Interaction of HIF1α and β-catenin inhibits matrix metalloproteinase 13 expression and prevents cartilage damage in mice

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Short title: HIF1α-β-catenin inhibits MMP13 and OA

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Abstract:

Low oxygen tension (hypoxia) regulates chondrocyte differentiation and metabolism. Hypoxia-Inducible factor 1α (HIF1α) is a crucial hypoxic factor for chondrocyte growth and survival during development. The major metalloproteinase matrix metalloproteinase 13 (MMP13) is also associated with chondrocyte hypertrophy in adult articular cartilage, the lack of which protects from cartilage degradation and osteoarthritis in mice. MMP13 is upregulated by the Wnt/β-catenin signaling, a pathway involved in chondrocyte catabolism and osteoarthritis (OA). We studied the role of HIF1α in regulating Wnt signaling in cartilage and OA. We used mice with conditional knockout of HIF1α ($\Delta HIF1\alpha^{chon}$) with joint instability. Specific loss of HIF1α exacerbated MMP13 expression and cartilage destruction. Analysis of Wnt signaling in hypoxic chondrocytes showed that HIF1α lowered transcription factor 4 (TCF4)–β-catenin transcriptional activity and inhibited MMP13 expression. Indeed, HIF1α interacting with β-catenin displaced TCF4 from *MMP13* regulatory sequences. Finally, $\Delta HIF1\alpha^{chon}$ mice with OA that were injected intra-articularly with PKF118-310, an inhibitor of TCF4–β-catenin interaction, showed less cartilage degradation and reduced MMP13 expression in cartilage. Therefore, HIF1α–β-catenin interaction is a negative regulator of Wnt signaling and MMP13 transcription, thus reducing catabolism in OA. Our study contributes to the understanding of the role of HIF1α in OA and highlights the HIF1α–β-catenin interaction, thus providing new insights into the impact of hypoxia in articular cartilage.

Keywords: Hypoxia-Inducible factor 1α, β-catenin, matrix metalloproteinase 13, cartilage, osteoarthritis, chondrocyte

Significance statement:

Hypoxia-Inducible factor 1α (HIF1α) is an important factor for cell growth and survival. It modulates Wnt signaling regulating cell differentiation and fate. Osteoarthritis (OA) is an increasingly frequent joint disorder characterized by progressive cartilage breakdown in which Wnt/β-catenin signaling triggers matrix metalloproteinase 13 (MMP13) expression and chondrocyte catabolism. Here we demonstrate HIF1α inhibits β-catenin signaling by blocking tcf4-β-cat interaction and downregulates MMP13 expression, thereby alleviating cartilage lesions, whereas the transcription factor 4 (TCF4)–β-catenin signaling induces an OA phenotype in mice. In OA joints, PKF-118-310, a small molecule that blocked TCF4–β-catenin interaction, significantly reduced the progression of OA cartilage lesions. Thus, blockade of TCF4–β-catenin signaling by HIF1α represents a promising strategy to prevent articular cartilage loss in OA.

\Body

Introduction

Low oxygen tension (hypoxia) orchestrates several cell functions and is critical in health and disease (1-4). Hypoxia-inducible factor 1α (HIF1α) is an essential factor to maintain chondrocyte homeostasis and allow cell differentiation (5, 6). HIF1 is a heterodimeric DNA-binding complex containing a constitutive HIF1β subunit and HIF1α subunit. In hypoxia, HIF1 binds to the hypoxia response elements of target genes, whereas in normoxia, HIF1α is hydroxylated, thereby leading to its degradation. Indeed, HIF1α hydroxylation is recognised by the von Hippel–Lindau tumor suppressor protein (pVHL), an E3 ubiquitin ligase that targets HIFα for proteolysis in the proteasome (7). The HIF1α pathway interacts with different cell signaling pathways, among them Wnt signaling. Indeed, HIF1α interacts with β-catenin in regulating cell growth and survival. In embryonic stem cells, HIF1α–β-catenin complexes upregulate lymphoid enhancer-binding factor 1 and transcription factor 1 (TCF1), which activates Wnt signaling (8), whereas in colorectal cancer cells, HIF1α blocks the TCF4–β-catenin interaction and transcriptional activity, thus inhibiting canonical Wnt signaling (9).

