FIH-1-Mint3 Axis Does Not Control HIF-1α Transcriptional Activity in Nucleus Pulposus Cells*

Yuichiro Hirose[‡§], Zariel I. Johnson[‡1], Zachary R. Schoepflin[‡1], Dessislava Z. Markova[‡], Kazuhiro Chiba[¶], Yoshiaki Toyama[§], Irving M. Shapiro[‡], and Makarand V. Risbud[‡2]

From the [‡]Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, the [§]Department of Orthopaedic Surgery, School of Medicine, Keio University, Tokyo 108-8345, Japan, and the [¶]Department of Orthopaedic Surgery, Kitasato University Kitasato Institute Hospital, Tokyo 108-8641, Japan

Background: Cells of the hypoxic nucleus pulposus (NP) require tightly regulated HIF-1 signaling for proper function.
Results: Although overexpressed FIH-1 can regulate HIF-1 activity in NP cells, silencing endogenous FIH-1 results in no change in HIF-1 target gene expression.
Conclusion: FIH-1 does not represent a major mechanism of controlling HIF-1-dependent transcription in NP cells.
Significance: This study describes a physiological adaptation of NP cells.

The objective of this study was to determine the role of FIH-1 in regulating HIF-1 activity in the nucleus pulposus (NP) cells and the control of this regulation by binding and sequestration of FIH-1 by Mint3. FIH-1 and Mint3 were both expressed in the NP and were shown to strongly co-localize within the cell nucleus. Although both mRNA and protein expression of FIH-1 decreased in hypoxia, only Mint3 protein levels were hypoxia-sensitive. Overexpression of FIH-1 was able to reduce HIF-1 function, as seen by changes in activities of hypoxia response element-luciferase reporter and HIF-1α-C-TAD and HIF-2α-TAD. Moreover, co-transfection of either full-length Mint3 or the N terminus of Mint3 abrogated FIH-1-dependent reduction in HIF-1 activity under both normoxia and hypoxia. Nuclear levels of FIH-1 and Mint3 decreased in hypoxia, and the use of specific nuclear import and export inhibitors clearly showed that cellular compartmentalization of overexpressed FIH-1 was critical for its regulation of HIF-1 activity in NP cells. Interestingly, microarray results after stable silencing of FIH-1 showed no significant changes in transcripts of classical HIF-1 target genes. However, expression of several other transcripts, including those of the Notch pathway, changed in FIH-1-silenced cells. Moreover, co-transfection of Notch-ICD could restore suppression of HIF-1-TAD activity by exogenous FIH-1. Taken together, these results suggest that, possibly due to low endogenous levels and/or preferential association with substrates such as Notch, FIH-1 activity does not represent a major mechanism by which NP cells control HIF-1-dependent transcription, a testament to their adaptation to a unique hypoxic niche.

The intervertebral disc is a complex tissue that permits a range of motion between adjacent vertebrae and accommodates biomechanical forces. It consists of an outer fibrocartilagenous annulus fibrosus (AF)[3] that encloses gel-like nucleus pulposus (NP). Although NP is completely avascular, blood vessels infiltrate only the superficial region of the end plates and the outer third of annulus fibrosus, making this tissue hypoxic (1, 2). However, during degeneration and herniation, vascular ingrowth can be seen into the tissue, altering its oxemic status (3).

NP cells have adapted to survive in this hypoxic niche through robust expression of hypoxia-inducible factor (HIF-1α), a transcription factor responsive to local oxygen tension (4). Importantly, HIF-1α is critical for maintenance of NP cell survival, glycolytic metabolism, and functional activities, including proteoglycan-rich matrix synthesis (5–9). HIF is a basic helix-loop-helix transcription factor comprising a constitutively expressed β subunit and an α subunit that undergoes degradation by both oxygen-dependent and -independent pathways (10, 11). When stabilized, the α subunit dimerizes with the β subunit and binds to hypoxia response elements (HREs) in the promoter of target genes. In most cells, HIF activity is regulated by the action of prolyl hydroxylases (PHDs) and factor inhibiting HIF-1 (FIH-1). In oxygen-rich conditions, PHDs modify proline residues in the oxygen-dependent degradation domain of HIF-1α, resulting in recognition of the modified HIF-1α by von Hippel-Lindau protein (12, 13). von Hippel-Lindau protein exhibits E3 ubiquitin ligase activity that leads to degradation of the modified HIF-1α by the proteasome system (14). Although PHDs are expressed in the hypoxic NP in HIF-dependent fashion, only PHD2 controls HIF-α degradation to a limited extent (10, 15).

