (A) PANTHER pathway analysis of upstream transcriptional regulators identified in microarray data comparing articular cartilage of 8-week old male *Anp32a*-deficient to wild-type mice ($n = 4$ per group) using Ingenuity Pathway Analysis. (B) Real-time PCR analysis of direct Wnt target genes *Tcf1*, *Ccnd1*, *cMyc* and *Axin2* in control and *Anp32a* knockdown (KD) ATDC5 cells ($P = 0.0068$ by MANOVA; *Ccnd1* and *Axin2* showed ≥0.9 correlation with *Tcf1* and were not included in the model, mean ± SD of three replicates). (C) Real-time PCR analysis of chondrogenic differentiation markers collagen 2 (*Col2a1*), aggrecan (*Acan*) and collagen 10 (*Col10a1*) in control and *Anp32a* KD ATDC5 cells [$P < 0.0001$ (*Col2a1*), $P = 0.007$ (*Acan*), $P < 0.0001$ (*Col10a1*) by 2-way ANOVA for interaction between silencing and time, mean ± SD of three replicates]. (D) Alcian blue (AB), safranin O (SO), picrosirius red (SR) at day 14 (D14) and alizarin red (AR) staining at day 21 (D21) showing reduced proteoglycan deposition (AB, SO), collagen content (SR) and mineralization (AR) in *Anp32a* KD ATDC5 cells during chondrogenesis. (E) AB staining demonstrating rescue of chondrogenic differentiation in *Anp32a* KD ATDC5 cells by treatment with Wnt inhibitor XAV939 (XAV).

partially suppressed in *Anp32a* KD micromasses compared with controls ($F_{1,4} = 24.841$ $P = 0.009$ by 2-way ANOVA for control vs KD cells) [Supplementary Fig. 1(B)], treatment of *Anp32a* KD micromasses with antioxidant N-acetylcysteine (NAC) did not rescue chondrogenic marker expression [Supplementary Fig. 2(A)]. However, blockade of ß-catenin dependent Wnt signaling with XAV939 (XAV)[11] showed rescue effects on chondrogenic differentiation in *Anp32a* KD cells [Fig. 1(E), Supplementary Fig. 2(B)]. XAV is a tankyrase inhibitor that stimulates ß-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex. Collectively, these results indicate that active Wnt signaling contributes to detrimental effects *Anp32a* loss in this cartilage differentiation model.

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

We then investigated whether Wnt signaling is similarly dysregulated in articular cartilage of *Anp32a*$^{-/-}$ mice, both at the gene and protein levels. Messenger RNA amounts of direct Wnt target genes were up-regulated in *Anp32a*$^{-/-}$ mice compared to wild-types (Pillai = 0.788, $F_{3,11} = 13.61$, $P = 0.0005$ by MANOVA) [Fig. 2(A)]. Furthermore, protein reactivity of Wnt target gene TCF1 in immunohistochemistry was increased in articular cartilage from *Anp32a*$^{-/-}$ mice, in healthy young mice at 8 weeks [2.4 fold (95% CI:2.11–3.67; $P < 0.001$ by *t*-test], after induction of osteoarthritis using the destabilization of the medial meniscus (DMM) model (DMM) [3.8 fold (95%CI:2.51–5.69; $P = 0.008$ by *t*-test] and upon ageing (12 months) [2.1 fold (95%CI:1.34–3.17; $P = 0.01$ by *t*-test] [Fig. 2(B)]. In human articular chondrocytes, expression of Wnt target genes was up-regulated upon siRNA-mediated *Anp32a* silencing [Fig. 2(C)]. *Anp32a*-silenced human articular chondrocytes showed stronger induction of the Wnt/β-catenin pathway reporter TOP-FLASH than control cells, upon recombinant WNT3A stimulation [Fig. 2(D)]. All these findings indicate that ANP32A negatively regulates Wnt signaling in articular cartilage.

In patients with knee and hip osteoarthritis, *ANP32A* expression was down-regulated in damaged compared to preserved areas of articular cartilage[1]. Conversely, expression of Wnt direct target genes was up-regulated in damaged areas ($P < 0.0001$, Benjamini-Hochberg adjusted paired *t*-test) [Fig. 2(E) and Supplementary Fig. 3(A)]. Expression of *ANP32A* and several Wnt target genes negatively correlated in human osteoarthritis cartilage (Spearman correlation $r = -0.36$, $P = 0.0173$) [Fig. 2(F) and Supplementary Fig. 3(B)]. These data suggest that the link between ANP32A and Wnt signaling may be clinically relevant in osteoarthritis.

Active Wnt signaling triggers ANP32A nuclear translocation

We next sought to investigate the molecular mechanism via which ANP32A negatively regulates Wnt signaling. The core of the canonical or Wnt/β-catenin cascade is the regulation of ß-catenin

