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EXTENDED REPORT

# Tyrosine kinase Fyn promotes osteoarthritis by activating the β-catenin pathway

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## ABSTRACT

**Objectives** To investigate the role of tyrosine kinase Fyn in the development of osteoarthritis (OA) and the underlying mechanisms, and to define whether targeting Fyn could prevent OA in mice.

**Methods** Cartilage samples from normal and aged mice were analysed with proteome-wide screening. Fyn expression was examined with immunofluorescence in human and age-dependent or experimental mouse OA cartilage samples. Experimental OA in Fyn-knockout mice was induced by destabilisation of the medial meniscus. Primary cultured mouse chondrocytes were treated with proinflammatory cytokine interleukin-1β. The inhibitor of Src kinase family, AZD0530 (saracatinib), and inhibitor of Fyn, PP1, were used to treat experimental OA in mice.

**Results** Fyn expression was markedly upregulated in human OA cartilage and in cartilage from aged mice and those with post-traumatic OA. Fyn accumulates in articular chondrocytes and interacts directly with and phosphorylates β-catenin at Tyr142, which stabilises β-catenin and promotes its nuclear translocation. The deletion of Fyn effectively delayed the development of post-traumatic and age-dependent OA in mice. Fyn inhibitors AZD0530 and PP1 significantly attenuated OA progression by blocking the β-catenin pathway and reducing the levels of extracellular matrix catabolic enzymes in the articular cartilage.

**Conclusions** Fyn accumulates and activates β-catenin signalling in chondrocytes, accelerating the degradation of the articular cartilage and OA development. Targeting Fyn is a novel and potentially therapeutic approach to the treatment of OA.

## INTRODUCTION

Osteoarthritis (OA) is an age-related or post-traumatic degenerative joint disease that is characterised by the loss of articular cartilage, the hypertrophic differentiation of chondrocytes, subchondral bone remodelling, synovial inflammation and osteophyte formation.[1 2] Despite the identified risk factors, which include ageing, previous joint injury, obesity, genetics, sex and anatomical factors related to joint shape and alignment, the exact pathogenesis of OA remains undefined.[3 4] The well-accepted pathogenic mechanisms of OA include the increased production of matrix-degrading enzymes, such as the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), the degradation of the cartilage-specific extracellular matrix (ECM) and chondrocyte apoptosis.[5–8] There is still no effective disease-modifying treatment for OA and a novel drug for its treatment is urgently required. Therefore, understanding the mechanisms that control chondrocyte hypertrophy and regulate the expression of ECM-degradation-related genes is important for developing effective therapies for OA.

Accumulating data suggest that canonical WNT/β-catenin signalling plays an important role in regulating the pathogenesis of OA.[9–13] Increased levels of β-catenin have been reported in chondrocytes within areas of degenerated cartilage. The activation of β-catenin signalling in articular chondrocytes in adult mice leads to premature chondrocyte differentiation and the development of an OA-like phenotype.[14] Furthermore, the activation of β-catenin in mature cartilage cells stimulates their hypertrophy, matrix mineralisation and the expression of MMP13 and vascular endothelial growth factor.[15–18] However, the mechanism by which WNT/β-catenin is activated during OA and cartilage degeneration has not been fully clarified.

In this study, we show that the non-receptor tyrosine kinase Fyn, a member of the Src family kinases (SFKs), accumulates in the cartilage of aged mice, mice with post-traumatic OA and human OA cartilage. Fyn directly phosphorylates and stabilises β-catenin, promoting its nuclear translocation and activation, leading to the enhanced expression of collagen X, MMP13 and ADAMTS5, and the accelerated degradation of the ECM. We thus identify, for the first time, the critical role of Fyn in OA through its activation of β-catenin signalling and provide a potential novel therapeutic target for OA.

## RESULTS

### Fyn accumulates in articular cartilage of aged mice, mice with post-traumatic OA and humans with OA

OA is strongly linked to ageing, but the mechanism of this link is poorly understood. We first used proteome-wide screening to identify proteins involved in cartilage degeneration by comparing the articular cartilage from young (2-month-old) and aged (12-month-old) mice. Among the 5015 proteins identified, 303 proteins were upregulated and 115 proteins were downregulated (>1.5-fold) in the cartilage of the aged mice compared with that of the young mice (online supplementary table 1). The tyrosine kinase Fyn, but no other member of the SFK family (including Fgr, Lyn, Csk and Hck), was the most strongly upregulated protein