Cartilage loss characterizes osteoarthritis (OA), one of the most frequent joint disorders, but available treatments are poorly efficient to prevent joint destruction (10, 11). Therefore, the need for novel drug targets to treat OA is paramount. Matrix metalloproteinase 13 (MMP13) triggers the degradation of articular cartilage. Indeed, chondrocyte-specific deletion of MMP13 alleviated OA in mice; the Wnt family members were candidates for the regulation of MMP13 expression in chondrocytes because its expression was increased in chondrocytes from mice with conditional activation of β-catenin (12). Cumulative data showed that Wnt activity is low

under physiological conditions, and activation of Wnt signalling contributes to cartilage breakdown in OA (13, 14). The modulation of Wnt inhibitors had significant effects on chondrocyte catabolism of mice. Indeed, loss of sclerostin enhanced cartilage degradation (15) and the overexpression of Dkk-1 alleviated OA (14). Despite the hypoxic status of cartilage (16), the involvement of hypoxia in regulating Wnt signalling and MMP13 expression in cartilage is still unclear.

We studied the role of HIF1α in regulating Wnt signaling in cartilage of mice with conditional knockout of HIF1α (ΔHIF1α^{chon}) and induced OA. Hypoxia maintained low Wnt/β-catenin signaling via HIF1α, which lowered MMP13 expression, then prevented chondrocyte catabolism and cartilage loss. We here therefore highlight the role of HIF1α in cartilage remodeling and loss during OA and provide a novel mechanism of microenvironmental regulation of Wnt signaling.

Results

HIF1α deletion in chondrocytes enhanced OA development and MMP13 expression in mice.

Articular chondrocytes are physiologically in a hypoxic state that might be altered in OA (5). To monitor oxygen tensions in OA chondrocytes, we administered a hypoxyprobe (pimonidazole hydrochloride) to control and OA mice. Hypoxia levels were markedly decreased in all layers of the OA articular cartilage, as shown by immunohistochemistry and by the decreased number of hypoxic cells (Fig. 1A). HIF1α was expressed in undamaged articular cartilage at baseline but its level decreased significantly in OA joints along with the increased cartilage damage (Fig. 1B). Noteworthy, the number of Hif1α expressing cells was steadily downregulated after

destabilization of the medial meniscus (4 and 6 weeks) whereas OA damage increased in a time dependent manner.

To determine the role of HIF1α in OA development, we generated mice with inducible conditional knockout of HIF1α by mating COL2-Cre^{ERT} mice with HIF1α$^{fl/fl}$ mice in which the recombination was induced by tamoxifen. We first verified that the Cre-lox recombination occurred correctly in cartilage; COL2-Cre^{ERT}; R26R and R26R mice were injected with tamoxifen as controls. β-galactosidase was expressed in the articular cartilage of COL2-Cre^{ERT};R26R mice, thus tamoxifen induced Cre-lox recombination in chondrocytes (Supplementary Fig. S1). OA was induced in ΔHIF1α^{Chon} mice 1 week after tamoxifen injections. OA cartilage lesions were increased in ΔHIF1α^{Chon} mice as showed by the OARSI score (Fig. 1C). As HIF1α is a survival factor, we assessed chondrocyte apoptosis in OA in the absence of HIF1α. The number of apoptotic cells was increased in ΔHIF1α^{Chon} mice although the OA score remained unchanged (Fig. 1D). TUNEL positive cells were further increased in ΔHIF1α^{Chon} mice with DMM. As previously described, the expression of MMP13 was induced in OA mice. This increase is enhanced in ΔHIF1α^{Chon} mice along with the exacerbated cartilage loss (Fig. 1E). Thus, OA is associated with loss of hypoxia in articular cartilage, decreased HIF1α protein levels and increased MMP13 expression and cartilage loss in mice. To rule out the hypothesis that ΔHIF1α^{Chon} could induce EPAS-1 (Hif2α) expression and therefore contribute to the phenotype, we found that EPAS-1 was expressed at the same level in HIF1α $^{fl/fl}$ mice and ΔHIF1α^{Chon} mice suggesting the absence of compensatory increase of EPAS1 (Figure S2A).