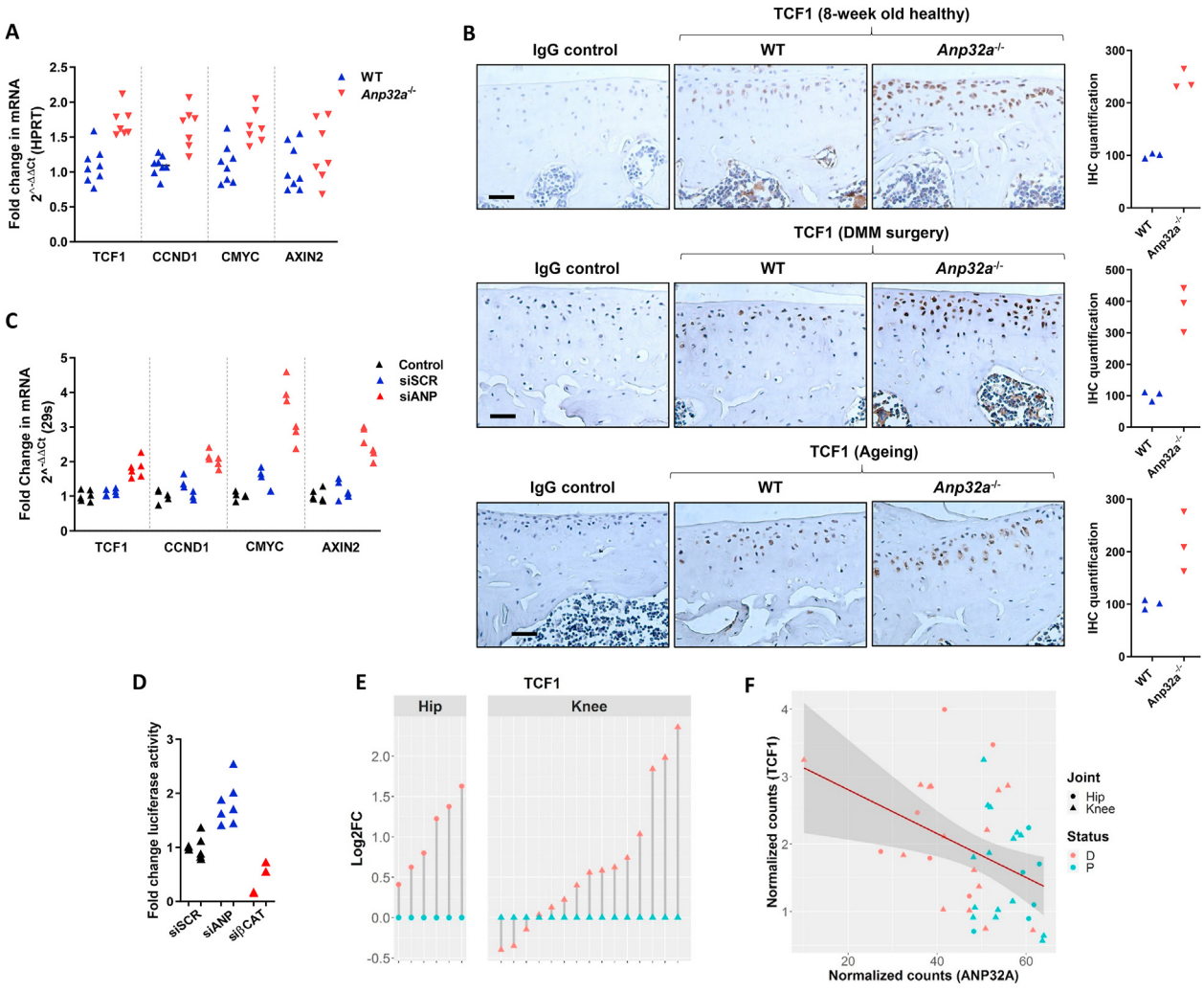

Fig. 2

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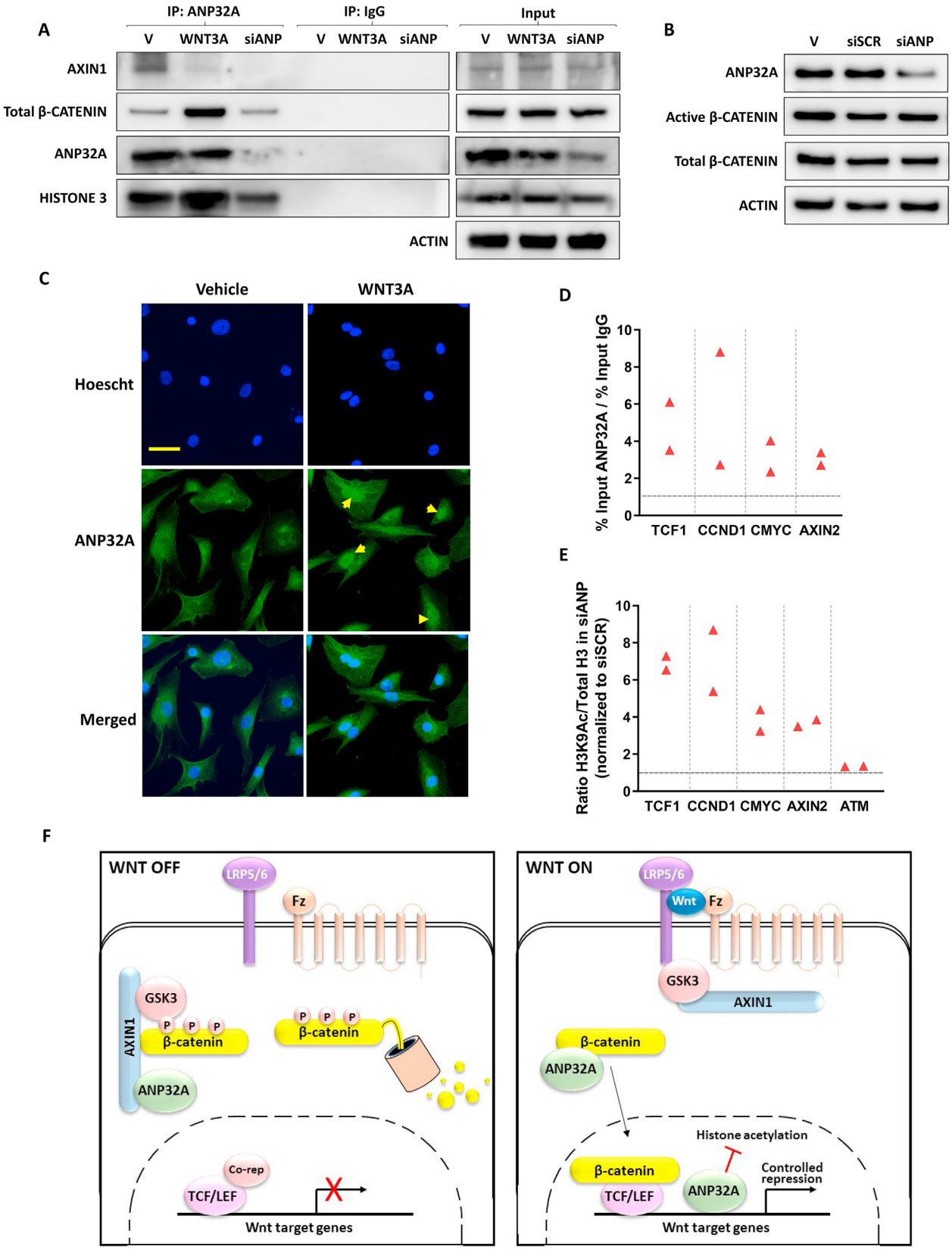
***Anp32a*-deficiency leads to Wnt signaling hyper-activation in articular cartilage**. (A) Real-time PCR analysis of Wnt target gene expression in articular cartilage from 8-week old male wild-type (WT) and *Anp32a*-deficient (*Anp32a*$^{-/-}$) mice ($P = 0.0005$ by MANOVA – *cMyc* showed ≥0.9 correlation with *Tcf1* and was not included in the model, $n = 8$ and 7 per group). (B) Immunohistochemical staining and quantification for TCF1 protein in male 8-week old, osteoarthritic and female ageing WT and *Anp32a*$^{-/-}$ mice (representative images of three different mice per group) ($P < 0.001$, $P = 0.008$, $P = 0.01$ by *t*-test). Scale bar 50 μm. (C) Real-time PCR analysis of Wnt target gene expression in human articular chondrocytes transfected with siRNA targeting *ANP32A* (siANP32A) or scrambled siRNA (siSCR) ($n = 2$ donors – mean ± SD of three replicates). (D) TOP/FOP-reporter assay in human articular chondrocytes transfected with siANP32A, siRNA targeting ß-catenin (siβCAT) or siSCR, treated with recombinant WNT3A ($n = 2$ biologically independent experiments, mean ± SD of three replicates). (E–F) *TCF1* expression (E) and correlation with *ANP32A* expression (F) by RNA sequencing in paired preserved and damaged cartilage from hips (○) and knees (Δ) from osteoarthritis patients [log2-fold change (Log2FC) of damaged (D) vs preserved (P)) ($n = 21$, $P < 0.0001$, Benjamini-Hochberg adjusted paired *t*-test (E), Spearman correlation $r = -0.36$, $P = 0.0173$ (F)].