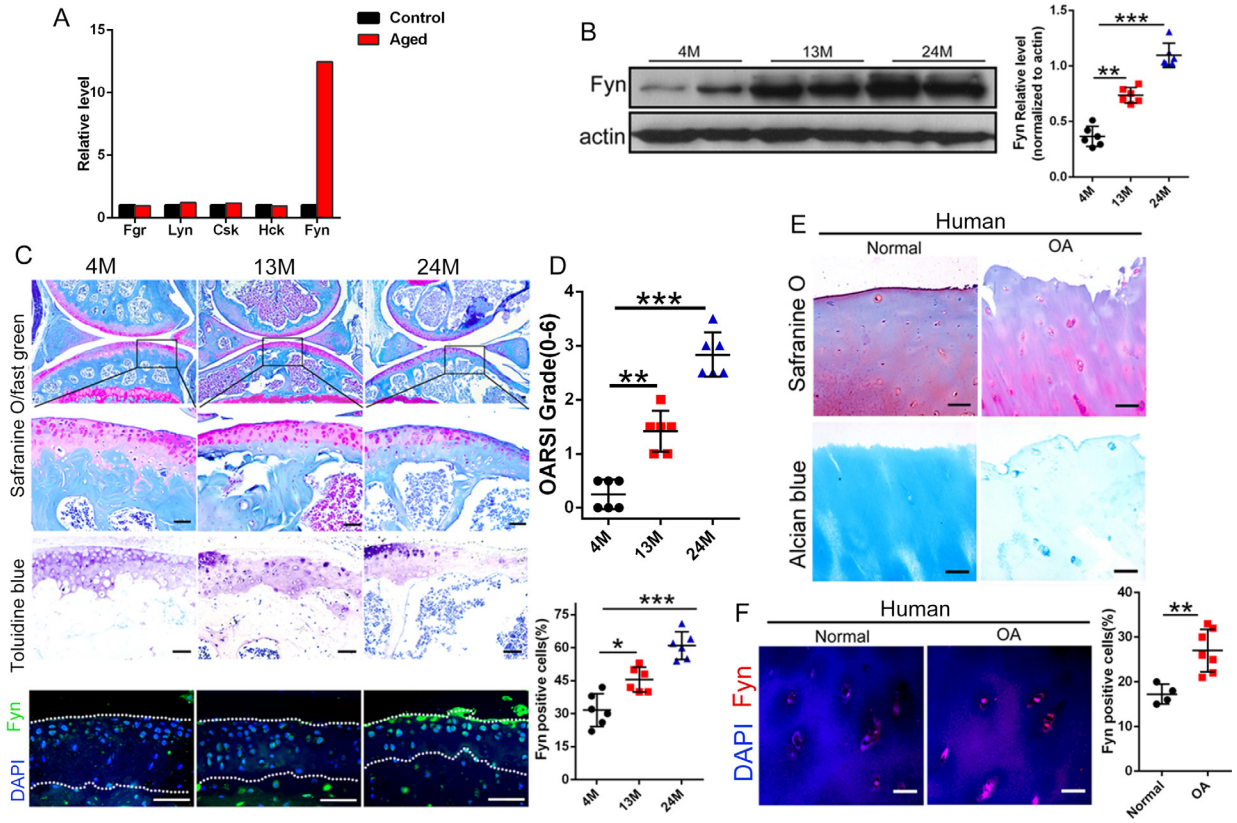


**Figure 1** Fyn accumulates in articular cartilage of aged mice and patients with osteoarthritis (OA). (A) Quantitative analysis of Src family kinases expression with a proteome-wide screen for the proteins involved in cartilage degeneration by analysing the articular cartilage from young (2-month-old) and aged (12-month-old) mice. (B) Immunoblotting analysis (left) and quantification (right) of Fyn in dissected articular cartilage from mice 4, 13 and 24 months old. Data are representative of three independent experiments, **P<0.01, ***P<0.001. (C) Upper: Safranin O and Fast Green staining of sagittal sections of joints from mice collected at 4, 13 and 24 months of age; proteoglycan (red) and bone (blue). The tibia medial compartments of the mice are shown at higher resolution. Middle: toluidine blue staining of sagittal sections of joints from aged mice. Lower: immunofluorescence analyses (left) and quantification (right) of Fyn (green) in articular chondrocytes; n=6. *P<0.05, ***P<0.001. Scale bars, 50 µm. (D) Osteoarthritis Research Society International (OARSI) grades for mice 4, 13 and 24 months old, n=6. **P<0.01, ***P<0.001. (E) Safranin O and Fast Green staining (upper) and Alcian Blue staining (lower) of cartilage from normal humans and those with OA. Scale bars, 50 µm. (F) Immunofluorescence analysis (left) and quantification (right) of Fyn (red) in cartilage chondrocytes from normal humans (n=4) and patients with OA (n=7); **P<0.01. Scale bars, 50 µm. All data are shown as means±SD.

(12.47-fold) in the aged cartilage (figure 1A). The marked increased Fyn in aged cartilage was confirmed with a western blotting analysis of the articular cartilage from mice aged 4, 13 and 24 months (figure 1B). The accumulation of Fyn was accompanied by the degeneration of the cartilage and increased Osteoarthritis Research Society International (OARSI) grades in the aged mice (figure 1C,D). Immunofluorescent staining confirmed that Fyn accumulates in the articular chondrocytes of aged mice (figure 1C).

We then assessed the expression of Fyn in a surgically induced post-traumatic OA mouse model. Consistent with our previous finding, Fyn expression was low in the undamaged articular cartilage at baseline, but its level increased dramatically in OA mice along with the increased cartilage damage (4 and 8 weeks after surgery performed to destabilise the medial meniscus (DMM surgery)) (online supplementary figure 1A–D). To determine whether Fyn levels were elevated in human OA articular cartilage, we compared Fyn expression in aged (67.00±3.03 years) human OA cartilage from subjects undergoing total knee replacement and normal cartilage samples from young (30.25±8.18 years) traffic incident victims with no history of arthritic disease. Marked elevation of Fyn levels was detected in the aged and OA cartilage chondrocytes with an immunofluorescence analysis, together with the degeneration and loss of structure in the OA cartilage (figure 1E,F). Taken together, our findings demonstrate that the Src kinase family member Fyn accumulates in degenerated and damaged articular cartilage in aged mice and patients with OA.

## Deletion of Fyn prevents the development of post-traumatic and aged-related OA in mice

To determine the significance of Fyn accumulation in the articular chondrocytes of degenerated or damaged cartilage, we used Fyn-knockout mice (Fyn-KO) from the Jackson Laboratory (*Fyn*$^{tm1Sor/J}$, stock no. 012468). In gross appearance, the Fyn-KO mice were slightly smaller than the control mice (online supplementary figure 2A), and their body weights and limb lengths were also slightly lower (online supplementary figure 2B,C). The deletion of Fyn in the cartilage was confirmed in 8-week-old mice with Fyn staining and western blotting (figure 2A,B). No discernible differences in the morphology or organisation of the articular cartilage or growth plates were apparent between the Fyn-KO mice and the littermate control mice at 8 weeks of age (online supplementary figure 2D–F). These results suggest that the deletion of *Fyn* did not induce skeletal developmental abnormalities in mice.