HIF1α inhibits *MMP13* expression and the transcription of Wnt targets. HIF1α is a major HIF that regulates chondrocyte metabolism (17). We first assessed the role of hypoxia in the

metabolic effects of Wnt signaling in primary chondrocytes. As expected in normoxia, Wnt3a reduced proteoglycan release and Col2A expression while increasing Mmp13 expression (Fig. 2A and 2B). In contrast, under hypoxic conditions, Wnt3a failed to modulate the proteoglycan release as well as the expression of catabolic markers. Furthermore target genes such as *Axin* and *Wisp1* were not regulated by Wnt3a in hypoxia (Fig 2C). To investigate whether HIF1α modulate *MMP13* expression induced by Wnt, we first deleted HIF1α chondrocytes and analyzed the expression of *MMP13* and Wnt target genes under Wnt stimulation. Using siRNA silencing, there was no effect of HIF1α knock-down in COL2A and Mmp13 in normoxia (Fig. 2D). Furthermore, HIF1α knockdown has no effect on the nuclear translocation of EPAS1 (Fig. S2B). Loss of HIF1α promoted the Wnt-induced *Mmp13* expression and the transcriptional Wnt targets (Fig 2E) and confirmed by Cre-Lox recombination (fig. 2F and 2G). This was not observed when EPAS1 was knocked-down (Fig. S2C).

To confirm that HIF1α inhibits *MMP13* expression by blocking transcription of Wnt targets, we stabilized HIF1α in normoxic chondrocytes upon *VHL* deletion using Cre-Lox recombination. HIF1α increase with VHL deletion was confirmed by Western blot analysis (Fig. 2H) and led to increased *MMP1,3 Axin,* and *Wisp1 expression* in normoxia (Fig. 2I). Moreover, stabilization of HIF1α did not affect the transcription of the anabolic marker *COL2A1* (Fig. 2I). Thus the regulation of anabolic markers is independent of the HIF1α pathway**.** Because loss of VHL stabilizes both HIF1 and Epas1, we used an overexpression of constitutive stabilized HIF1 in chondrocytes (18). We found that HIF1α abolished Mmp13 expression induced by Wnt (Fig. 2J). Thus, HIF1α alone is able to inhibit Wnt-induced *MMP13* expression.

HIF1α–β-catenin interaction reduced TCF4 binding to the MMP13 regulatory region. Upon Wnt pathway activation, β-catenin accumulates in the cytoplasm, translocates into the nucleus, binds to TCF transcription factor and activates the transcription of target genes (19). Wnt activation increases the levels of catabolic markers under normoxia (16). We then investigated whether the translocation of β-catenin is reduced in hypoxia. Wnt3a promoted β-catenin translocation into the nucleus and its protein expression regardless of O_2 level (Fig. 3A, Fig. 3B). Thus, hypoxia reduced transcriptional activity of β-catenin independently of nuclear β-catenin translocation.

We investigated whether HIF1α inhibits Wnt activity by direct interaction between HIF1α and β-catenin. In hypoxia, HIF1α and β-catenin co-localized in the nucleus of Wnt3a-induced chondrocytes (fig 4A), suggesting a possible interaction within nuclear complexes. Indeed, co-immunoprecipitation in nuclear extracts assay revealed HIF1α–β-catenin interaction complexes in hypoxia (Fig. 4B). However, EPAS1 failed to co-immunoprecipitate with β-catenin in normoxia and hypoxia (Fig. S2D). We next assessed the impact of HIF1α–β-catenin interaction on the transcriptional activity of TCF4–β-catenin complexes. In chondrocyte stimulated by Wnt3a, the formation of TCF4–β-catenin nuclear complexes was decreased in hypoxia compared to normoxia (Fig. 4B). These data suggest that under hypoxia, β-catenin may bind preferentially to HIF1α rather than TCF4, thus reducing Wnt/β-catenin signaling.