The transcriptional activity of HIF-1/2α is mediated by the C-terminal transactivation domain (C-TAD) that binds essen-

[3] The abbreviations used are: AF, annulus fibrosus; NP, nucleus pulposus; HIF, hypoxia-inducible factor; FIH-1, factor inhibitory to HIF-1α; Mint3, amyloid β A4 precursor protein-binding family A member 3; HRE, hypoxia response element; PHD, prolyl hydroxylase; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; TAD, transactivation domain; C-TAD, C-terminal TAD; ICD, intracellular domain; ST, sense transcript.

tial transcriptional co-activators p300/CBP (16–18). An aspar-agine residue within the C-TAD of HIF-1α (Asn[803]) and of HIF-2α (Asn[851]) is a target for hydroxylation by FIH-1, a non-redundant asparaginyl hydroxylase that, similar to PHD, requires molecular oxygen, α-ketogluratate, and ascorbate as its substrates. This post-translational modification of a specific Asn residue prevents binding to p300/CBP, thereby suppressing HIF-1/2 transcriptional activity (18, 19). Noteworthy, because the K_m of FIH-1 for oxygen is significantly lower than that of PHD1–3, even under conditions of moderate hypoxia, such as those present in the NP, FIH-1 activity is preserved (20). Thus, controlling expression/activity of FIH-1 is one of the important ways cells control HIF transcriptional activity. For example, in macrophages, FIH-1 activity is suppressed by an X11 protein family member, Mint3/APBA3 (21–23), through its N-terminal domain that binds and sequesters FIH-1. This interaction limits the ability of FIH-1 to hydroxylate and block HIF-1 function (23); as a consequence of this high HIF-1 activity, macrophages generate most of their ATP through glycolysis.

Although the PHD-dependent regulation of the activity of HIF in NP cells has received some attention, the role of FIH-1 and Mint3 in NP cells is completely unknown. Therefore, the major goal of this study is to delineate the role of FIH-1 and Mint3 in regulating HIF activity in NP cells. Our results clearly show that, although the Mint3 or FIH-1 system is capable of controlling HIF-1 function, due to the low endogenous levels of both of these proteins and/or preferential binding of FIH-1 with substrates such as Notch, they are likely to play a limited role in controlling HIF-1 transcriptional activity in physiologically hypoxic NP cells.

MATERIALS AND METHODS

Plasmids and Reagents—For transactivation studies of HIF-1α and HIF-2α, the binary Gal4 reporter plasmids (HIF-1α-C-TAD, amino acids 740–826; HIF-2α-TAD, amino acids 819–870) were provided by Dr. Nianli Sang (Thomas Jefferson University, Philadelphia, PA). Backbone plasmid pM (Clontech) contains no transactivation domain (TAD) but expresses the Gal4 DNA binding domain. pFR-Luc (Stratagene) reporter contains the yeast Gal4-binding site upstream of a minimal promoter and the firefly luciferase gene. Expression constructs of pcDNA3.1-FIH-1 were provided by Dr. Richard Bruick (University of Texas Southwestern Medical Center). Constructs, pcDNA3.1-Mint3, pcDNA3.1-Mint3-NT (amino acids 1–214), pcDNA3.1-Mint3-CT (amino acids 215–575), lentiviral Sh-FIH-1, Sh-Mint3, and FL-Mint3 were kindly provided by Dr. Sakamoto (University of Tokyo, Japan) (23). Notch1-ICD (catalog no. 15131) by Connie Cepko, Notch2-ICD (catalog no. 20184) by Raphael Kopan, HRE-Luc (catalog no. 26731) by Navdeep Chandel and psPAX2 (catalog no. 12260), pMD2.G (catalog no. 12259), pMDLg/pRRE (catalog no. 12251), and pRSV/Rev (catalog no. 12253) by Didier Trono were obtained from Addgene. pRL-TK (Promega) containing the *Renilla reniformis* luciferase gene was used as an internal transfection control.