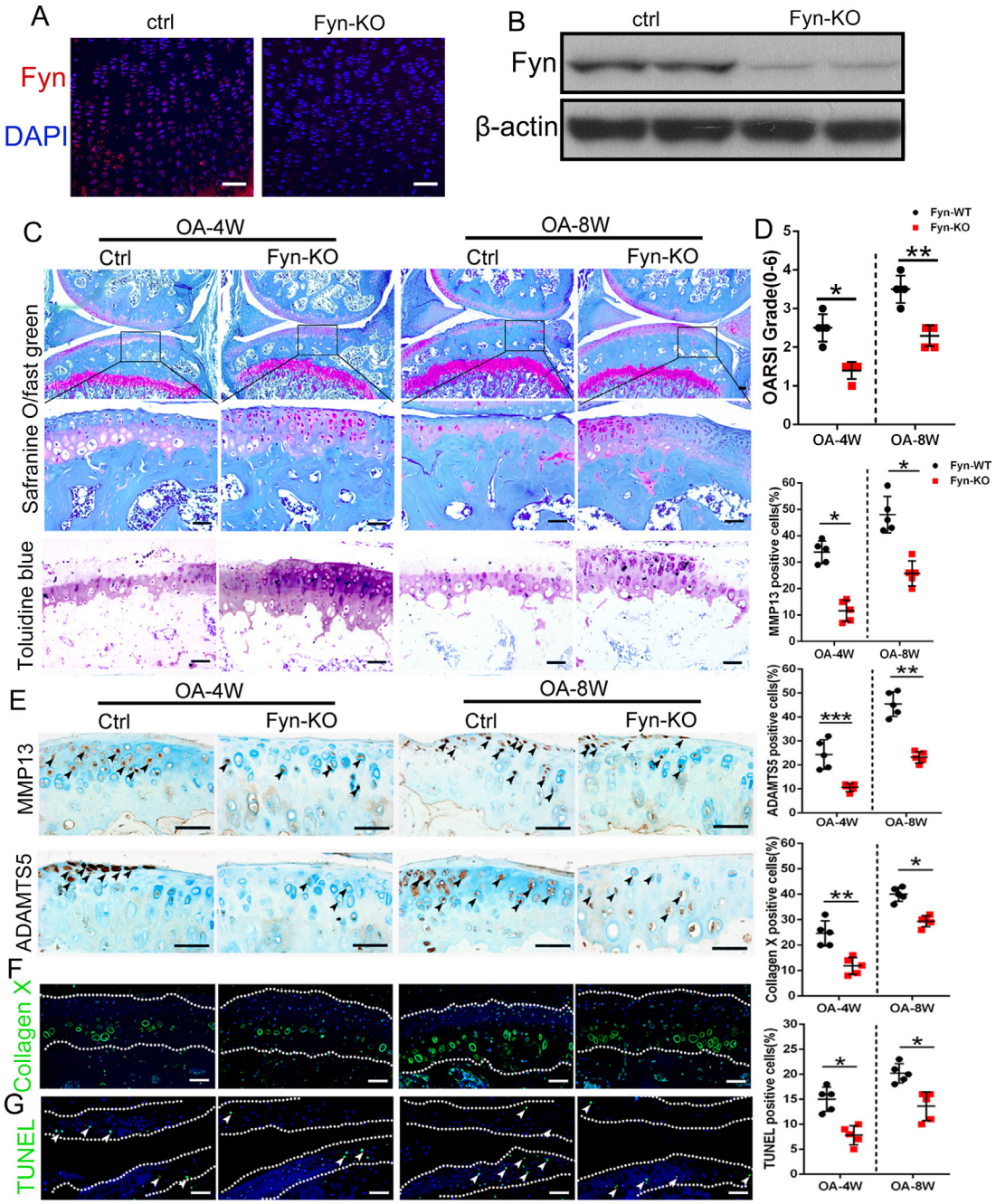


**Figure 2** Deletion of Fyn delays development of post-traumatic osteoarthritis (OA) in mice. (A) Immunofluorescence analysis of Fyn (red) expression in joints of control and Fyn-knockout (Fyn-KO) mice. Data are representative of three independent experiments. (B) Immunoblotting analysis of Fyn levels in cartilage from control and Fyn-KO mice; the β-actin level was used as the internal control. Data are representative of three independent experiments. (C) Safranin O/Fast Green (upper) and toluidine blue staining (lower) of joints from control and Fyn-KO mice at 4 and 8 weeks after surgery performed to destabilise the medial meniscus (DMM surgery). n=5. Scale bars, 50 µm. (D) Osteoarthritis Research Society International (OARSI) grades for the joints of control and Fyn-KO mice at 4 and 8 weeks after DMM surgery. n=5. (E) Immunostaining (left) and quantification (right) of matrix metalloproteinase 13 (MMP13) (upper) and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (lower) in the joint cartilage of control and Fyn-KO mice at 4 and 8 weeks after DMM surgery. Black arrowheads indicate positive cells. n=5. Scale bars, 50 µm. Immunofluorescence analysis (left) and quantification (right) of collagen X (F) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (G) in joint cartilage from control and Fyn-KO mice at 4 and 8 weeks after DMM surgery. White arrowheads indicate TUNEL-positive cells. n=5. Scale bars, 50 µm. *P<0.05, **P<0.01, ***P<0.001. All data are shown as means±SD.

We next examined the effects of Fyn deficiency on the development of post-traumatic and age-dependent OA. DMM surgery was performed on 6-week-old Fyn-KO mice and their littermate control mice (Control). The control mice developed moderate and severe OA at 4 and 8 weeks after surgery, respectively. Interestingly, the Fyn-KO mice showed markedly reduced cartilage degradation (figure 2C), significantly lower OARSI grades (figure 2D) and decreased synovial inflammation (online supplementary figure 3A) at both 4 and 8 weeks after DMM surgery. Expression of MMP13 and ADAMTS5 (figure 2E), the chondrocyte hypertrophic marker collagen X (figure 2F) and osteocalcin in the subchondral bone (online supplementary figure 3B)

was dramatically reduced in the cartilage of the Fyn-KO mice compared with that of the control mice. Moreover, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining showed that the number of apoptotic chondrocytes was significantly reduced in the articular cartilage of the Fyn-KO mice (figure 2G). We also noticed reduced cartilage degradation in 12-month-old Fyn-KO mice as compared with that in control mice (online supplementary figure 3C,D). Together, these data suggest that Fyn plays a role in OA development and that the loss of Fyn efficiently prevents the development of post-traumatic and age-dependent OA in mice.

### Fyn interacts with, phosphorylates (Tyr142) and stabilises β-catenin in chondrocytes

To explore the mechanism by which Fyn promotes the development of OA, we combined immunoprecipitation (IP) with a proteomic analysis to identify the Fyn-interacting proteins in the chondroprogenitor cell line ATDC5. Interestingly, the peptide sequence of the β-catenin protein was found in the purified complex, indicating that β-catenin is a potential binding partner of Fyn (figure 3A). The interaction between β-catenin and Fyn was confirmed in ATDC5 cells (figure 3B), primary chondrocytes (online supplementary figure 4A) and cartilage tissues (online supplementary figure 4B) with IP analyses. Double staining for β-catenin and Fyn also showed that β-catenin colocalises with Fyn in ATDC5 cells (figure 3C). Previous studies have demonstrated that tyrosine kinases, such as Fyn, phosphorylate β-catenin at multiple sites (including Tyr142) and promote the disassociation of the E-cadherin–catenin complex, resulting in the loss of cadherin-mediated cell–cell adhesion and an increase in cytoplasmic β-catenin. This represents an alternative mechanism for activating β-catenin signalling.[19 20] To determine whether Fyn phosphorylates β-catenin in chondrocytes, we conducted an in vitro Fyn kinase assay in ATDC5 cells. As shown in figure 3D, Fyn immunoprecipitated β-catenin phosphorylated at Tyr142 in vitro, and this phosphorylation was inhibited by the Fyn kinase inhibitor, PP1, or AZD0530. Importantly, the expressions of β-catenin and its phosphorylation (Tyr142) were