protein levels by a cytoplasmic destruction complex. In the absence of Wnt signaling, the destruction complex captures cytosolic ß-catenin, leading to its phosphorylation and subsequent degradation by the proteasome. Wnt receptor activation leads to functional inactivation of the destruction complex, resulting in ß-catenin accumulation and nuclear entry, where it binds to TCF/LEF transcription factors and regulates transcription of Wnt target genes[12]. A yeast two-hybrid screening demonstrated interactions between ANP32A and AXIN1, a central scaffold for the destruction complex[13]. We investigated whether ANP32A interacts with AXIN1 at the protein level in articular chondrocytes. We carried out co-immunoprecipitations of endogenous ANP32A from extracts of cells treated with recombinant WNT3A or vehicle control. This revealed that ANP32A bound AXIN1 in baseline conditions, and this association was abolished upon Wnt signaling activation by WNT3A [Fig. 3(A)].

These observations prompted us to investigate the functional relevance of the interaction of ANP32A with AXIN1 for the cytoplasmic stabilization of ß-catenin. Silencing of ANP32A did not affect ß-catenin protein levels (active nor total) in human articular


A
IP: ANP32A
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Input
V WNT3A siANP V WNT3A siANP V WNT3A siANP
AXIN1
Total β-CATENIN
ANP32A
HISTONE 3
ACTIN
B
V siSCR siANP
ANP32A
Active β-CATENIN
Total β-CATENIN
ACTIN
C
Vehicle
WNT3A
Hoescht
ANP32A
Merged
D
% Input ANP32A / % Input IgG
TCF1 CCND1 CMYC AXIN2
E
Ratio H3K9Ac/Total H3 in siANP (normalized to siSCR)
TCF1 CCND1 CMYC AXIN2 ATM
F
WNT OFF
LRP5/6
Fz
GSK3
AXIN1
β-catenin
ANP32A
Co-rep
TCF/LEF
Wnt target genes
WNT ON
Wnt
Histone acetylation
Controlled repression

Fig. 3

chondrocytes [Fig. (3(B)]. This suggests that ANP32A may regulate Wnt signaling downstream of ß-catenin stabilization. Remarkably, the dissociation of ANP32A from AXIN1 upon Wnt activation paralleled enhanced association of ANP32A with ß-catenin [Fig. 3(A)]. Then, we investigated whether Wnt activation triggers nuclear translocation of ANP32A, as it occurs for ß-catenin. We examined 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. 3(C)], in agreement with our previous data[1]. In contrast, ANP32A also localized in the nucleus after WNT3A treatment [Fig. 3(C)].

ANP32A represses Wnt target gene expression through histone acetylation masking

Next, we investigated how ANP32A represses Wnt target genes. ANP32A is a member of the inhibitor of histone acetyltransferase (INHAT) complex[14], which limits transcription by binding to histones, preferentially histone 3, and sterically hindering acetylation[15]. Hypoacetylation of histones is linked to condensed chromatin and transcriptional repression[16]. Wnt-induced ANP32A nuclear accumulation in human articular chondrocytes [Fig. 3(C)] was paralleled by increased interaction of ANP32A with histone 3 [Fig. 3(A)], indicating that ANP32A may repress Wnt target gene expression via its inhibitory role on histone acetylation. Chromatin-immunoprecipitation (ChIP)-qPCR analysis showed that ANP32A bound the chromatin at Wnt target gene promoters in human articular chondrocytes [Fig. 3(D)]. In these cells, ANP32A silencing resulted in increased H3K9 acetylation at Wnt target gene promoters [Fig. 3(E)]. Collectively, these results indicate that Wnt induces ANP32A nuclear internalization and chromatin binding to Wnt target gene promoters, with inhibition of histone acetylation, and thus, transcriptional repression of Wnt target genes [see Fig. 3(F)]. Of note, in *Anp32a*-silenced cells, we did not find increased H3K9 acetylation marks at the promoter of *Atm* [Fig. 3(E)], a gene positively regulated at the transcriptional level by ANP32A[1].

