



## Interaction of HIF1 $\alpha$ and $\beta$ -catenin inhibits matrix metalloproteinase 13 expression and prevents cartilage damage in mice

Wafa Bouaziz, Johanna Sigaux, Dominique Modrowski, Claire-Sophie Devignes, Thomas Funck-Brentano, Pascal Richette, Hang-Korng Ea, Sylvain Provot, Martine Cohen-Solal, Eric Haÿ

### ► To cite this version:

Wafa Bouaziz, Johanna Sigaux, Dominique Modrowski, Claire-Sophie Devignes, Thomas Funck-Brentano, et al.. Interaction of HIF1 $\alpha$  and  $\beta$ -catenin inhibits matrix metalloproteinase 13 expression and prevents cartilage damage in mice. Proceedings of the National Academy of Sciences of the United States of America, 2016, 113 (19), pp.5453-8. 10.1073/pnas.1514854113 . inserm-01310631

**HAL Id: inserm-01310631**

**<https://inserm.hal.science/inserm-01310631>**

Submitted on 2 May 2016

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## **Interaction of HIF1 $\alpha$ and $\beta$ -catenin inhibits matrix metalloproteinase 13 expression and prevents cartilage damage in mice**

Wafa Bouaziz<sup>1,2</sup>, Johanna Sigaux<sup>1,2,3</sup>, Dominique Modrowski<sup>1</sup>, Claire-Sophie Devignes<sup>1,2</sup>, Thomas Funck-Brentano<sup>2,3</sup>, Pascal Richette<sup>1,2,3</sup>, Hang-Korng Ea<sup>1,2,3</sup>, Sylvain Provot<sup>1</sup>, Martine Cohen-Solal<sup>1,2,3</sup> and Eric Hay<sup>1,2</sup>.

### **Institutions:**

1. INSERM UMR-S U1132, 2 rue Ambroise Paré, Paris (75010), France.
2. USPC-Paris-Diderot medical School, 10 avenue de Verdun, Paris (75010), France.
3. Lariboisière Hospital, 2, rue Ambroise Paré, Paris (75010), France.

**Short title:** HIF1 $\alpha$ - $\beta$ -catenin inhibits MMP13 and OA

### **Corresponding author**

Pr Martine Cohen-Solal

Mailing address: INSERM U1132, Lariboisière hospital, 2 rue Ambroise Paré, 75010 Paris, France

Phone number: +33-1-49-95-63-58

Fax number: +33-1-49-95-84-52

E-mail: martine.cohen-solal@inserm.fr

**Abstract:**

Low oxygen tension (hypoxia) regulates chondrocyte differentiation and metabolism. Hypoxia-Inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) 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/ $\beta$ -catenin signaling, a pathway involved in chondrocyte catabolism and osteoarthritis (OA). We studied the role of HIF1 $\alpha$  in regulating Wnt signaling in cartilage and OA. We used mice with conditional knockout of HIF1 $\alpha$  ( $\Delta$ HIF1 $\alpha^{\text{chon}}$ ) with joint instability. Specific loss of HIF1 $\alpha$  exacerbated MMP13 expression and cartilage destruction. Analysis of Wnt signaling in hypoxic chondrocytes showed that HIF1 $\alpha$  lowered transcription factor 4 (TCF4)- $\beta$ -catenin transcriptional activity and inhibited MMP13 expression. Indeed, HIF1 $\alpha$  interacting with  $\beta$ -catenin displaced TCF4 from *MMP13* regulatory sequences. Finally,  $\Delta$ HIF1 $\alpha^{\text{chon}}$  mice with OA that were injected intra-articularly with PKF118-310, an inhibitor of TCF4- $\beta$ -catenin interaction, showed less cartilage degradation and reduced MMP13 expression in cartilage. Therefore, HIF1 $\alpha$ - $\beta$ -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 HIF1 $\alpha$  in OA and highlights the HIF1 $\alpha$ - $\beta$ -catenin interaction, thus providing new insights into the impact of hypoxia in articular cartilage.