Isolation of NP Cells, Cell Treatments, and Hypoxic Culture—Rat and human NP cells were isolated and characterized as reported earlier (5). T/C-28, a human chondrocyte line, was kindly provided by Dr. Mary Goldring (Hospital for Special Surgery, Weill Cornell Medical College, New York) (24). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics. To investigate the effect of nuclear transport inhibitors, cells were treated with ivermectin (12.5 μM) or leptomycin B (10 ng/ml) for 16 or 24 h, respectively. Cells were cultured in a hypoxia work station (Invivo₂ 300, Ruskinn, Bridgend, UK) with a mixture of 1% O_2, 5% CO_2, and 94% N_2 for 24–72 h.

Immunohistological Studies—Freshly isolated rat spines were immediately fixed in 10% paraformaldehyde in PBS and then embedded in paraffin. Sagittal sections, 6–8 μm in thickness, were deparaffinized in xylene and rehydrated through graded ethanol and some sections were stained with Alcian blue, eosin, and hematoxylin. For localizing FIH-1 and Mint3, sections were incubated with either the anti-FIH-1 (1:100; Novus) or anti-Mint3 (1:50; Novus) antibodies in 5% goat serum in PBS at 4 °C overnight. After thoroughly washing the sections, the bound primary antibodies were incubated with Alexa Fluor-488-conjugated anti-rabbit (Invitrogen) secondary antibody at a dilution of 1:200 for 1 h at room temperature. Sections were visualized using a fluorescence microscope (Nikon, Japan).

Real-time RT-PCR Analysis—Total RNA was extracted from NP cells using RNAeasy minicolumns (Qiagen). Before elution from the column, RNA was treated with RNase-free DNase I (Qiagen). The purified, DNA-free RNA was converted to cDNA using EcoDry™ Premix (Clontech). Template cDNA and gene-specific primers were added to the SYBR Green master mixture (Applied Biosystems), and mRNA expression was quantified using the Step One Plus real-time PCR system (Applied Biosystems). Hprt1 and β-actin were used to normalize gene expression. Melting curves were analyzed to verify the specificity of the RT-PCR and the absence of primer dimer formation. Each sample was analyzed in duplicate and included a template-free control. All primers used were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Western Blotting—Cells were placed on ice immediately following treatment and washed with ice-cold Hanks' balanced salt solution. All wash buffers and final resuspension buffer included 1× protease inhibitor mixture (Roche Applied Science), NaF (5 mM), and Na_3VO_4 (200 μM). Nuclear and cytosolic proteins were prepared using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Total cell proteins were resolved on 10–12% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4 °C in 5% nonfat dry milk in TBST with the anti-FIH-1 (1:2000; Novus Biologicals), anti-human Mint3 (1:800; BD Biosciences), anti-rat Mint3 (1:1000; Novus Biologicals), anti-β-tubulin (1:4000; Developmental Studies Hybridoma Bank), anti-GAPDH (1:4000; Novus Biologicals), or anti-Lamin A/C (1:1000; Cell Signaling). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

HIF-1α Function in NP Cells Is Not Controlled by FIH-1-Mint3

Transfections and Dual Luciferase Assay—Cells were transferred to 48-well plates at a density of 2×10^4 cells/well 1 day before transfection. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. For measuring the effect of hypoxia or ivermectin and leptomycin B treatment on HRE-Luc activity, 24 h after transfection, the cells in some wells were treated with ivermectin or leptomycin B or moved to the hypoxia work station. The next day, the cells were harvested, and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurements of firefly and *Renilla* luciferase activities. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20, Turner Designs, CA).

Lentiviral Production and Transduction—HEK 293T cells were seeded in 10-cm plates (1.0×10^6 cells/plate) in DMEM with 10% heat-inactivated FBS 1 day before transfection. Cells were transfected with 12 μg of Sh-FIH-1, Sh-Mint3, or FL-Mint3 plasmids along with 2 μg of pMD2G, 2 μg of pMDLg/pRRE, and 2 μg of pRSV/Rev using calcium phosphate solution. After 16 h, transfection medium was removed and replaced with DMEM with 10% heat-inactivated FBS and penicillin-streptomycin. Lentiviral particles were harvested at 48 and 60 h post-transfection. NP cells were plated in DMEM with 10% heat-inactivated FBS 1 day before transduction. Cells in 10-cm plates were transduced with 8 ml of conditioned medium containing viral particles along with 6 μg/ml Polybrene. After 24 h, conditioned medium was removed and replaced with DMEM with 10% heat-inactivated FBS. Cells were harvested for mRNA or protein 5 days after viral transduction.