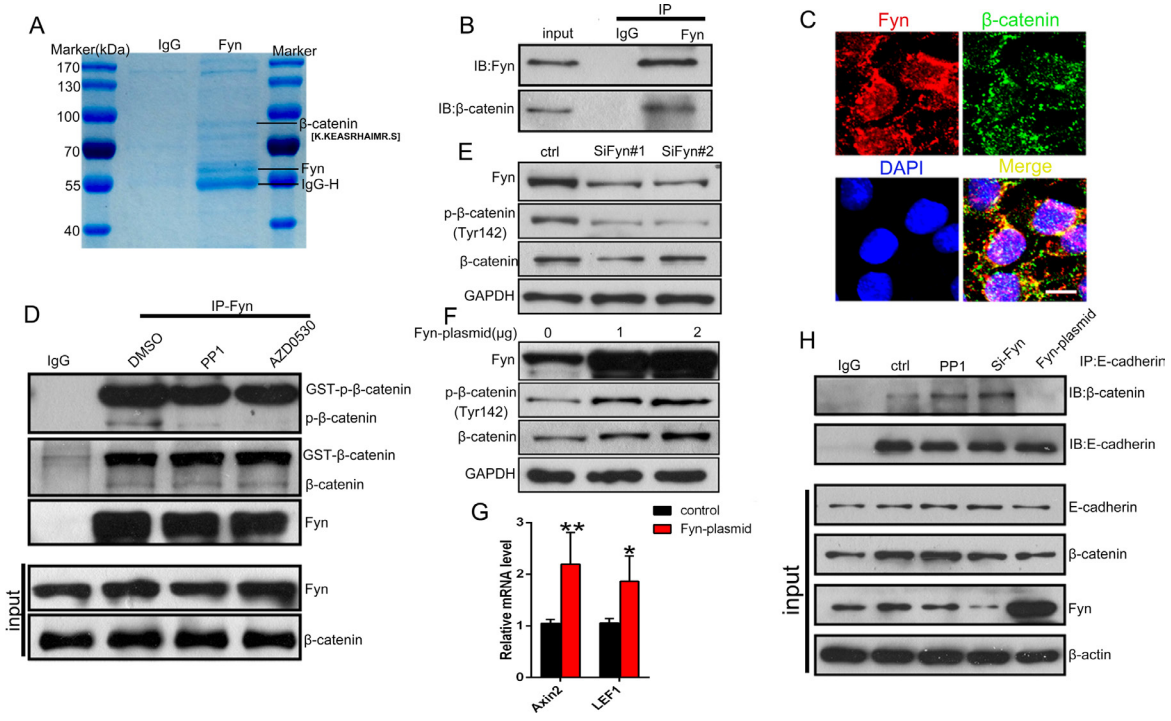


**Figure 3** Fyn interacts with, phosphorylates (Tyr142) and stabilises β-catenin in chondrocytes. (A) ATDC5 cell extracts were subjected to immunoprecipitation with an anti-Fyn antibody. The immunoprecipitate–protein complex was separated with SDS–PAGE and the gel was then stained with Coomassie Brilliant Blue. The proteins were digested in-gel with proteases and identified with an LC–MS/MS analysis. The identified peptide sequence of β-catenin is shown (K.KEASRHAIMR.S). (B) Fyn was immunoprecipitated from ATDC5 cells with an anti-Fyn antibody. The presence of β-catenin and Fyn in these immunoprecipitates was evaluated with immunoblotting. Data are representative of three independent experiments. (C) ATDC5 cells were fixed for immunofluorescence analysis. Fyn was detected with a primary anti-Fyn antibody and Alexa-Fluor-594-conjugated goat anti-mouse IgG antibody, and β-catenin was detected with a primary anti-β-catenin antibody and Alexa-Fluor-488-conjugated goat anti-rabbit IgG antibody. Representative cells in the same field from each experimental group are shown. Scale bar, 10 μm. (D) ATDC5 cells were pretreated with PP1, AZD0530 or DMSO for 24 hours, and then immunoprecipitated with an anti-Fyn antibody. The precipitated Fyn was assayed for kinase activity against recombinant GST-tagged β-catenin. Data are representative of three independent experiments. (E) ATDC5 cells were transfected with negative control siRNA or two Fyn-specific small interfering RNAs (siRNAs) and different amounts (1 or 2 μg) of Fyn-encoding plasmid (F) for 60 hours. Expression levels of Fyn, β-catenin, p-β-catenin (Tyr142) and GAPDH were detected with immunoblotting. Data are representative of three independent experiments. (G) Relative mRNA expression levels of two Wnt canonical targets Axin2 and LEF1 in ATDC5 cells after transfected with a Fyn-encoding plasmid (1 μg). n=3, *P<0.05, **P<0.01. All data are shown as means±SD. (H) ATDC5 cells were pretreated with DMSO (ctrl) or PP1 for 24 hours, and transfected with siRNA or Fyn-encoding plasmid for 60 hours. The cell extracts were subjected to immunoprecipitation with an anti-E-cadherin antibody, and the levels of β-catenin and E-cadherin were analysed with immunoblotting. Data are representative of three independent experiments. DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; IB, immunoblotting; IP, immunoprecipitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