**Keywords:** Hypoxia-Inducible factor 1 $\alpha$ ,  $\beta$ -catenin, matrix metalloproteinase 13, cartilage, osteoarthritis, chondrocyte

**Significance statement:**

Hypoxia-Inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) 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/ $\beta$ -catenin signaling triggers matrix metalloproteinase 13 (MMP13) expression and chondrocyte catabolism. Here we demonstrate HIF1 $\alpha$  inhibits  $\beta$ -catenin signaling by blocking tcf4- $\beta$ -cat interaction and downregulates MMP13 expression, thereby alleviating cartilage lesions, whereas the transcription factor 4 (TCF4)- $\beta$ -catenin signaling induces an OA phenotype in mice. In OA joints, PKF-118-310, a small molecule that blocked TCF4- $\beta$ -catenin interaction, significantly reduced the progression of OA cartilage lesions. Thus, blockade of TCF4- $\beta$ -catenin signaling by HIF1 $\alpha$  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 1 $\alpha$  (HIF1 $\alpha$ ) is an essential factor to maintain chondrocyte homeostasis and allow cell differentiation (5, 6). HIF1 is a heterodimeric DNA-binding complex containing a constitutive HIF1 $\beta$  subunit and HIF1 $\alpha$  subunit. In hypoxia, HIF1 binds to the hypoxia response elements of target genes, whereas in normoxia, HIF1 $\alpha$  is hydroxylated, thereby leading to its degradation. Indeed, HIF1 $\alpha$  hydroxylation is recognised by the von Hippel–Lindau tumor suppressor protein (pVHL), an E3 ubiquitin ligase that targets HIF $\alpha$  for proteolysis in the proteasome (7). The HIF1 $\alpha$  pathway interacts with different cell signaling pathways, among them Wnt signaling. Indeed, HIF1 $\alpha$  interacts with  $\beta$ -catenin in regulating cell growth and survival. In embryonic stem cells, HIF1 $\alpha$ – $\beta$ -catenin complexes upregulate lymphoid enhancer-binding factor 1 and transcription factor 1 (TCF1), which activates Wnt signaling (8), whereas in colorectal cancer cells, HIF1 $\alpha$  blocks the TCF4– $\beta$ -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  $\beta$ -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 HIF1 $\alpha$  in regulating Wnt signaling in cartilage of mice with conditional knockout of HIF1 $\alpha$  ( $\Delta$ HIF1 $\alpha^{\text{chon}}$ ) and induced OA. Hypoxia maintained low Wnt/ $\beta$ -catenin signaling via HIF1 $\alpha$ , which lowered MMP13 expression, then prevented chondrocyte catabolism and cartilage loss. We here therefore highlight the role of HIF1 $\alpha$  in cartilage remodeling and loss during OA and provide a novel mechanism of microenvironmental regulation of Wnt signaling.

## Results

### **HIF1 $\alpha$ 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 hypoxyp probe (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). HIF1 $\alpha$  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 Hif1 $\alpha$  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 HIF1 $\alpha$  in OA development, we generated mice with inducible conditional knockout of HIF1 $\alpha$  by mating COL2-Cre<sup>ERT</sup> mice with HIF1 $\alpha$ <sup>fl/fl</sup> mice in which the recombination was induced by tamoxifen. We first verified that the Cre-lox recombination occurred correctly in cartilage; COL2-Cre<sup>ERT</sup>; R26R and R26R mice were injected with tamoxifen as controls.  $\beta$ -galactosidase was expressed in the articular cartilage of COL2-Cre<sup>ERT</sup>;R26R mice, thus tamoxifen induced Cre-lox recombination in chondrocytes (Supplementary Fig. S1). OA was induced in  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice 1 week after tamoxifen injections. OA cartilage lesions were increased in  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice as showed by the OARSI score (Fig. 1C). As HIF1 $\alpha$  is a survival factor, we assessed chondrocyte apoptosis in OA in the absence of HIF1 $\alpha$ . The number of apoptotic cells was increased in  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice although the OA score remained unchanged (Fig. 1D). TUNEL positive cells were further increased in  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice with DMM. As previously described, the expression of MMP13 was induced in OA mice. This increase is enhanced in  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice along with the exacerbated cartilage loss (Fig. 1E). Thus, OA is associated with loss of hypoxia in articular cartilage, decreased HIF1 $\alpha$  protein levels and increased MMP13 expression and cartilage loss in mice. **To rule out the hypothesis that  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> could induce EPAS-1 (Hif2 $\alpha$ ) expression and therefore contribute to the phenotype, we found that EPAS-1 was expressed at the same level in HIF1 $\alpha$ <sup>fl/fl</sup> mice and  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> mice suggesting the absence of compensatory increase of EPAS1 (Figure S2A).**