MMP13 is targeted by both HIF and Wnt signaling (20-24). To better characterize the role of HIF1α–β-catenin interaction in inhibiting Wnt/β-catenin signaling and *MMP13* expression under hypoxia, we assessed TCF4 binding to the *MMP13* regulatory region by CHIP assay (Fig. 4C). We used a *MMP13* regulatory 3' region downstream of coding area that includes Wnt responsive

elements (WREs) (24) (Fig. 4C). TCF4 binding to WREs was decreased in hypoxia compared to normoxia (Fig. 4C). To confirm the role of HIF1α in inhibiting TCF4 binding to WREs, we overexpress the stabilized form of HIF1α in normoxic chondrocytes and found that TCF4 binding to WREs was abolished (Fig. 4C). Therefore, TCF4 binding to the *MMP13* regulatory region was reduced by HIF1α. We further investigated the transcriptional activity of *MMP13* under hypoxia. The regulatory region was cloned downstream of a luciferase reporter gene and the plasmid was transfected in C3H10 cells. This assay revealed downregulation of Wnt3a-induced luciferase activity when cultured under hypoxia (Fig. 4D), and confirmed the functional regulation of this sequence by hypoxia and Wnt3a.

PKF118-310 reduced OA progression in ΔHIF1α^{Chon} mice. Given the sequestration of β-catenin by HIF1α, we hypothesized that HIF1α deletion may increase TCF4–β-catenin complex level, thereby leading to chondrocyte catabolism. To verify this hypothesis, we deleted HIF1α in chondrocytes by siRNA silencing and blocked TCF4–β-catenin complexes by using PKF118-310 which blocks the interaction between TCF4 and β-catenin. In normoxia, PKF118-310 totally suppressed Wnt-induced Mmp13 and target genes expression (Fig. 4E). Loss of HIF1α increased the expression of *MMP13* and that of the bona fide Wnt target genes *Axin* and *Wisp1*. Hence, the PKF118-310 addition inhibited the increased expression of the canonical Wnt targets *MMP13, Axin* and *Wisp1* induced by loss of HIF1α. Thus, HIF1α prevented Wnt to induce *MMP13* expression by blocking TCF4–β-catenin complexes.

In OA mice, conditional loss of HIF1α increased MMP13 expression and cartilage lesions (Fig. 5A). Increased TCF4–β-catenin complex level may promote the phenotype observed in

ΔHIF1α^{Chon} mice. Articular injection of the β-catenin inhibitor PKF118-310 in ΔHIF1α^{Chon} mice prevented cartilage lesions and reduced MMP-13 expression as compared with PBS treatment. Thus, PKF118-310 administration in ΔHIF1α^{Chon} mice blocked the increased MMP13 expression and cartilage damage (Fig. 5A and 5B). Moreover, PKF118-310 resulted in a reduced number of apoptotic cells in OA mice (Fig. 5C). Loss of HIF1α enhanced cartilage damage by increasing TCF4–β-catenin complexes, which activated MMP13 expression.