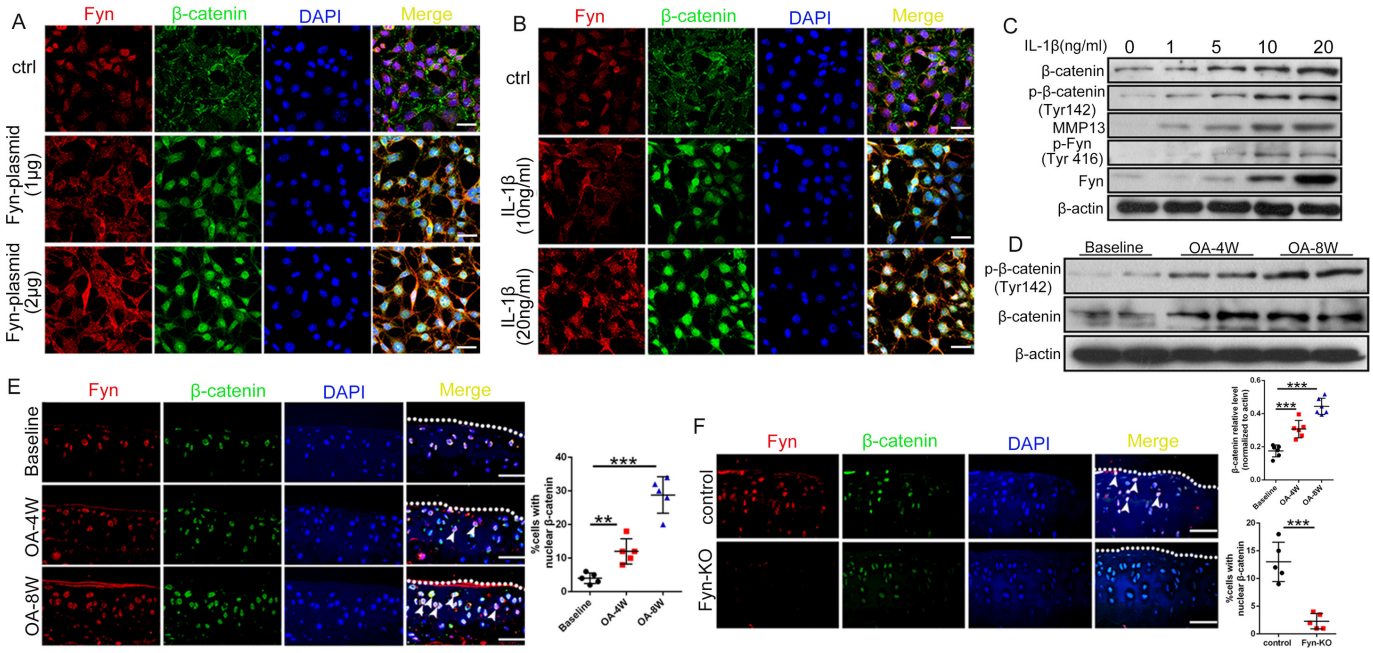


**Figure 4** Fyn promotes the nuclear translocation of β-catenin and activates the β-catenin pathway in chondrocytes during osteoarthritis (OA) development. (A) Representative images of immunofluorescence for Fyn (red) and β-catenin (green) in ATDC5 cells after transfection with a Fyn-encoding plasmid (0, 1 or 2 μg) for 60 hours. Scale bar, 10 μm. (B) Representative images of immunofluorescence for Fyn (red) and β-catenin (green) in ATDC5 cells after treatment with different concentrations of IL-1β (0, 10 or 20 ng/mL) for 24 hours. Scale bar, 10 μm. (C) Immunoblotting analysis of β-catenin, p-β-catenin (Tyr142), matrix metalloproteinase 13 (MMP13), p-Fyn (Tyr416), Fyn, and β-actin in ATDC5 cells after incubation with different concentrations of IL-1β (0, 1, 5, 10 or 20 ng/mL) for 24 hours. Data are representative of three independent experiments. (D) Immunoblotting analysis of β-catenin, p-β-catenin (Tyr142) and β-actin in cartilage from sham-treated C57BL/6J mice or C57BL/6J mice at 4 or 8 weeks after induction of traumatic OA. Expression of β-catenin was quantified. Data are representative of three independent experiments. ***P<0.001. (E) Representative images of immunofluorescence (left) for Fyn (red) and β-catenin (green) and quantification (right) of cells with nuclear β-catenin in articular chondrocytes from sham-treated C57BL/6J mice or C57BL/6J mice 4 or 8 weeks after induction of traumatic OA. n=5. **P<0.01, ***P<0.001. Scale bar, 50 μm. (F) Representative images of immunofluorescence (left) for Fyn (red) and β-catenin (green) and quantification (right) of cells with nuclear β-catenin in articular chondrocytes from control and Fyn-KO mice. n=5. ***P<0.001. Scale bar, 50 μm. White arrows indicate β-catenin-positive nuclei (E and F). All data are shown as means±SD.

diminished by *Fyn* knockdown with a Fyn-directed small interfering RNAs (siRNAs) (figure 3E, online supplementary figure 4C), but were enhanced by Fyn overexpression from a Fyn-encoding plasmid in ATDC5 cells (figure 3F, online supplementary figure 4D). Additionally, the mRNA levels of Wnt canonical target Axin2 and LEF1 were also increased as Fyn was enhanced in ATDC5 cells (figure 3G), suggesting that Fyn activated β-catenin signalling. To verify the critical role of Fyn in the β-catenin signalling pathway, the formation of the cadherin–catenin complex was examined in ATDC5 cells. The inhibition of Fyn by PP1 or the downregulation of Fyn expression with siRNAs stabilised the cadherin–catenin complex, whereas Fyn overexpression disrupted the complex (figure 3H). Taken together, these data suggest that Fyn interacts with, phosphorylates (Tyr142) and stabilises β-catenin, causing the dissociation of the cadherin–catenin complex in chondrocytes.

### Fyn activates the β-catenin pathway and promotes OA development

To evaluate the role of Fyn in β-catenin signalling in chondrocytes during OA, we doubly stained chondrocytes for Fyn and β-catenin in vitro and in vivo. When we transfected ATDC5 cells with increasing amounts of a Fyn-encoding plasmid (0, 1 or 2 μg), we observed that β-catenin translocated from the cell membrane to the nucleus as the level of Fyn increased (figure 4A). We then treated the ATDC5 cells with the proinflammatory factor interleukin 1β (IL-1β; 0, 10 and 20 ng/mL) to mimic the degradation of the ECMs and proteoglycans in OA.[21] IL-1β dose-dependently induced the accumulation of Fyn and β-catenin in both the cytoplasm and nuclei of the cells (figure 4B) and increased the levels of Fyn expression and phosphorylation (Tyr416), β-catenin expression and phosphorylation (Tyr142), and MMP13 expression (figure 4C). Similar results were observed in IL-1β-stimulated primary chondrocytes (online supplementary figure 5A,B). We then assessed the levels of β-catenin and its phosphorylation in cartilage from aged mice and mice with post-traumatic OA. As expected, the levels of β-catenin and its phosphorylation increased with ageing (online supplementary figure 5C) and the exacerbation of OA (figure 4D). Consistent with this, double staining for Fyn and β-catenin demonstrated the accumulation and nuclear translocation of β-catenin in the chondrocytes of aged mice (online supplementary figure 5D) and mice with surgically induced OA (figure 4E). Furthermore, the levels of β-catenin and its nuclear localisation were markedly lower in the articular chondrocytes of post-traumatic Fyn-KO mice than in the control mice (figure 4F). Wnt3a is an activator of canonical WNT/β-catenin pathway. Knockdown of Fyn did not reduce the Wnt3a-upregulated β-catenin (online supplementary figure 4E). Although WNT antagonist DKK-1 or SFRP1 prevented Wnt3a-induced accumulation of β-catenin, they did not affect upregulation of β-catenin caused by overexpression of Fyn in ATDC5 cells (online supplementary figure 4F). Thus, our results