**HIF1 $\alpha$  inhibits *MMP13* expression and the transcription of Wnt targets.** HIF1 $\alpha$  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 HIF1 $\alpha$  modulate *MMP13* expression induced by Wnt, we first deleted HIF1 $\alpha$  chondrocytes and analyzed the expression of *MMP13* and Wnt target genes under Wnt stimulation. Using siRNA silencing, there was no effect of HIF1 $\alpha$  knock-down in COL2A and Mmp13 in normoxia (Fig. 2D). Furthermore, HIF1 $\alpha$  knockdown has no effect on the nuclear translocation of EPAS1 (Fig. S2B). Loss of HIF1 $\alpha$  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 HIF1 $\alpha$  inhibits *MMP13* expression by blocking transcription of Wnt targets, we stabilized HIF1 $\alpha$  in normoxic chondrocytes upon *VHL* deletion using Cre-Lox recombination. HIF1 $\alpha$  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 HIF1 $\alpha$  did not affect the transcription of the anabolic marker *COL2A1* (Fig. 2I). Thus the regulation of anabolic markers is independent of the HIF1 $\alpha$  pathway. Because loss of *VHL* stabilizes both HIF1 and *Epas1*, we used an overexpression of constitutive stabilized HIF1 in chondrocytes (18). We found that HIF1 $\alpha$  abolished Mmp13 expression induced by Wnt (Fig. 2J). Thus, HIF1 $\alpha$  alone is able to inhibit Wnt-induced *MMP13* expression.

### **HIF1 $\alpha$ – $\beta$ -catenin interaction reduced TCF4 binding to the MMP13 regulatory region.** Upon

Wnt pathway activation,  $\beta$ -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  $\beta$ -catenin is reduced in hypoxia. Wnt3a promoted  $\beta$ -catenin translocation into the nucleus and its protein expression regardless of O<sub>2</sub> level (Fig. 3A, Fig. 3B). Thus, hypoxia reduced transcriptional activity of  $\beta$ -catenin independently of nuclear  $\beta$ -catenin translocation.

We investigated whether HIF1 $\alpha$  inhibits Wnt activity by direct interaction between HIF1 $\alpha$  and  $\beta$ -catenin. In hypoxia, HIF1 $\alpha$  and  $\beta$ -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 HIF1 $\alpha$ – $\beta$ -catenin interaction complexes in hypoxia (Fig. 4B). However, EPAS1 failed to co-immunoprecipitate with  $\beta$ -catenin in normoxia and hypoxia (Fig. S2D). We next assessed the impact of HIF1 $\alpha$ – $\beta$ -catenin interaction on the transcriptional activity of TCF4– $\beta$ -catenin complexes. In chondrocyte stimulated by Wnt3a, the formation of TCF4– $\beta$ -catenin nuclear complexes was decreased in hypoxia compared to normoxia (Fig. 4B). These data suggest that under hypoxia,  $\beta$ -catenin may bind preferentially to HIF1 $\alpha$  rather than TCF4, thus reducing Wnt/ $\beta$ -catenin signaling.

MMP13 is targeted by both HIF and Wnt signaling (20-24). To better characterize the role of HIF1 $\alpha$ – $\beta$ -catenin interaction in inhibiting Wnt/ $\beta$ -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 HIF1 $\alpha$  in inhibiting TCF4 binding to WREs, we overexpress the stabilized form of HIF1 $\alpha$  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 HIF1 $\alpha$ . 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  $\Delta$ HIF1 $\alpha^{\text{Chon}}$  mice.** Given the sequestration of  $\beta$ -catenin by HIF1 $\alpha$ , we hypothesized that HIF1 $\alpha$  deletion may increase TCF4– $\beta$ -catenin complex level, thereby leading to chondrocyte catabolism. To verify this hypothesis, we deleted HIF1 $\alpha$  in chondrocytes by siRNA silencing and blocked TCF4– $\beta$ -catenin complexes by using PKF118-310 which blocks the interaction between TCF4 and  $\beta$ -catenin. In normoxia, PKF118-310 totally suppressed Wnt-induced *Mmp13* and target genes expression (Fig. 4E). Loss of HIF1 $\alpha$  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 HIF1 $\alpha$ . Thus, HIF1 $\alpha$  prevented Wnt to induce *MMP13* expression by blocking TCF4– $\beta$ -catenin complexes.