Targeting Wnt hyper-activation and oxidative stress downstream of ANP32A deficiency protects against different features of osteoarthritis

Next, we investigated therapeutic implications of our findings for osteoarthritis. *Anp32a* deficiency results in hyper-activation of Wnt signaling in articular cartilage [Fig. 2(B)], concomitantly with an increase in oxidative stress[1]. We evaluated the effects of treatment with a Wnt inhibitor and an antioxidant, individually or in combination, on osteoarthritis, using the DMM mouse model of the disease[17] that mimics mechanisms and features of posttraumatic osteoarthritis in humans[18]. In this model, we previously showed that *Anp32a*$^{-/-}$ mice have more severe cartilage damage and oxidative stress compared to wild-type and sham-operated *Anp32a*$^{-/-}$ mice[1]. In a new experiment, we demonstrate Wnt hyper-activation in the articular cartilage of DMM-operated *Anp32a*$^{-/-}$ mice [Fig. 2(B)]. After DMM surgery, *Anp32a*$^{-/-}$ mice were intra-articularly injected with Wnt inhibitor XAV and given NAC via the drinking water [Fig. 4(A)]. XAV treatment did not provide cartilage protection [OARSI score difference between means −0.344 (95%CI:-0.735–0.048)] [Fig. 4(B)], although it effectively inhibited Wnt hyper-activation in articular cartilage [TCF1 5.36-fold decreased between XAV and vehicle (V) (95%CI:3.86–7.46) by two-way ANOVA] [Fig. 4(C)]. Of note, decreased severity of OA in the NAC group also decreased Wnt activation [TCF1 2.47-fold decreased NAC vs V (95% CI:1.78–3.43)] and combination treatment decreased TCF1 6.98 fold (95%CI:5.03–9.72). Combination therapy was not considered different from XAV treatment alone [TCF1 1.30-fold decreased XAV/NAC vs XAV (95%CI:0.94–1.81)] whereas combination was more effective than NAC alone [TCF1 2.9-fold decreased XAV/NAC vs NAC (95%CI:2.03–3.93)]. However, Wnt inhibition prevented osteophyte formation in the osteoarthritis model in *Anp32a*$^{-/-}$ mice [osteophyte score difference between the means 0.336 (95%CI:0.089–0.585), $F_{1,28} = 7.69$ $P = 0.0098$ by two-way ANOVA] [(Fig. 4(D)]. In contrast, NAC protected the *Anp32a*$^{-/-}$ mice against cartilage damage [OARSI score difference between means 0.806 (95%CI:0.415–1.20), $F_{1,28} = 8.48$ $P = 0.007$ by two-way ANOVA] but had no effect on osteophyte formation [osteophyte score difference between the means 0.163 (95%CI:-0.086–0.411)] [Fig. 4(B-D)]. Combination of XAV and NAC improved both cartilage damage and osteophyte formation [Fig. 4(B-D)]. Image analysis showed that XAV treatment effectively inhibited Wnt hyper-activation in osteophytes [AXIN2 4.28-fold decreased XAV vs V (95%CI:3.08–5.96) by two-way ANOVA] [Fig. 4(E)]. Of note, decreased severity of OA in the NAC group also decreased Wnt activation in osteophytes [AXIN2 1.50-fold decreased NAC vs V (95%CI:1.08–2.08)] and combination treatment decreased AXIN2 3.57-fold (95%CI:2.57–4.50). Combination therapy was not different from XAV treatment alone [AXIN2 staining 1.20-fold increased XAV/NAC vs XAV (95% CI:0.86–1.70)] but combination was more effective than NAC alone [AXIN2 2.39-fold decreased XAV/NAC vs NAC (95% CI:1.72–3.32)]. These observations suggest that pathological consequences of ANP32A deficiency may hinge on different pathways depending on the tissue involved, oxidative stress in articular cartilage and hyper-activation of Wnt signaling in osteophyte formation.

ANP32A negatively regulates Wnt signaling in heart and hippocampus

As demonstrated above, ANP32A interacts with distinct components of the Wnt signaling pathway. Highly-connected nodes

ANP32A represses Wnt target genes by histone acetylation masking in human articular chondrocytes. (A) Co-immunoprecipitation (Co-IP) with anti-ANP32A antibody showing ANP32A-AXIN1 binding in untreated cells, abolished upon Wnt activation by recombinant WNT3A. Wnt activation increases ANP32A-β-catenin and -histone-3 binding. ANP32A silencing (siANP32A) shows specificity of anti-ANP32A antibody. (B) Immunoblot of ANP32A, active and total ß-catenin (with actin as loading control) in chondrocytes transfected with *ANP32A* (siANP32A) or scrambled siRNA (siSCR). (C) Immunofluorescent staining of ANP32A (green) and Hoechst nuclear counterstaining (blue) in chondrocytes upon WNT3A treatment. Representative images are shown ($n = 3$ (A), $n = 2$ (B)). Scale bar = 10 μm. (D) Chromatin-immunoprecipitation quantitative PCR (ChIP-qPCR) analysis of ANP32A Wnt target (*Tcf1*, *Cnnd1, cMyc, Axin2*) gene promoter chromatin binding in chondrocytes with high endogenous Wnt signaling, and (E) of acetylated H3K9 (H3K9Ac) on Wnt target and *Atm* gene promoters. Data expressed as ratio of H3K9Ac and total Histone-3 (H3) in siANP32A normalized to siSCR cells ($n = 2$ biologically independent experiments (D–E)). (F) Scheme Wnt transcriptional response regulation by ANP32A. In basal conditions, ANP32A interacts with AXIN1 in the destruction complex. Upon Wnt activation, ANP32A dissociates from AXIN1, associates with ß-catenin, translocates to the nucleus and represses Wnt targets via blocking histone acetylation.