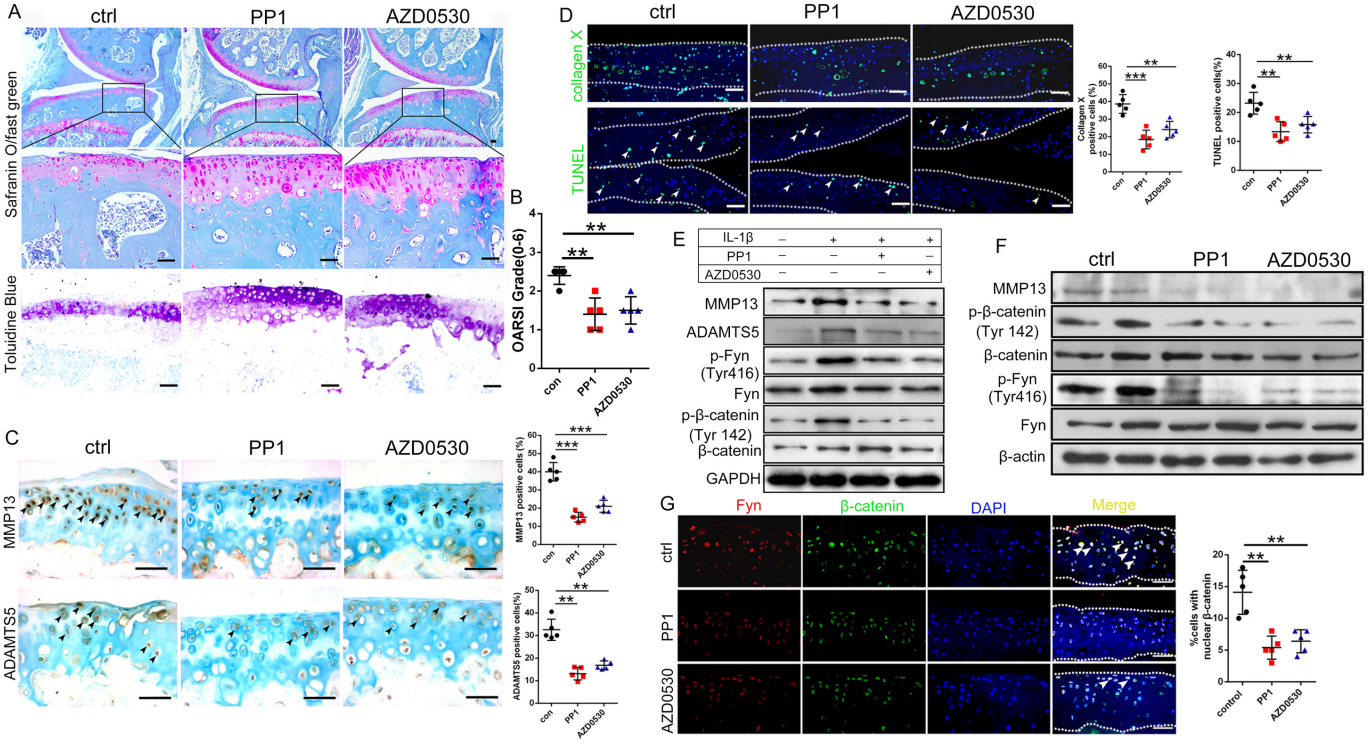


**Figure 5** Fyn inhibitors AZD0530 and PP1 prevent surgically induced OA in mice by inhibiting β-catenin signalling. (A) Safranin O/Fast Green staining (upper), toluidine blue staining (lower) and Osteoarthritis Research Society International (OARSI) grades (right) (B) of joints from mice that underwent surgery to destabilise the medial meniscus (DMM mice) treated with vehicle, PP1 or AZD0530 for 4 weeks. n=5, **P<0.01. Scale bar, 50 µm. (C) Representative images of immunostaining (left) and quantification (right) of matrix metalloproteinase 13 (MMP13) (upper) and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (lower) in joint cartilage from DMM mice treated with vehicle, PP1 or AZD0530 for 4 weeks. n=5, **P<0.01, ***P<0.001. Scale bar, 50 µm. (D) Representative images of immunofluorescence analysis (left) and quantification (right) of collagen X (upper) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (lower) in joint cartilage from DMM mice treated with vehicle, PP1 or AZD0530 for 4 weeks. White arrowheads indicate positive cells. n=5. **P<0.01, ***P<0.001. Scale bar, 50 µm. (E) Immunoblotting analysis of MMP13, ADAMTS5, p-Fyn (Tyr416), Fyn, p-β-catenin (Tyr142), β-catenin and GAPDH in primary chondrocytes after incubation with vehicle, IL-1β, IL-1β plus PP1 or IL-1β plus AZD0530 for 24 hours. Data are representative of three independent experiments. (F) Immunoblotting analysis of the indicated proteins in articular chondrocytes from DMM mice treated with vehicle, PP1 or AZD0530 for 4 weeks. Data are representative of three independent experiments. (G) Representative images of immunofluorescence (left) for Fyn (red) and β-catenin (green) and quantification (right) of cells with nuclear β-catenin in articular chondrocytes from DMM mice treated with vehicle, PP1 or AZD0530 for 4 weeks. White arrows indicate β-catenin in the nuclei. n=5 per group. **P<0.01. Scale bar, 50 µm. All data are shown as means±SD. GADPH, glyceraldehyde-3-phosphate dehydrogenase .

demonstrate that Fyn activates the β-catenin pathway independent of Wnt signalling and enhances the nuclear translocation of β-catenin in articular cartilage chondrocytes during the development of OA.

We next investigated whether Fyn accumulation promotes OA development through the β-catenin pathway. ICG-001 is a selective low-molecular-weight inhibitor that antagonises β-catenin/TCF-mediated transcription.[22 23] After the articular injection of ICG-001 for 4 or 8 weeks, ICG-001 treatment markedly reduced cartilage damage, maintained the proteoglycan in the articular cartilage (online supplementary figure 6A) and reduced the arthritis grades during the development of OA (online supplementary figure 6B). The expression levels of MMP13 and ADAMTS5 also decreased correspondingly in the degenerated cartilage after ICG-001 injection (online supplementary figure 6C,D). Subsequently, we measured the effects of Fyn, the β-catenin inhibitor ICG-001 and the β-catenin activator SKL2001 on IL-1β-treated ATDC5 cells. Fyn overexpression reinforced the effects of IL-1β on the upregulation of MMP13 and ADAMTS5, whereas treatment with ICG-001 counteracted the Fyn-promoted expression of both enzymes induced by IL-1β (online supplementary figure 6E). SKL2001 upregulated the IL-1β-stimulated expression of MMP13 and ADAMTS5 in chondrocytes, whereas Fyn knockdown downregulated it (online supplementary figure 6F). These results demonstrate that the β-catenin pathway contributes to Fyn-stimulated MMP13 and ADAMTS5 expression in chondrocytes and the development of OA in mice.

### Fyn inhibitors AZD0530 and PP1 inhibit β-catenin signalling and prevent OA development in mice

To investigate the potential use of Fyn as a therapeutic target for OA, we examined the effects of chemical inhibitors of Fyn on the pathogenesis and progression of OA. AZD0530 (also known as 'saracatinib') and PP1 are selective inhibitors of Src kinases (including Fyn), which were used in the treatment of cancer. These two compounds inhibited the kinase activity of Fyn and blocked Fyn phosphorylation (p-Fyn) in ATDC5 cells transfected with Fyn-encoding plasmids (online supplementary figure 7A,B). C57BL/6J mice treated with DMM surgery were orally administered AZD0530 or intraperitoneally injected with PP1 every day, and their joints were collected 4 weeks after surgery. The mice