In OA mice, conditional loss of HIF1 $\alpha$  increased *MMP13* expression and cartilage lesions (Fig. 5A). Increased TCF4– $\beta$ -catenin complex level may promote the phenotype observed in

$\Delta$ HIF1 $\alpha^{\text{Chon}}$  mice. Articular injection of the  $\beta$ -catenin inhibitor PKF118-310 in  $\Delta$ HIF1 $\alpha^{\text{Chon}}$  mice prevented cartilage lesions and reduced MMP-13 expression as compared with PBS treatment. Thus, PKF118-310 administration in  $\Delta$ HIF1 $\alpha^{\text{Chon}}$  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 HIF1 $\alpha$  enhanced cartilage damage by increasing TCF4- $\beta$ -catenin complexes, which activated MMP13 expression.

## Discussion

Hypoxia is a characteristic of physiological articular cartilage (5, 25). We addressed the specific function of HIF1 $\alpha$  in chondrocytes and in OA in mice. With inducible conditional knockout of HIF1 $\alpha$  in mice, we showed that HIF1 $\alpha$  alleviated OA development by downregulating MMP13 through inhibition of  $\beta$ -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 HIF1 $\alpha$  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 HIF1 $\alpha$  is necessary to maintain a physiologic chondrocyte microenvironment and function. Consistent with our data, the inhibition of HIF1 $\alpha$  by 2-methoxyestradiol induced an OA phenotype in mice (5), which suggests that HIF1 $\alpha$  modulation is an important event that triggers chondrocyte differentiation in OA. With our conditional HIF1 $\alpha$ -knockout mouse model, we show that chondrogenic HIF1 $\alpha$  directly maintains cartilage metabolism. Moreover, we show that HIF1 $\alpha$  is also a physiological anti-apoptotic factor in articular cartilage as its loss enhanced chondrocyte apoptosis. Six weeks

after HIF1 $\alpha$  deletion, the increase of apoptotic chondrocyte did not induce cartilage lesion as chondrocyte apoptosis alone is not sufficient 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  $\Delta$ HIF1 $\alpha^{\text{chon}}$  mice results of the double function of HIF1 $\alpha$  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/ $\beta$ -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 HIF1 $\alpha$ -dependent manner (25). Because MMP13 is an important target of both HIF signaling and canonical Wnt pathways (20-24), we assessed whether HIF1 $\alpha$  regulates MMP13 and Wnt signaling to prevent OA. Here we demonstrated that hypoxia downregulated canonical Wnt signaling, thereby preventing chondrocyte catabolism. HIF1 $\alpha$  deficiency exacerbated cartilage catabolism, thus HIF1 $\alpha$  is necessary to prevent OA development. We show that Wnt-induced MMP13 expression was promoted in  $\Delta$ HIF1 $\alpha$  chondrocytes but was blunted by stabilized HIF1 $\alpha$  in chondrocytes. Taken together, we demonstrate that HIF1 $\alpha$  downregulates the transcription of *MMP13* driven by canonical Wnt signaling.

Given the critical role of  $\beta$ -catenin to drive Wnt signaling, understanding the role of HIF1 $\alpha$ - $\beta$ -catenin interaction may reveal novel mechanisms in regulating Wnt signaling. Because HIF1 $\alpha$  inhibits TCF4- $\beta$ -catenin interaction and transcriptional activity (9), we investigated whether HIF1 $\alpha$  modulates MMP13 through a Wnt/ $\beta$ -catenin pathway. Hypoxia promoted the translocation of  $\beta$ -catenin and binding to HIF1 $\alpha$  but not MMP13 expression. These results are consistent with transcriptional blockade of  $\beta$ -catenin activity despite the stabilization of  $\beta$ -catenin in the nucleus. Our study brings new insights into the protein regulation of HIF1 $\alpha$  and  $\beta$ -catenin interaction (9) and its impact on *MMP13* transcription. Under hypoxia, HIF1 $\alpha$ - $\beta$ -catenin complexes are preferentially formed, which results in a lower TCF4- $\beta$ -catenin complex level and therefore markedly reduced TCF4 binding to the *MMP13* regulatory region. Interestingly, when stabilized in normoxic chondrocytes, HIF1 $\alpha$  blocked TCF4 binding to the *MMP13* regulatory region. Furthermore, the inhibition of TCF4- $\beta$ -catenin complexes by PKF118-310 abolished Wnt3a-induced *MMP13* expression in  $\Delta$ HIF1 $\alpha$  chondrocytes. These results confirm the role of TCF4- $\beta$ -catenin complexes triggering *MMP13* expression in  $\Delta$ HIF1 $\alpha$  chondrocytes. Thus, HIF1 $\alpha$  is a nuclear negative regulator of TCF4- $\beta$ -catenin complexes that inhibits the shift toward a catabolic phenotype in chondrocytes.