within a signaling pathway, which moreover are ubiquitously expressed like ANP32A[19,20] (Human Protein Atlas - http://www.proteinatlas.org), may regulate such cascade in multiple tissues[21]. Thus, we investigated whether ANP32A's regulatory role on Wnt signaling is relevant beyond the joint. We analyzed data from the Human Protein Atlas to identify tissues and organs with inverse relationship between expression of ANP32A and Wnt target gene TCF1, which may indicate an underlying regulatory link. Although there was no ubiquitous relationship between these factors, our analysis identified brain and muscle including the heart, as organs of interest [Fig. 5(A)].

In heart tissue, Wnt signaling was enhanced in *Anp32a*$^{-/-}$ mice compared to controls, at the gene expression (Pillai = 0.492, $F_{3,12} = 3.877$, $P = 0.038$ by MANOVA) and protein levels [TCF1 5.7 fold increased (95%CI:3.58–9.91; $P = 0.0005$ by *t*-test] [Fig. 5(B and C)]. *Anp32a* was down-regulated in a study exploring key deregulated genes and pathways involved in cardiac hypertrophy[22]. Conversely, Wnt signaling is activated during heart failure and cardiac hypertrophy[23,24]. Hearts from 20-week-old male *Anp32a*$^{-/-}$ mice were enlarged compared to controls [Fig. 5(D)]. In *Anp32a*$^{-/-}$ animals, total heart weight as well as heart weight expressed relative to body weight were higher than in wild-types [heart weight/body weight ratio difference between means 2.313 (95% CI:1.859–2.767), $P < 0.0001$ by *t*-test] [Fig. 5(D) and Supplementary Table 2). The expression of hypertrophy markers natriuretic peptide precursor A *(Nppa)* and skeletal muscle *a*-actin *(Acta1)* was up-regulated in *Anp32a*$^{-/-}$ compared to control mice [Fig. 5(E)] [fold change difference between means 2.85 (95%CI:1.64–4.92), $t_{15} = 4.10$ $P < 0.0011$ and 2.19 (95%CI:1.22–3.95), $P < 0.0124$ by *t*-test] and histology showed increased amounts of fibrotic tissue [% fibrosis difference between means 2.145 (95%CI:1.548–3.282), $P = 0.0005$ by *t*-test] [Fig. 5(F)]. Collectively, our data show that lack of ANP32A potentially triggers the development of spontaneous cardiac hypertrophy, which may result from an excessive activation of Wnt signaling.

As suggested by the Protein Atlas data, Wnt target gene expression was up-regulated in brain from *Anp32a*$^{-/-}$ mice compared to controls (Pillai = 0.666, $F_{4,10} = 4.978$, $P = 0.018$ by MANOVA) [Supplementary Fig. 4(A)], demonstrating that ANP32A also negatively regulates Wnt signaling in this organ. ANP32A dysregulation has been linked to Alzheimer's disease with ANP32A increased in human brains from Alzheimer's disease patients[25,26]. In a mouse model of this disease, ANP32A elevation in the hippocampus correlates with learning deficits, and downregulating ANP32A rescues synaptic plasticity and memory loss[27,28]. Overexpression of ANP32A in hippocampus induced memory deficits in mice[29]. Conversely, loss of Wnt signaling plays a critical role in Alzheimer's disease and emerging studies suggest that restoring Wnt signaling may be a promising therapeutic strategy[30–32]. We found that Wnt signaling was enhanced in the hippocampus of *Anp32a*$^{-/-}$ mice [TCF1 3.60 fold increased (95%CI:2.59–5.23; $P = 0.0007$ by *t*-test] [Supplementary Fig. 4(B)]. Therefore, our insights suggest that ANP32A's detrimental role in the pathogenesis of Alzheimer's disease may be linked to a decline in the activation of Wnt signaling.

Discussion

Our findings reveal that ANP32A is a converging node regulating the transcriptional responses of two central cascades in cell biology: Wnt signaling as identified here, and oxidative stress as previously shown[1]. Thus, ANP32A is proposed as a key regulator of a complex network of pathways that protect against osteoarthritis. We provide evidence that the regulatory role of ANP32A on the Wnt transcriptional response is likely not restricted to the joint, but also seems to function in tissues where dysregulation of *ANP32A* expression has been linked to disease, namely heart and hippocampus.

The Wnt/β-catenin pathway has been extensively implicated in osteoarthritis[4–6]. In humans, polymorphisms in genes involved in Wnt signaling, particularly in extracellular inhibitor *SFRP3* and epigenetic modulator *DOT1L*, are associated with increased susceptibility to osteoarthritis[33,34]. In rodent models, loss of molecules that suppress Wnt signaling triggers osteoarthritis[35–38]. Furthermore, mechanical injury and inflammation are potent inducers of Wnt signaling in cartilage[39].

Mechanistically, we show that ANP32A regulates Wnt signaling by interacting with chromatin and inhibiting histone acetylation at Wnt target gene promoters, thereby resulting in gene repression. This repressive function is in line with its earlier defined role as part of the INHAT complex, a multiprotein complex that sterically inhibits histone acetyltransferases by binding to histone tails[14,40,41]. Notably, this molecular mechanism contrasts with our previous findings for the role for ANP32A as positive transcriptional regulator of the *Atm* gene and a report indicating that ANP32A enhances gene transcription of interferon-stimulated genes[1,42]. Collectively, these observations indicate that ANP32A can either activate or repress gene expression programs.