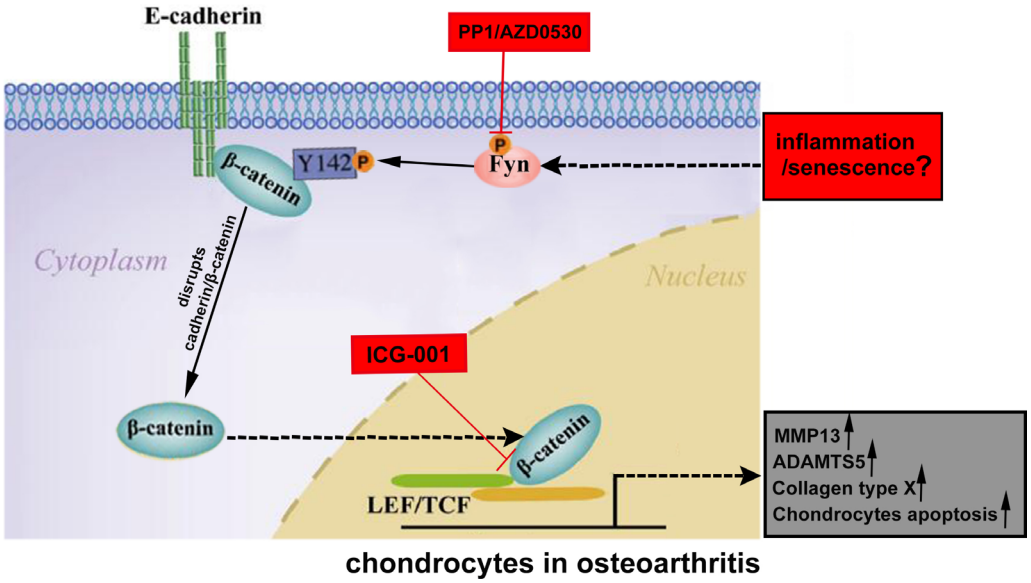


**Figure 6** Proposed model for the role of Fyn in the development of OA. ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs 5; LEF/TCF, lymphoid-enhancing factor/T-cell factor; MMP13, matrix metalloproteinase 13.

treated with AZD0530 or PP1 showed no significant adverse effects and no increase in mortality compared with the controls (body weight shown in online supplementary figure 7C). As expected, AZD0530 and PP1 significantly reduced p-Fyn level in the chondrocytes of the cartilage, confirming its inhibitory effect on Fyn (online supplementary figure 7D). Notably, the AZD0530 and PP1 treatments markedly reduced the destruction of the cartilage (figure 5A), OARSI grades (figure 5B) and synovial inflammation (online supplementary figure 7E) compared with those of the vehicle-treated mice. The levels of MMP13, ADAMTS5 and collagen X, the number of TUNEL-positive chondrocytes and osteocalcin expression in subchondral bone were also significantly reduced in the inhibitor-treated mice (figure 5C,D, online supplementary figure 7F). These results suggest that the inhibition of Fyn kinase activities prevents the development of post-traumatic OA in mice.

We next determined whether AZD0530 or PP1 ameliorates the progression of OA by reducing the phosphorylation of β-catenin. After stimulation with IL-1β, ATDC5 cells were treated with or without AZD0530 or PP1. As indicated in figure 5E, western blotting showed that the upregulation of MMP13 and ADAMTS5 caused by IL-1β stimulation was abolished by AZD0530 or PP1, and the increase in p-β-catenin (Tyr142) was also inhibited. We confirmed the reduced expression of p-β-catenin (Tyr142) with immunofluorescence staining (online supplementary figure 7G). We next measured the expression of p-β-catenin (Tyr142) in cartilage samples from mice with post-traumatic OA treated with or without AZD0530 or PP1. A western blotting analysis confirmed that the expressions of MMP13 and p-Fyn were lower and the expression of p-β-catenin (Tyr142) was significantly reduced in the AZD0530-treated and PP1-treated mice (figure 5F). We also noted that the colocalisation of Fyn and β-catenin in the nuclei of chondrocytes in OA was abolished by treatment with AZD0530 or PP1 (figure 5G). Incubation of human OA cartilage explants with AZD0530 or PP1 also decreased β-catenin expression in the nuclei of chondrocytes (online supplementary figure 7H). Collectively, these findings suggest that AZD0530 and PP1 prevent the development of OA by inhibiting the expression of Fyn-induced phosphorylation β-catenin.

## DISCUSSION

There are currently no effective disease-modifying drugs approved for the treatment of OA. A comprehensive understanding of the pathogenesis in OA is essential for the development of specific and effective drugs to prevent and treat this disease.[24 25] The roles of SFKs in many diseases have been described, including cancer and neurological disorders, and they are therefore emerging as novel pharmacological targets.[26–28] However, to the best of our knowledge, the present study is the first to demonstrate the essential role of the SFK family member Fyn in cartilage degeneration and the development of OA. We found that Fyn accumulates in the degenerated and damaged articular cartilage of ageing mice, mice with experimental OA and humans with OA. We also found that Fyn deficiency protected mice against age-related and trauma-induced cartilage degradation and the development of OA. Importantly, the disruption of Fyn or its chemical inhibition with AZD0530 or PP1 effectively prevented the development of OA in mice, implying that Fyn is potentially a novel therapeutic target for OA, and that the use of drugs AZD0530 and PP1, which inhibit Fyn kinase activity, might be extended to the treatment of OA. We propose a model in which Fyn activates β-catenin signalling, and the accumulation of Fyn, and the tyrosine phosphorylation and nuclear translocation of β-catenin play vital roles in regulating the expression of matrix-degrading enzymes and the development of OA (figure 6). However, the mechanism underlying the accumulation of Fyn in OA and aged articular cartilage remains to be identified.

The activation of β-catenin in articular chondrocytes constitute a pathological mechanism for the conversion of normal articular chondrocytes into terminally differentiated chondrocytes during the development of OA, which is associated with the activation of chondrocyte-maturation genes and matrix degradation.[29] However, the mechanism of β-catenin activation is not fully understood. In the canonical WNT/β-catenin pathway, the activation of WNT signalling leads to the inhibition of GSK-3β activity and β-catenin degradation, resulting in the accumulation of cytoplasmic (signalling) β-catenin.[19] We screened for Fyn-interacting proteins and demonstrated that Fyn interacts with,

phosphorylates β-catenin at Tyr142 and stabilises it independent of Wnt signalling in chondrocytes. Previous studies have shown that tyrosine kinases can phosphorylate β-catenin, releasing it from the cadherin complex and leading to the accumulation of β-catenin in the cytoplasm, which represents an alternative mechanism by which β-catenin signalling is activated.[30] We also confirmed that Fyn phosphorylates β-catenin, thus releasing it from the cadherin complex in chondrocytes, leading to the accumulation and nuclear translocation of β-catenin and the activation of chondrocyte-maturation genes and matrix degradation, which promote the development of OA. This evidence suggests that Fyn activates β-catenin signalling via a Wnt-independent mechanism to promote the pathogenesis and progression of OA.

The established mechanisms that inhibit the development of OA largely depend on the inhibition of enzymes that catabolise cartilage.[2] We found that the deletion of Fyn inhibited the expression of cartilage-catabolising enzymes and attenuated OA in mice. Therefore, further investigation of the targeted inhibition of Fyn for the prevention and treatment of OA is warranted. AZD0530 (saracatinib) is an inhibitor of the SFKs, blocking Src with low (nanomolar) potency and also displaying activity against Fyn.[31] It was originally developed to treat various types of cancer, but was discontinued in phase II trials for its lack of efficacy. PP1 was identified as a high-potency inhibitor of Fyn and acts as a competitive inhibitor of ATP binding. Currently, both AZD0530 and PP1 are mainly under investigation for the treatment of solid tumours, lung cancer and Alzheimer's disease.[32] In this study, we have demonstrated that OA progression can be prevented by targeting Fyn with these two inhibitors. Moreover, the Fyn-stimulated accumulation and nuclear translocation of β-catenin is also inhibited by AZD0530 and PP1. OA-related catabolic enzymes and chondrocyte apoptosis in the articular cartilage decreased significantly after treatment with either inhibitor. This is the first report of the role of SFKs in OA, and the use of SFK and Fyn inhibitors may be successfully extended to its treatment.