Discussion

Hypoxia is a characteristic of physiological articular cartilage (5, 25). We addressed the specific function of HIF1α in chondrocytes and in OA in mice. With inducible conditional knockout of HIF1α in mice, we showed that HIF1α alleviated OA development by downregulating MMP13 through inhibition of β-catenin transcriptional activity in chondrocytes. The hypoxic avascular nature of the articular cartilage maintains the chondrocyte phenotype and homeostasis. Indeed, we observed that hypoxia and HIF1α were downregulated in OA cartilage. Our findings are consistent with lower chondrocyte hypertrophy during hypoxia, which may contribute to the maintenance of cartilage homeostasis (26-28). Moreover, we show that HIF1α is necessary to maintain a physiologic chondrocyte microenvironment and function. Consistent with our data, the inhibition of HIF1α by 2-methoxyestradiol induced an OA phenotype in mice (5), which suggests that HIF1α modulation is an important event that triggers chondrocyte differentiation in OA. With our conditional HIF1α-knockout mouse model, we show that chondrogenic HIF1α directly maintains cartilage metabolism. Moreover, we show that HIF1α is also a physiological anti-apoptotic factor in articular cartilage as its loss enhanced chondrocyte apoptosis. Six weeks

after HIF1α deletion, the increase of apoptotic chondrocyte did not induce cartilage lesion as chondrocyte apoptosis alone is not sufficient to induce to induce cartilage lesion (15). Here, the increase in procatabolic enzymes in addition with increased apoptosis can exacerbate the cartilage lesion during OA. Finally the cartilage erosion observed in OA ΔHIF1α^{Chon} mice results of the double function of HIF1α in articular cartilage as a Wnt inhibitor and an anti-apoptotic protein. However, we cannot discard that Epas1 could be involved in cartilage erosion, since it was described as an inhibitor of survival and catabolic activator (22, 29).

Wnt/β-catenin signaling is one of the key pathways involved in OA (30). Its activation triggers the osteoarthritic differentiation of chondrocytes and OA in mice (16, 31). Thus, understanding the molecular regulators of Wnt signaling is of great therapeutic interest. Oxygen level is an important regulator of Wnt activity (9, 32). Indeed, high oxygen level promotes Wnt signaling and the differentiation of stem cells (33). Hypoxia inhibited the destruction of human cartilage explants by reducing MMP13 production in a HIF1α-dependent manner (25). Because MMP13 is an important target of both HIF signaling and canonical Wnt pathways (20-24), we assessed whether HIF1α regulates MMP13 and Wnt signaling to prevent OA. Here we demonstrated that hypoxia downregulated canonical Wnt signaling, thereby preventing chondrocyte catabolism. HIF1α deficiency exacerbated cartilage catabolism, thus HIF1α is necessary to prevent OA development. We show that Wnt-induced MMP13 expression was promoted in ΔHIF1α chondrocytes but was blunted by stabilized HIF1α in chondrocytes. Taken together, we demonstrate that HIF1α downregulates the transcription of *MMP13* driven by canonical Wnt signaling.

Given the critical role of β-catenin to drive Wnt signaling, understanding the role of HIF1α–β-catenin interaction may reveal novel mechanisms in regulating Wnt signaling. Because HIF1α inhibits TCF4–β-catenin interaction and transcriptional activity (9), we investigated whether HIF1α modulates MMP13 through a Wnt/β-catenin pathway. Hypoxia promoted the translocation of β-catenin and binding to HIF1α but not MMP13 expression. These results are consistent with transcriptional blockade of β-catenin activity despite the stabilization of β-catenin in the nucleus. Our study brings new insights into the protein regulation of HIF1α and β-catenin interaction (9) and its impact on *MMP13* transcription. Under hypoxia, HIF1α–β-catenin complexes are preferentially formed, which results in a lower TCF4–β-catenin complex level and therefore markedly reduced TCF4 binding to the *MMP13* regulatory region. Interestingly, when stabilized in normoxic chondrocytes, HIF1α blocked TCF4 binding to the *MMP13* regulatory region. Furthermore, the inhibition of TCF4–β-catenin complexes by PKF118-310 abolished Wnt3a-induced *MMP13* expression in ΔHIF1α chondrocytes. These results confirm the role of TCF4–β-catenin complexes triggering *MMP13* expression in ΔHIF1α chondrocytes. Thus, HIF1α is a nuclear negative regulator of TCF4–β-catenin complexes that inhibits the shift toward a catabolic phenotype in chondrocytes.