Our preclinical data in the *Anp32a*-deficient mouse model show that combinatorial treatment with a Wnt inhibitor and an antioxidant leads to increased therapeutic efficacy in osteoarthritis. We did not perform these dual therapy experiments in wild-type animals as our previous study indicated that NAC had no significant impact in the DMM model, in contrast to XAV-939 treatment[43,44]. Hence, we did not anticipate beneficial effects of adding NAC to XAV-939 intra-articular treatment in wild-type animals. Cartilage damage in the absence of ANP32A is linked to oxidative stress, yet joint remodeling with osteophyte formation appears to be Wnt dependent. Antioxidants provide protection for cartilage in translationally relevant pre-clinical settings[45,46]. In our previous study, NAC treatment showed protective effects for osteoarthritis in *Anp32a*-deficient mice[1]. However, combination of antioxidants and Wnt inhibitors for this disease has never been explored. The additional effects of the dual therapy strategy are translationally relevant for disease management as it demonstrates potential of combination therapies for osteoarthritis with different interventions simultaneously targeting distinct disease-associated networks and disease manifestations. Given the complexity of osteoarthritis, single target therapies will likely not halt all disease features. Yet, the specific effect of this combination therapy in wild-type animals remains unknown, and further translation to humans will require additional approaches, in particular seeking for situations where dual targeting could be relevant.

We combined oral antioxidant administration with intra-articular administration of a Wnt inhibitor in *Anp32a*-deficient mice. The risk of systemic toxicity has prompted a paradigm shift in osteoarthritis drug development with redirection of attention to benefits of localized vs systemic treatment[47]. However, locally-administered pharmacological agents in the synovial joint might not easily reach cartilage regions closer to the subchondral bone, as dense extracellular matrix in articular cartilage restricts penetration and diffusion of solutes[48]. Systemic administration of well tolerated pharmacological agents such as NAC might add benefits to local administration within the joint, as therapeutic effects would not be only dependent on diffusion into the cartilage from the synovial fluid. Once in the bloodstream, small molecules can diffuse from the blood vessels in the subchondral bone into calcified and non-calcified articular cartilage[48,49].

We show that Wnt signaling is enhanced in the heart of *Anp32a*-deficient mice. We examined heart tissue as Wnt target gene *TCF1*