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

catenin and Wnt signaling and is required to block cartilage degradation. The use of HIF1 $\alpha$  agonists might be a useful strategy for treating cartilage lesions in OA.

## Methods

**Harvesting, expansion and transfection of primary chondrocytes.** HIF1 $\alpha^{fl/fl}$  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-HIF1 $\alpha$ , mouse primary anti- $\beta$ -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 HIF1 $\alpha$  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 HIF1 $\alpha$ , R26R and COL2Cre<sup>ERT</sup> mice were supplied by the Jackson Laboratory. At 9 weeks of age, COL2Cre<sup>ERT</sup>;fl/fl HIF1 $\alpha$  and COL2-Cre<sup>ERT</sup>;R26R, fl/fl HIF1 $\alpha$  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  $\Delta$ HIF1 $\alpha$ <sup>chon</sup> mice (n = 8-10 per group) and fl/fl HIF1 $\alpha$  littermates (n = 9–12 per group) in the right knee, with sham operation performed in the left knee.  $\Delta$ HIF1 $\alpha$ <sup>chon</sup> and fl/fl HIF1 $\alpha$  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 (Hypoxyprom, 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- $\mu$ m-thick sagittal sections of medial femorotibial joints were collected at 3 depths at 70- $\mu$ m 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  $\pm$  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.

## References

1. Maes C, Carmeliet G, & Schipani E (2012) Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol* 8(6):358-366.
2. Maes C, *et al.* (2012) VEGF-independent cell-autonomous functions of HIF-1 $\alpha$  regulating oxygen consumption in fetal cartilage are critical for chondrocyte survival. *J Bone Miner Res* 27(3):596-609.
3. Semenza GL (2013) HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest* 123(9):3664-3671.
4. Gezer D, Vukovic M, Soga T, Pollard PJ, & Kranc KR (2014) Concise review: genetic dissection of hypoxia signaling pathways in normal and leukemic stem cells. *Stem Cells* 32(6):1390-1397.
5. Gelse K, *et al.* (2008) Role of hypoxia-inducible factor 1  $\alpha$  in the integrity of articular cartilage in murine knee joints. *Arthritis Res Ther* 10(5):R111.
6. Dunwoodie SL (2009) The role of hypoxia in development of the Mammalian embryo. *Dev Cell* 17(6):755-773.
7. Weidemann A & Johnson RS (2008) Biology of HIF-1 $\alpha$ . *Cell Death Differ* 15(4):621-627.
8. Mazumdar J, *et al.* (O<sub>2</sub> regulates stem cells through Wnt/ $\beta$ -catenin signalling. *Nat Cell Biol* 12(10):1007-1013.
9. Kaidi A, Williams AC, & Paraskeva C (2007) Interaction between  $\beta$ -catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol* 9(2):210-217.