Immunofluorescence Microscopy—Human and rat NP cells were plated in a collagen-coated 4-well chamber slide (8×10^3/well). Cells were fixed with 4% paraformaldehyde, permeabilized with ice-cold 100% methanol for 10 min, and blocked with PBS containing 10% goat serum for 1 h at room temperature. Human cells are incubated with antibodies against FIH-1 (1:50), Mint3 (1:50), RCAS1 (Golgi marker, Cell Signaling; 1:100), and EEA1 (endosome marker, Cell Signaling; 1:200), at 4 °C overnight. Rat cells were incubated with either FIH-1 (1:100) or Mint3 (1:100, Novus) antibodies. After washing with PBS, the cells were incubated with Alexa Fluor-488 conjugated anti-rabbit (Invitrogen) and Alexa Fluor-594-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch) at a dilution of 1:100 for 1 h at room temperature. The images were taken with a Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc., Melville, NY) or Fluoview confocal system (Olympus, Japan). ImagePro 5.0 software (Media Cybermetics, Inc., Rockville, MD) was used to capture and analyze human NP cell images.

Microarray Analysis—Amplification of cDNA was performed using the Ovation Pico WTA-system V2 RNA amplification system (NuGen Technologies, Inc.). Briefly, 50 ng of total RNA was reverse transcribed using a chimeric cDNA/mRNA primer, and a second complementary cDNA strand was synthesized. Purified cDNA was then amplified with ribo-SPIA enzyme and SPIA DNA/RNA primers (NuGEN Technologies, Inc.). Amplified ST-DNA was purified with Qiagen MinElute reaction cleanup kit. The concentration of purified ST-cDNA was measured using the nanodrop. 2.5 μg of ST-cDNAs were fragmented and chemically labeled with biotin to generate biotinylated ST-cDNA using FL-Ovation cDNA biotin module V2 (NuGen Technologies, Inc.). Affymetrix gene chips (human gene 1.0 ST array (Affymetrix, Santa Clara, CA)) were hybridized with fragmented and biotin-labeled target (2.5 μg) in 110 μl of hybridization mixture. Target denaturation was performed at 99 °C for 2 min and then 45 °C for 5 min, followed by hybridization for 18 h. Arrays were then washed and stained using a Gene Chip Fluidic Station 450, and hybridization signals were amplified using antibody amplification with goat IgG and anti-streptavidin biotinylated antibody. Chips were scanned on an Affymetrix Gene Chip Scanner 3000, using Command Console software. Background correction and normalization were done using Iterative Plier 16 with GeneSpring version 12.0 software (Agilent, Palo Alto, CA). A 1.5-fold differentially expressed gene list was generated.

Statistical Analysis—All measurements were performed at least in triplicate, and data are presented as mean ± S.E. Differences between groups were analyzed by Student's *t* test and one-way analysis of variance (*, $p < 0.05$).