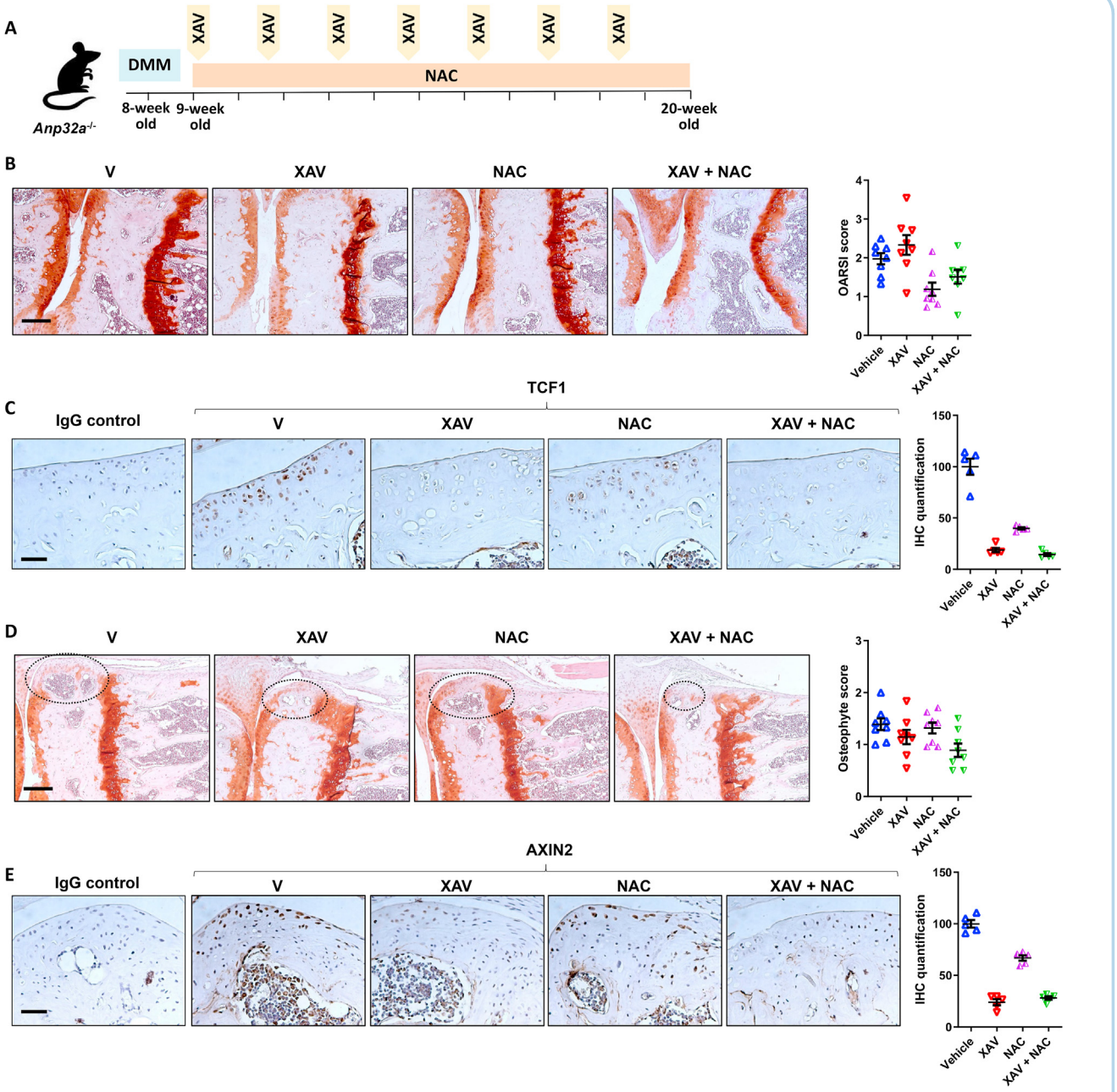

Fig. 4

Osteoarthritis and Cartilage

ANP32A protects against different features of osteoarthritis by controlling distinct pathways. (A) Outline of *in vivo* interventions against osteoarthritis in 8-week-old *Anp32a*-deficient (*Anp32a*$^{-/-}$) male mice subjected to destabilization of the medial meniscus (DMM) surgery. One week after injury, mice were injected intra-articularly with vehicle or Wnt inhibitor XAV939 (XAV) every 10 days for a total of 7 times. Mice were treated orally with vehicle, XAV or NAC alone or in combination. Knee joints were collected 12 weeks after surgery. (B) Hematoxylin-safranin-O-stained sections and quantification by OARSI severity grade demonstrating NAC protects against articular cartilage damage: [$P = 0.007$ for main effect NAC by two-way ANOVA, $n = 8$ per group]. (C) Immunohistochemical staining and quantification for TCF1 in articular cartilage of *Anp32a*$^{-/-}$ mice in the DMM model treated or not with XAV or NAC (representative images of $n = 5$ mice per group) [$P = 0.0010$ for interaction between treatments and control by two-way ANOVA, $n = 5$ per group]. (D) Hematoxylin-safranin-O-stained sections and quantification of osteophytes demonstrating that Wnt inhibition protects against osteophyte formation [$P = 0.0098$ for main effect XAV by two-way ANOVA, $n = 8$ per group]. (E) Immunohistochemical staining and quantification for AXIN2 in developing osteophytes in *Anp32a*$^{-/-}$ mice in the DMM model treated or not with XAV or NAC (representative images of $n = 5$ mice per group) [$P = 0.002$ for interaction between treatments and control by two-way ANOVA, $n = 5$ per group]. Scale bar: 200 μm (B–D) 50 (C–E) μm.