10. Arden N & Nevitt MC (2006) Osteoarthritis: epidemiology. *Best Pract Res Clin Rheumatol* 20(1):3-25.
11. Murray CJ, *et al.* (2010) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380(9859):2197-2223.
12. Wang M, *et al.* (2011) Recent progress in understanding molecular mechanisms of cartilage degeneration during osteoarthritis. *Ann N Y Acad Sci* 1240:61-69.
13. Zhu M, *et al.* (2009) Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. *J Bone Miner Res* 24(1):12-21.
14. Funck-Brentano T, *et al.* (2014) Dkk-1-mediated inhibition of Wnt signaling in bone ameliorates osteoarthritis in mice. *Arthritis Rheumatol* 66(11):3028-3039.
15. Bouaziz W, *et al.* (2015) Loss of sclerostin promotes osteoarthritis in mice via beta-catenin-dependent and -independent Wnt pathways. *Arthritis Res Ther* 17:24.
16. Yuasa T, Otani T, Koike T, Iwamoto M, & Enomoto-Iwamoto M (2008) Wnt/beta-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration. *Lab Invest* 88(3):264-274.
17. Wu L, *et al.* (2012) Insights on biology and pathology of HIF-1alpha/-2alpha, TGFbeta/BMP, Wnt/beta-catenin, and NF-kappaB pathways in osteoarthritis. *Curr Pharm Des* 18(22):3293-3312.
18. Yan Q, Bartz S, Mao M, Li L, & Kaelin WG, Jr. (2007) The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. *Mol Cell Biol* 27(6):2092-2102.
19. Clevers H & Nusse R (2012) Wnt/beta-catenin signaling and disease. *Cell* 149(6):1192-1205.
20. Leeman MF, Curran S, & Murray GI (2002) The structure, regulation, and function of human matrix metalloproteinase-13. *Crit Rev Biochem Mol Biol* 37(3):149-166.
21. Leeman MF, McKay JA, & Murray GI (2002) Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer. *J Clin Pathol* 55(10):758-762.
22. Saito T, *et al.* (2010) Transcriptional regulation of endochondral ossification by HIF-2alpha during skeletal growth and osteoarthritis development. *Nat Med* 16(6):678-686.
23. Saito T & Kawaguchi H (2010) HIF-2alpha as a possible therapeutic target of osteoarthritis. *Osteoarthritis Cartilage* 18(12):1552-1556.

24. Yun K & Im SH (2007) Transcriptional regulation of MMP13 by Lef1 in chondrocytes. *Biochem Biophys Res Commun* 364(4):1009-1014.
25. Thoms BL, Dudek KA, Lafont JE, & Murphy CL (2013) Hypoxia promotes the production and inhibits the destruction of human articular cartilage. *Arthritis Rheum* 65(5):1302-1312.
26. Leijten JC, Moreira Teixeira LS, Landman EB, van Blitterswijk CA, & Karperien M (2012) Hypoxia inhibits hypertrophic differentiation and endochondral ossification in explanted tibiae. *PLoS One* 7(11):e49896.
27. Provot S, *et al.* (2007) Hif-1alpha regulates differentiation of limb bud mesenchyme and joint development. *J Cell Biol* 177(3):451-464.
28. Portron S, *et al.* (2015) Inverse regulation of early and late chondrogenic differentiation by oxygen tension provides cues for stem cell-based cartilage tissue engineering. *Cell Physiol Biochem* 35(3):841-857.
29. Ryu JH, *et al.* (2012) Hypoxia-inducible factor-2alpha regulates Fas-mediated chondrocyte apoptosis during osteoarthritic cartilage destruction. *Cell Death Differ* 19(3):440-450.
30. Loeser RF (2013) Osteoarthritis year in review 2013: biology. *Osteoarthritis Cartilage* 21(10):1436-1442.
31. Nalesso G, *et al.* (2011) WNT-3A modulates articular chondrocyte phenotype by activating both canonical and noncanonical pathways. *J Cell Biol* 193(3):551-564.
32. Mitani T, Harada N, Nakano Y, Inui H, & Yamaji R (2012) Coordinated action of hypoxia-inducible factor-1alpha and beta-catenin in androgen receptor signaling. *J Biol Chem* 287(40):33594-33606.
33. Hakim F, *et al.* (2014) High oxygen condition facilitates the differentiation of mouse and human pluripotent stem cells into pancreatic progenitors and insulin-producing cells. *J Biol Chem* 289(14):9623-9638.
34. Kadri A, *et al.* (2008) Osteoprotegerin inhibits cartilage degradation through an effect on trabecular bone in murine experimental osteoarthritis. *Arthritis Rheum* 58(8):2379-2386.
35. Glasson SS CM, Van Den Berg WB, Little CB. (2010) The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage*. 18(3):17-23.