We further demonstrated that HIF1α signaling protected against cartilage damage by blocking TCF4–β-catenin. Indeed, PKF118-310 administration reduced cartilage breakdown and the expression of MMP13 observed in $\Delta HIF1\alpha^{Chon}$ mice. Our findings support a pro-catabolic role of Wnt/β-catenin signaling in OA and bring new insights in the modulation of Wnt/β-catenin signaling by hypoxia. Furthermore, we provide evidence that HIF1α is a potent inhibitor of β-

catenin and Wnt signaling and is required to block cartilage degradation. The use of HIF1α agonists might be a useful strategy for treating cartilage lesions in OA.

Methods

Harvesting, expansion and transfection of primary chondrocytes. HIF1α$^{fl/fl}$ and VHL$^{fl/fl}$ mice (Jackson Laboratory) were used for *in vitro* experiments involving chondrocyte cultures. Chondrocytes were harvested from 6-day-old mice and cultured with 10% fetal bovine serum (50% confluence). Recombination, transient overexpression and plasmid (18) information are detailed in the supporting file.

Real-time PCR. Real-time PCR involved use of SYBR green (Applied Biosystems) in (5 to 8) independent experiments. Averaged Ct values were normalized to the averaged Ct value of *RPL13A*. Adjusted average Ct values were used to calculate relative expression versus control. Primer sequences are detailed in supplemental data.

Immunocytochemistry. Cells were cultured in 24-well plates containing cover glasses, fixed for 15 min with 4% formaldehyde (w/v). Cultures were saturated with 3% BSA for 60 min at room temperature, then incubated with the antibodies rabbit primary anti-HIF1α, mouse primary anti-β-catenin and anti- EPAS1 (All Santa Cruz Biotechnology) for 1 h. Cultures were incubated for 60 min with Alexa dye 488-conjugated rabbit secondary antibody or Cy3 dye-conjugated mouse secondary antibody. Cells were observed with Axio Observer Z1 (Zeiss).

Quantification of proteoglycan release, western blots analysis and immunoprecipitation. Proteoglycan release was measured in the supernatant by colorimetric method. Whole cell lysates and nuclear extracts were prepared and proteins were extracted (n = 3 independent

experiments). Immunoprecipitation, cloning and reporter gene assay are described in the supporting file.

Mice. To evaluate the expression of HIF1α during OA, we induced joint instability in 10-week-old male C57BL6 mice (Janvier, France) by destabilization of the medial meniscus (DMM) of the right knee, with sham operation performed on the left knee as described (34). Mice were killed at weeks 0 (n=3), 4 (n=5) and 6 (n=5) after OA induction. fl/fl HIF1α, R26R and COL2Cre^{ERT} mice were supplied by the Jackson Laboratory. At 9 weeks of age, COL2Cre^{ERT};fl/fl HIF1α and COL2-Cre^{ERT};R26R, fl/fl HIF1α and R26R mice were injected with tamoxifen (1 mg/10 g weight) (Sigma) daily for 5 days. Joint instability was induced in 10-week-old male ΔHIF1α^{chon} mice (n = 8-10 per group) and fl/fl HIF1α littermates (n = 9–12 per group) in the right knee, with sham operation performed in the left knee. ΔHIF1α^{chon} and fl/fl HIF1α were injected intra-articularly once a week with PKF118-310 or phosphate buffered saline (PBS; n=8 per group). Mice were killed 6 weeks after surgery. This time point is suitable to quantify the expression of MMP13 in the remaining cartilage. To monitor hypoxia level in healthy and OA cartilage, we injected the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe, USA) in mice. Male FVB mice (Jackson laboratory) at 16 weeks old were intraperitoneally injected with 0.6 mg pimonidazole/10 g weight and killed 17 h later. Mice were treated in accordance with the Guidelines for Animal Experimentation issued by the local Committee (Ethics Committee Lariboisière-Villemin no. CEEALV/2012-02-01, Paris).