In conclusion, in this study, we identified Fyn as a novel target for the prevention and treatment of OA. We also established links between Fyn, β-catenin and OA by demonstrating that the Fyn accumulated in articular chondrocytes during OA interacts directly with and phosphorylates β-catenin, promoting its nuclear translocation and inducing OA-related gene expression. An inhibitor of SFKs, AZD0530, and an inhibitor of Fyn, PP1, delay the development of OA and are therefore potential drugs for the treatment of this disease.

## MATERIALS AND METHODS

Detailed experimental procedures are described in online supplementary materials and methods, including human and experimental OA, animals, histology, immunohistochemistry, cell line and primary chondrocyte culture, iTRAQ labelling and LC–MS/MS proteomics, siRNA and plasmid, antibodies, immunoblotting, immunoprecipitation, TUNEL staining, drug treatment and statistical analysis.

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**Contributors** KL: study design, data acquisition, data analysis, wrote the manuscript, DMM experiments. YueZ, YuwZ, WJ and JS: data analysis, discussion of results, confocal imaging and analysis. SX, QS and DC: data acquisition, histochemistry and western blotting analysis. BH, AL and ML: data acquisition. JS and YJ: statistical analyses. XB: study design, manuscript correction.

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## REFERENCES

1 Glyn-Jones S, Palmer AJR, Agricola R, *et al*. Osteoarthritis. *The Lancet* 2015;386:376–87.
2 Heinegård D, Saxne T. The role of the cartilage matrix in osteoarthritis. *Nat Rev Rheumatol* 2011;7:50–6.
3 Loeser RF, Collins JA, Diekman BO. Ageing and the pathogenesis of osteoarthritis. *Nat Rev Rheumatol* 2016;12:412–20.
4 Prieto-Alhambra D, Judge A, Javaid MK, *et al*. Incidence and risk factors for clinically diagnosed knee, hip and hand osteoarthritis: influences of age, gender and osteoarthritis affecting other joints. *Ann Rheum Dis* 2014;73:1659–64.
5 Glasson SS, Askew R, Sheppard B, *et al*. Erratum: Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 2007;446:102–02.
6 van den Berg WB. Osteoarthritis year 2010 in review: pathomechanisms. *Osteoarthritis Cartilage* 2011;19:338–41.
7 Majumdar MK, Askew R, Schelling S, *et al*. Double-knockout of ADAMTS-4 and ADAMTS-5 in mice results in physiologically normal animals and prevents the progression of osteoarthritis. *Arthritis Rheum* 2007;56:3670–4.
8 Neuhold LA, Killar L, Zhao W, *et al*. Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J Clin Invest* 2001;107:35–44.
9 Blom AB, Brockbank SM, van Lent PL, *et al*. Involvement of the Wnt signaling pathway in experimental and human osteoarthritis: prominent role of Wnt-induced signaling protein 1. *Arthritis Rheum* 2009;60:501–12.
10 Miclea RL, Siebelt M, Finos L, *et al*. Inhibition of Gsk3β in cartilage induces osteoarthritic features through activation of the canonical Wnt signaling pathway. *Osteoarthritis Cartilage* 2011;19:1363–72.
11 Oh H, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. *Arthritis Rheum* 2012;64:2568–78.
12 Thysen S, Luyten FP, Lories RJ. Loss of Frzb and Sfrp1 differentially affects joint homeostasis in instability-induced osteoarthritis. *Osteoarthritis Cartilage* 2015;23:275–9.
13 Bouaziz W, Funck-Brentano T, Lin H, *et al*. Loss of sclerostin promotes osteoarthritis in mice via β-catenin-dependent and -independent Wnt pathways. *Arthritis Res Ther* 2015;17:24.
14 Zhu M, Tang D, Wu Q, *et al*. Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. *J Bone Miner Res* 2009;24:12–21.
15 Yuasa T, Otani T, Koike T, *et al*. Wnt/beta-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration. *Lab Invest* 2008;88:264–74.
16 Kawaguchi H. Regulation of osteoarthritis development by Wnt-beta-catenin signaling through the endochondral ossification process. *J Bone Miner Res* 2009;24:8–11.
17 Lories RJ, Corr M, Lane NE. To Wnt or not to Wnt: the bone and joint health dilemma. *Nat Rev Rheumatol* 2013;9:328–39.
18 Monteagudo S, Lories RJ. Cushioning the cartilage: a canonical Wnt restricting matter. *Nat Rev Rheumatol* 2017;13:670–81.
19 Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004;303:1483–7.
20 Lilien J, Balsamo J. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. *Curr Opin Cell Biol* 2005;17:459–65.
21 Mengshol JA, Vincenti MP, Coon CI, *et al*. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum* 2000;43:801.
22 Emami KH, Nguyen C, Ma H, *et al*. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. *Proc Natl Acad Sci U S A* 2004;101:12682–7.
23 Beyer C, Reichert H, Akan H, *et al*. Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. *Ann Rheum Dis* 2013;72:1255–8.
24 Wieland HA, Michaelis M, Kirschbaum BJ, *et al*. Osteoarthritis—an untreatable disease? *Nat Rev Drug Discov* 2005;4:331–44.

25 Berenbaum F. Osteoarthritis year 2010 in review: pharmacological therapies. *Osteoarthritis Cartilage* 2011;19:361–5.
26 Kaufman AC, Salazar SV, Haas LT, *et al*. Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. *Ann Neurol* 2015;77:953–71.
27 Um JW, Nygaard HB, Heiss JK, *et al*. Alzheimer amyloid-β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat Neurosci* 2012;15:1227–35.
28 Lu KV, Zhu S, Cvrljevic A, *et al*. Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients. *Cancer Res* 2009;69:6889–98.
29 Wu Q, Zhu M, Rosier RN, *et al*. Beta-catenin, cartilage, and osteoarthritis. *Ann N Y Acad Sci* 2010;1192:344–50.
30 Piedra J, Miravet S, Castaño J, *et al*. p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. *Mol Cell Biol* 2003;23:2287–97.
31 Chang YM, Bai L, Liu S, *et al*. Src family kinase oncogenic potential and pathways in prostate cancer as revealed by AZD0530. *Oncogene* 2008;27:6365–75.
32 Kumar A, Jaggi AS, Singh N. Pharmacology of Src family kinases and therapeutic implications of their modulators. *Fundam Clin Pharmacol* 2015;29:115–30.

# Tyrosine kinase Fyn promotes osteoarthritis by activating the β-catenin pathway

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