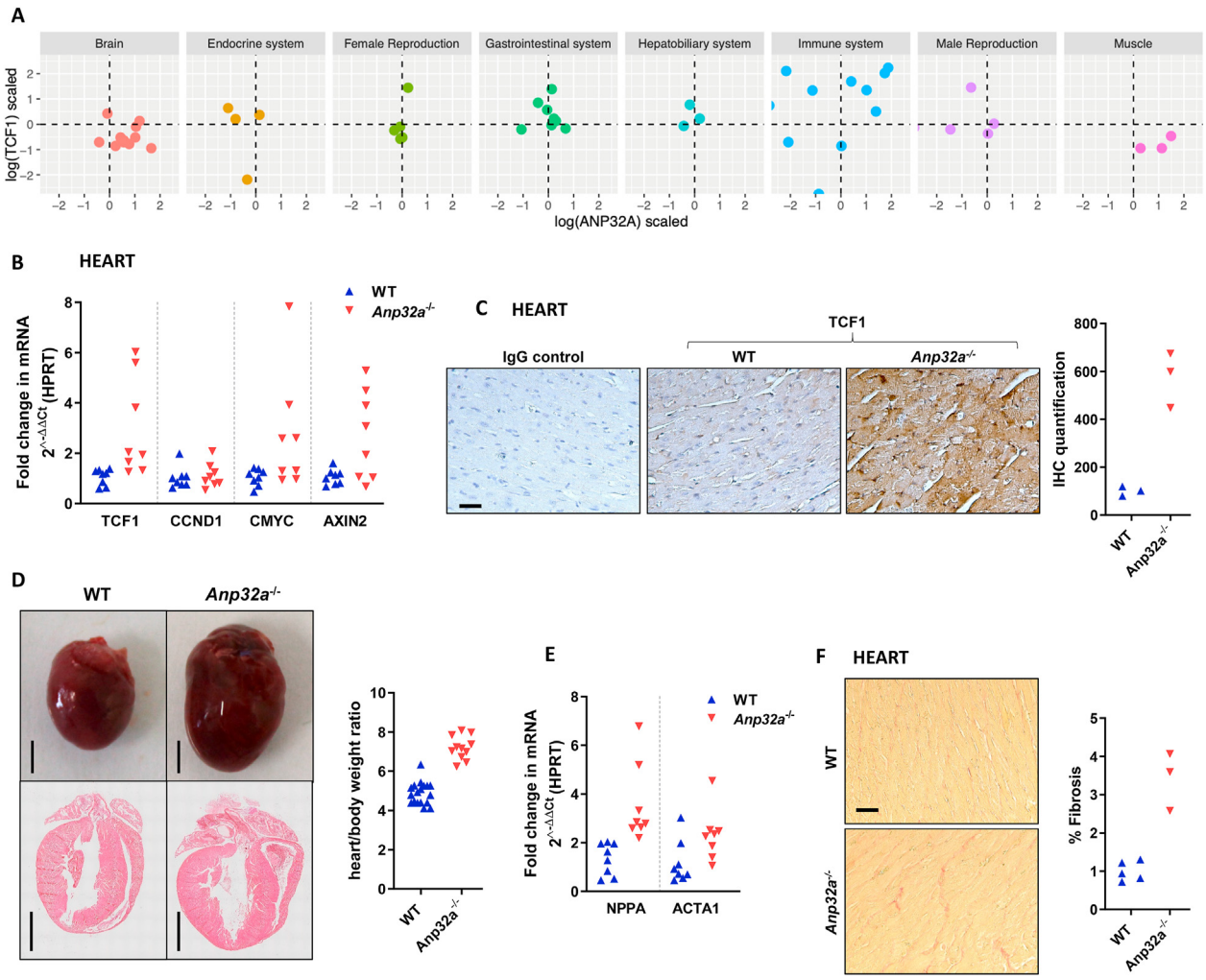

Fig. 5

Osteoarthritis and Cartilage

Regulation of Wnt signaling by ANP32A in tissues beyond cartilage. (A) Selected Protein Atlas data showing the inverse relationship between ANP32A and Wnt target gene TCF1 in brain and muscle tissues compared to other systems. Data are presented as scaled variables. (B) Real-time PCR analysis of Wnt target gene expression ($n = 8$) in heart from 20-week-old male WT and *Anp32a*$^{-/-}$ mice ($P = 0.038$ by MANOVA - *Axin2* data did not show homogeneity of variance and were not included in the model). (C) Immunohistochemical staining and quantification for TCF1 in 20-week-old male WT and *Anp32a*$^{-/-}$ mice hearts ($P = 0.0005$ by *t*-test). Scale bar 50 μm. (D) Macroscopic and Hematoxylin-Eosin stained hearts demonstrating cardiac hypertrophy in *Anp32a*$^{-/-}$ compared to WT mice and heart weight/body weight ratio in 20-week old male WT and *Anp32a*$^{-/-}$ mice ($P < 0.0001$ by *t*-test). Scale bar 2 mm. (E) Real-time PCR analysis of hypertrophy markers *natriuretic peptide precursor A (Nppa)* and *skeletal muscle α-actin (Acta1)* ($n = 8$) in heart from 20-week old male WT and *Anp32a*$^{-/-}$ mice ($P = 0.0011$ (*Nppa*), $P = 0.0125$ (*Acta1*) by *t*-test). (F) Picrosirius red staining showing increased amounts of fibrotic tissue in hearts from 20-week-old *Anp32a*$^{-/-}$ mice compared to WT ($P = 0.0005$ by *t*-test). Scale bar 50 μm.

and *ANP32A* expression showed an inverse relationship, and *Anp32a* expression was reported to be downregulated in cardiac hypertrophy[22]. Besides increased Wnt target gene expression, loss of *Anp32a* resulted in an increased heart/body weight ratio in mice and more fibrosis. Wnt signaling is activated in cardiac hypertrophy and several studies have reported anti-hypertrophic effects for Wnt inhibitors[23]. Our findings suggest that ANP32A may protect against the development of cardiac hypertrophy via limiting Wnt signaling. Thus, increasing ANP32A expression may be beneficial in this context.

We further show that Wnt signaling is enhanced in hippocampus of *Anp32a*-deficient mice. In a genetic mouse model of Alzheimer's disease, downregulating ANP32A restored synaptic plasticity and memory loss[27,28]. The pathological role of ANP32A in Alzheimer's disease contrasts with its protective role in cerebellar ataxia[1], suggesting that ANP32A has tissue-specific functions within different brain regions. In line with ANP32A's pathological involvement in Alzheimer's disease, ANP32A is reported to be increased in brain of Alzheimer's disease patients and in disease mouse models[25,26]. Overexpression of ANP32A in hippocampus

induced memory impairments in mice[29]. ANP32A's pathological roles were not previously linked to a deficit in Wnt signaling, which has been extensively demonstrated to contribute to cognitive decline in Alzheimer's disease[30,31]. Thus, our study potentially sheds light on the molecular mechanism underlying ANP32A's link to Alzheimer's disease, and supports that targeting ANP32A may prevent memory deficits by restoring the Wnt signaling balance, often lost in the ageing brain[43].

This study has limitations worth mentioning. *In vivo* experiments were performed with mice with a global deletion of the *Anp32a* gene as a conditional *Anp32a* allele is currently unavailable. Although unlikely, features reported here may not primarily be caused by the absence of ANP32A in the particular explored tissue, but secondary to other events occurring in other tissues. Thus, this study stimulates research to corroborate the mechanisms reported here in a more tissue-specific manner. Of note, despite the important role of Wnt signaling during development, we did not see striking phenotypic abnormalities in the genetic model. We hypothesize that during development, loss of *Anp32a* can be compensated by other molecules that regulate Wnt signaling, or that there is some degree of functional redundancy with related family members ANP32B and ANP32D. In addition, translation of preclinical interventions in mouse models, in particular of osteoarthritis, has been challenging. Many factors may play a role in this. Among these the existence of multiple human osteoarthritis endophenotypes is not well represented in the post-traumatic DMM model, and the impact of ageing on the chondrocyte's identity and molecular program is challenging to mimic in mice.

In conclusion, our study identifies that ANP32A controls the transcriptional response of Wnt signaling in cartilage, heart and hippocampus, suggesting ANP32A as a therapeutic target for osteoarthritis and other diseases associated with dysregulation in this central signaling cascade. This insight, together with our previous report discovering ANP32A as regulator of the antioxidant defense, position ANP32A as a critical node regulating diverse key signaling networks in the cell. As ANP32A expression is dysregulated in several diseases, further research should focus on factors that control ANP32A expression. Notably, our study provides preclinical evidence for augmented therapeutic efficacy of a combinatorial treatment with Wnt inhibitors and antioxidants in osteoarthritis. Future studies could explore this combination in large animal models for osteoarthritis, and in other pathologies in which Wnt hyper-activation and oxidative stress simultaneously occur.

Author contributions

R.J.L. and S.M. planned the study and designed all the *in vitro*, ex vivo, and *in vivo* experiments. F.M.F.C. performed the animal experiments. S.M., X.W., A.R. and T.P. performed the *in vitro* experiments. R.C.A. and I.M. performed the analysis of the RAAK study. R.J.L. is responsible for all the other statistical analyses. A.S provided essential materials. S.M., R.J.L. and F.M.F.C. wrote the manuscript.

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Conflict of interest

Leuven Research and Development, the technology transfer office of KU Leuven, has received consultancy, speaker fees and research grants on behalf of R.J.L. from Abbvie, Amgen (formerly Celgene), Boehringer-Ingelheim, Eli-Lilly, Galapagos, Janssen, Kabi-Fresenius, MSD, Novartis, Pfizer, Samumed, Sandoz and UCB. The other authors declare that they have no competing financial interests.

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Supplementary data

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