Histology. Knees were fixed in 4% PFA for 24 h at 4°C and decalcified in Osteosoft at room temperature for 4 days, tissue was embedded in paraffin. Serial 5-µm-thick sagittal sections of medial femorotibial joints were collected at 3 depths at 70-µm intervals. Sections of tibias and

femurs were stained with Safranin-O. The OARSI scoring method was used for both tibias and femurs, with a total severity score ranging from 0 to 12 (35). Immunohistochemistry methods are described in the supporting file.

Statistical analysis. Data are reported as mean ± SEM. Statistical analyses involved ANOVA and the Mann-Whitney test with Statview (SAS Inst., Cary, NC). $P<0.05$ was the threshold of statistical significance.

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Figure legends:

Figure 1: Hypoxia and hypoxia-inducible factor 1α (HIF1α) are reduced in cartilage of mice with osteoarthritis (OA).

A. Immunohistofluorescence staining of hypoxyprobe adducts in healthy and OA wild-type mouse cartilage. Hypoxyprobe adducts were revealed in hypoxic cells (P_{O2} <10 mm Hg) by a fluorescein-conjugated monoclonal antibody (HP-FITC-MAb). Graph shows proportion of hypoxic positive cells in cartilage of the tibial plateau and internal femoral condyle. Data are mean ± SEM. *, $p < 0.05$ compared to control (n = 7 animals/group). Bar: 100 µm**. B.** Upper panel: HIF1α immunostaining and OA score (Safranin-O staining) in joints of control mice at 0, 4 and 6 weeks post OA induction. Graphs show percentage of HIF1α(+) cells and OA score in articular cartilage of the tibial plateau and internal femoral condyle. Bar: 100 µm. *, $p < 0.05$ compared to control (n = 7 animals/group) **C.** Safranin-O staining and D. TUNEL assay of $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mouse joints with OA or sham operation (ct) at Week 6. Bar, 100 µm. OA score in OA and sham-operated knees of $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mice (Week 6). *, $p < 0.05$ compared to control. #, $p < 0.05$ (n = 8–11 animals/group). **E.** Immunostaining for matrix metalloproteinase 13 (MMP13) in $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mouse joints (Week 6) (Bar, 100 µm) and quantification. *, $p < 0.05$ compared to control. #, $p < 0.05$ (n = 8–11 animals/group).

Figure 2: HIF1α inhibits the transcription of Wnt targets in Wnt3a-induced chondrocytes.

A. Proteoglycan release in chondrocyte culture media (n = 9). qPCR analysis of relative gene expression in primary chondrocytes with 21% and 1% O_2 for: **B.** anabolic marker (collagen 2A , *COL2A*) and catabolic marker (*MMP13*) (n = 7); and **C.** direct transcriptional targets of Wnt3a (*Axin* and *Wisp1*) (n = 14). **D. E**xpression of anabolic and catabolic genes (*COL2A* and *MMP13*)

with HIF1α siRNA silencing (n = 6); **E.** direct transcriptional targets of Wnt3a (*Axin* and *Wisp1*) with HIF1α siRNA silencing (n = 6); **F.** catabolic marker *MMP13* in HIF1α-lacking chondrocytes (Cre-Lox recombination in vitro) (n = 5); and **G.** direct transcriptional targets of Wnt3a (*Axin* and *Wisp1*) in HIF1α-lacking chondrocytes (Cre-Lox recombination in vitro) (n = 5). Data are mean ± SEM. *, $p < 0.05$ compared to control, # $p<0.05$. **H.** Western blot analysis of HIF1α expression in von Hippel–Lindau tumor suppressor protein (VHL)-lacking chondrocytes (with 21% O_2), and quantification (n = 3). qPCR analysis of relative gene expression in VHL-lacking chondrocytes (Cre-Lox recombination in vitro) for: **I.** anabolic marker *COL2A*; catabolic marker *MMP13*; and direct transcriptional targets of Wnt (*Axin* and *Wisp1*) (with 21% O_2). **J**. Western blot analysis of stabilized HIF1α expression using a tag antibody (HA) in primary chondrocytes and qPCR analysis of relative gene expression of MMP13. Data are mean ± SEM (n = 5 experiments); *, $p < 0.05$ compared to control; # $p<0.05$.