RESULTS

FIH-1 and Mint3 Are Expressed in Both Rat and Human NP Tissues and Are Co-localized—To investigate expression of FIH-1 and Mint3 in the intervertebral disc, we stained rat NP tissues with antibodies against FIH-1 and Mint3 (Fig. 1*A*). Fig. 1*A* shows that FIH-1 and Mint3 proteins are expressed in both NP and AF tissues. Real-time RT-PCR analysis shows lower expression of both *Mint3* and *Fih-1* mRNAs in AF tissue than in NP (Fig. 1*B*). We also measured expression of FIH-1 and Mint3 protein in NP tissues isolated from three rats by Western blot analysis (Fig. 1*C*). All three samples expressed both FIH-1 and Mint3. Then to investigate the cellular localization of FIH-1 and Mint3 in NP cells, we immunostained NP cells with anti-Mint3 and anti-FIH-1 along with antibodies directed against RCAS1, a Golgi marker, or EEA1, an early endosome marker. Surprisingly, Mint3 was strongly co-localized with FIH-1 in the cell nucleus. On the other hand, although some localization of Mint3 was seen in Golgi, little or no staining was seen in early endosomes (Fig. 1*D*).
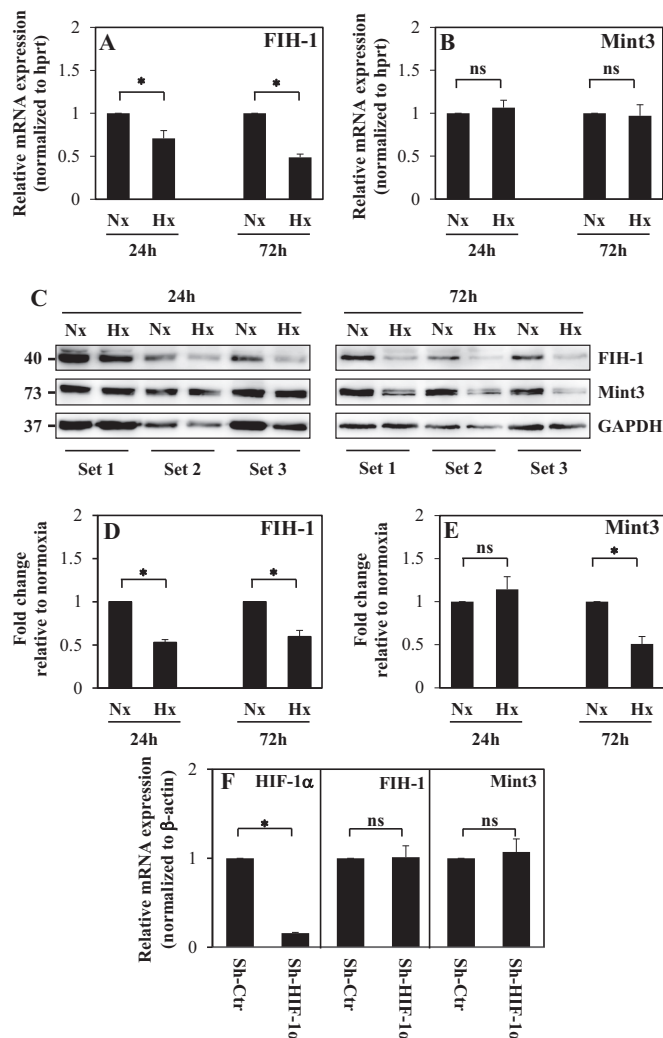
Hypoxia Represses FIH-1 and Mint3 Expression in HIF-1-independent Fashion in NP Cells—To evaluate the effect of hypoxia on FIH-1 and Mint3 levels in NP cells, we measured their expression in NP cells under hypoxia using real-time RT-PCR and Western blot analysis. Our results show that although mRNA expression of *Fih-1* was suppressed by hypoxia, *Mint3* expression was not affected (Fig. 2, *A* and *B*). Interestingly, Western blot and subsequent densitometric analysis shows that protein expressions of both FIH-1 and Mint3 are significantly down-regulated by 72 h in hypoxia (Fig. 2, *C–E*); hypoxic decrease in FIH-1 levels is evident even at 24 h. We then investigated if HIF-1 played a role in hypoxic suppression of FIH-1 mRNA. For this purpose, we silenced HIF-1α in NP cells by lentiviral delivery of shRNA and measured FIH-1 and Mint3 expression. Real-time RT-PCR analysis shows that knockdown of HIF-1α does not affect mRNA levels of *FIH-1* or *MINT3* in NP cells (Fig. 2*F*).
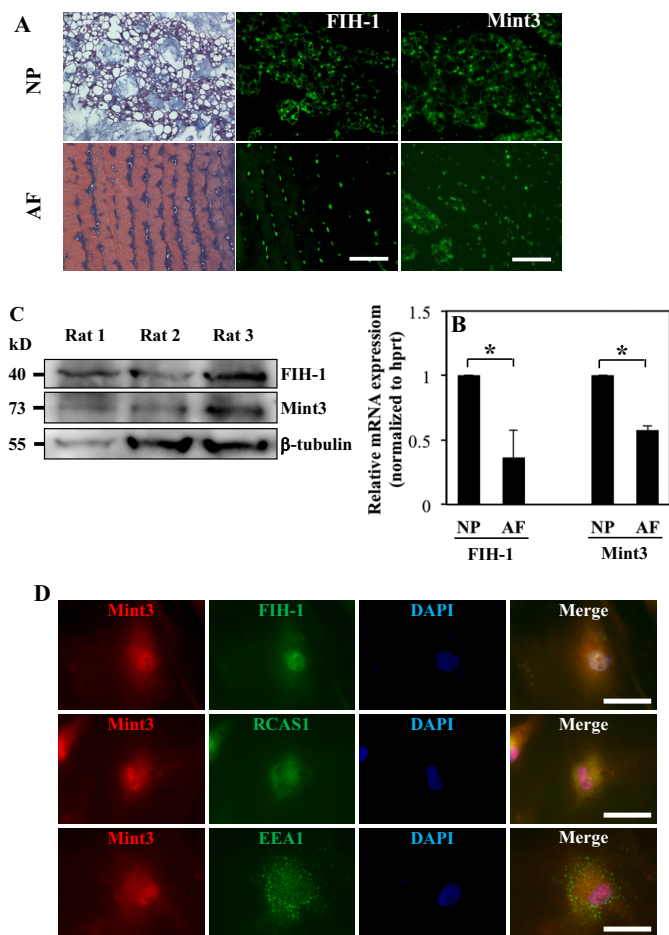
FIGURE 1. **FIH-1 and Mint3 are expressed in rat NP tissues.** *A,* immunohistochemistry of FIH-1 and Mint3 in rat intervertebral disc tissues. Sagittal sections of the intervertebral disc of a mature rat were treated with either FIH-1 or Mint3 antibodies. FIH-1 and Mint3 proteins were expressed in both NP and AF tissues. *Scale bar,* 100 μm. *B,* real-time RT-PCR analysis of FIH-1 and Mint3 in rat NP and AF tissues shows lower expression of both FIH-1 and Mint3 in AF tissues compared with NP. Data represent the mean ± S.E. (*error bars*) from three rats ($p < 0.1$). *C,* Western blot analysis of FIH-1 and Mint3 expression in NP tissues from three rats. All tissue samples expressed both the proteins. *D,* subcellular co-localization of FIH-1 and Mint3 in NP cells. Immunofluorescence shows that FIH-1 and Mint3 are primarily co-localized in the cell nucleus (*top*) and to a smaller extent in the Golgi (*middle*) but not in early endosomes (*bottom*). *Scale bars,* 50 μm.