## Figure legends:

### Figure 1: Hypoxia and hypoxia-inducible factor 1 $\alpha$ (HIF1 $\alpha$ ) 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 ( $P_{O_2} < 10$  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  $\pm$  SEM. \*,  $p < 0.05$  compared to control ( $n = 7$  animals/group). Bar: 100  $\mu$ m. **B.** Upper panel: HIF1 $\alpha$  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 HIF1 $\alpha$ (+) cells and OA score in articular cartilage of the tibial plateau and internal femoral condyle. Bar: 100  $\mu$ m. \*,  $p < 0.05$  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  $\mu$ m. 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  $\mu$ m) and quantification. \*,  $p < 0.05$  compared to control. #,  $p < 0.05$  ( $n = 8-11$  animals/group).

### Figure 2: HIF1 $\alpha$ 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.** Expression of anabolic and catabolic genes (*COL2A* and *MMP13*)

with HIF1 $\alpha$  siRNA silencing (n = 6); **E.** direct transcriptional targets of Wnt3a (*Axin* and *Wisp1*) with HIF1 $\alpha$  siRNA silencing (n = 6); **F.** catabolic marker *MMP13* in HIF1 $\alpha$ -lacking chondrocytes (Cre-Lox recombination in vitro) (n = 5); and **G.** direct transcriptional targets of Wnt3a (*Axin* and *Wisp1*) in HIF1 $\alpha$ -lacking chondrocytes (Cre-Lox recombination in vitro) (n = 5). Data are mean  $\pm$  SEM. \*, p < 0.05 compared to control, # p<0.05. **H.** Western blot analysis of HIF1 $\alpha$  expression in von Hippel–Lindau tumor suppressor protein (VHL)-lacking chondrocytes (with 21% O<sub>2</sub>), 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<sub>2</sub>). **J.** Western blot analysis of stabilized HIF1 $\alpha$  expression using a tag antibody (HA) in primary chondrocytes and qPCR analysis of relative gene expression of *MMP13*. Data are mean  $\pm$  SEM (n = 5 experiments); \*, p < 0.05 compared to control; # p<0.05.

**Figure 3: Wnt3a promotes  $\beta$ -catenin translocation into the nucleus in hypoxia and normoxia.**

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

**Figure 4: HIF1 $\alpha$  binds  $\beta$ -catenin and inhibits transcription factor 4 (TCF4) binding to *MMP13* regulatory region.**

**A.** Immunocytofluorescence staining of  $\beta$ -catenin and HIF1 $\alpha$  in hypoxic chondrocytes. (X63). (n = 3). **B.** Co-immunoprecipitation of  $\beta$ -catenin in nuclear protein extracts. Western blot (WB) analysis of protein levels of HIF1 $\alpha$ , TCF4 and  $\beta$ -catenin and quantification of HIF1 $\alpha$ - $\beta$ -catenin and TCF4- $\beta$ -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 HIF1 $\alpha$  siRNA silencing) with 21% and 1% O<sub>2</sub> for *MMP13* (n = 8) and direct transcriptional targets of Wnt (*Axin* and *Wisp1*) (n = 8). Data are mean  $\pm$  SEM. \*, p < 0.05 compared to control; # p<0.05.

**Figure 5: Loss of HIF1 $\alpha$  increases TCF4- $\beta$ -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  $\mu$ m. **B.** *MMP13* expression in HIF1 $\alpha^{fl/fl}$  and  $\Delta$ HIF1 $\alpha^{Chon}$  mice at Week 6 (immunohistochemistry). Bar 100  $\mu$ m 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  $\pm$  SEM, \*, p < 0.05, #: p < 0.05 compared to control Hif-1 $\alpha^{fl/fl}$  (n = 8–11 animals/group).

**Supplemental figures.**

**Figure S1:**  $\beta$ -galactosidase immunostaining in COL2-Cre<sup>ERT</sup>;R26R and R26R mice with injection of Tamoxifen (n=3).

**Figure S2**

**A.** EPAS1 expression in HIF1 $\alpha$ <sup>fl/fl</sup> and  $\Delta$ HIF1 $\alpha$ <sup>Chon</sup> 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 Hif1 $\alpha$  siRNA silencing (pixels) (n = 198-277). **C.** PCR analysis of relative gene expression in primary chondrocytes with 21% and 1% O<sub>2</sub> for Wnt targets (*Axin* and *Wisp1*) with EPAS1 siRNA silencing (n = 6). **D.** Co-immunoprecipitation of  $\beta$ -catenin in nuclear protein extracts. Western blot (WB) analysis of protein levels of EPAS1 and  $\beta$ -catenin.