Figure 3: Wnt3a promotes β-catenin translocation into the nucleus in hypoxia and normoxia.

A. Immunocytofluorescence staining of β-catenin in normoxic and hypoxic chondrocytes. Bar, 100 µm (n=4). Quantification of β-catenin translocation in chondrocytes: intensity of β-catenin signal into the nucleus of chondrocytes cultured in hypoxia and normoxia after Wnt3a stimulation (pixels) (n = 198-277). **B.** Western blot analysis of β-catenin protein level in normoxic and hypoxic chondrocytes and quantification. Data are mean ± SEM. *, $p < 0.05$ compared to control (Ct).

Figure 4: HIF1α binds β-catenin and inhibits transcription factor 4 (TCF4) binding to *MMP13* regulatory region.

A. Immunocytofluorescence staining of β-catenin and HIF1α in hypoxic chondrocytes. (X63). (n = 3). **B.** Co-mmunoprecipitation of β-catenin in nuclear protein extracts. Western blot (WB) analysis of protein levels of HIF1α, TCF4 and β-catenin and quantification of HIF1α–β-catenin and TCF4–β-catenin complexes. **C.** CHIP analysis of TCF4 binding to *MMP13* regulatory region. Sequence contains Wnt responsive elements. RNA Pol: RNA polymerase (positive control). IgG: mouse IgG (negative control). qPCR analysis of TCF4 binding to MMP13 regulatory regions (n = 3). *, $p < 0.05$ compared to control; # $p<0.05$. **D.** Luciferase reporter assay in C3H10 cells. Data are ratio of firefly luciferase to control (Renilla) luciferase activity (n=3). **E.** qPCR analysis of relative gene expression (with HIF1α siRNA silencing) with 21% and 1% O_2 for *MMP13* (n = 8) and direct transcriptional targets of Wnt (*Axin* and *Wisp1*) (n = 8). Data are mean ± SEM. *, $p < 0.05$ compared to control; # $p<0.05$.

Figure 5: Loss of HIF1α increases TCF4–β-catenin complexes and cartilage lesions in OA mice with control or PKF118-310 treatment.

A. Safranin-O staining and OA score of $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mouse knees after OA induction or control (Ct) at Week 6 after treatment or not with PKF118-310. Bar, 100 µm. **B.** MMP13 expression in $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mice at Week 6 (immunohistochemistry). Bar 100 µm and Quantification. C. TUNEL assay, quantification in $HIF1\alpha^{fl/fl}$ and $\Delta HIF1\alpha^{Chon}$ mouse joints with OA or control at Week 6. Data are mean ± SEM, *, $p < 0.05$, #: $p < 0.05$ compared to control $Hif\text{-}1\alpha^{fl/fl}$

(n = 8–11 animals/group).

Supplemental figures.

Figure S1: β-galactosidase immunostaining in COL2-Cre^{ERT};R26R and R26R mice with injection of Tamoxifen (n=3).

Figure S2

A. EPAS1 expression in HIF1α$^{fl/fl}$ and ΔHIF1α^{Chon} mice at Week 6 (immunohistochemistry) in control knees. **B.** Immunocytofluorescence staining of EPAS1 in normoxic and hypoxic chondrocytes (n=4). Quantification of EPAS1 translocation in chondrocytes: intensity of Epas1 signal into the nucleus of chondrocytes cultured in hypoxia and normoxia after Hif1α siRNA silencing (pixels) (n = 198-277). **C.** PCR analysis of relative gene expression in primary chondrocytes with 21% and 1% O_2 for Wnt targets (*Axin* and *Wisp1*) with EPAS1 siRNA silencing (n = 6). **D.** Co-mmunoprecipitation of β-catenin in nuclear protein extracts. Western blot (WB) analysis of protein levels of EPAS1 and β-catenin.