Both HIF-1 and HIF-2 Activity Is Controlled by Forced Expression of FIH-1 in NP Cells—Next, we examined the effect of forced expression of FIH-1 on HIF transactivation in rat NP cells by measuring the ability of HIF-1α-C-TAD and HIF-2α-TAD to recruit co-factors and initiate transcription using a GAL4 binary system (see schematic in Fig. 3, *A* and *B*). Fig. 3, *C* and *D,* shows that the transactivation of HIF-1α/HIF-2α-TAD, which contain crucial Asn[803] and Asn[851] (19), respectively, is significantly suppressed by exogenous FIH-1. Moreover, overexpression of FIH-1 results in decreased activity of the HIF-responsive HRE reporter (PGK1–3xHRE-Luc) under both normoxia and hypoxia (Fig. 3, *E* and *F*). Although HRE reporter activity in hypoxia was ~3-fold higher than basal activity in normoxia, it failed to reach the normoxic baseline following forced expression of FIH-1 (Fig. 3*F*).

Decrease in HIF Activity by Exogenous FIH-1 Is Rescued by Mint3 Overexpression—Next, we evaluated if Mint3 modulates HIF function by measuring the activity of HRE-Luc reporter

FIGURE 2. **FIH-1 and Mint3 levels are decreased in hypoxia in NP cells.** *A* and *B,* real-time RT-PCR analysis of hypoxic changes in FIH-1 and Mint3 mRNA expression in NP cells. FIH-1 mRNA expression was significantly down-regulated at 72 h under hypoxia (1% O$_2$), but Mint3 mRNA expression was not affected by hypoxia until 72 h. *C,* Western blot analysis of FIH-1 and Mint3 expression in NP cells cultured under hypoxia for 24–72 h. Both FIH-1 and Mint3 protein levels were decreased by hypoxia. *D* and *E,* multiple blots of the experiment as described in *C* above were quantified by densitometric analysis. GAPDH expression was used as a loading control and to calculate relative expression level in hypoxia compared with the normoxic level. Both FIH-1 and Mint3 protein levels were decreased after a 72-h hypoxic culture. *F,* real-time RT-PCR analysis of HIF-1α, FIH-1, and Mint3 expression in NP cells transduced with Sh-control or Sh-HIF-1α under hypoxia. Note that the significant reduction of HIF-1α was seen in the cells transduced by Sh-HIF-1α, but FIH-1 and Mint3 expressions were not affected by HIF-1α silencing. Data represent the mean ± S.E. (*error bars*) of three independent experiments. *, $p < 0.05$.

under normoxic and hypoxic conditions. Results show that overexpression of Mint3 increased HRE-Luc reporter activity under normoxia (Fig. 4*A*). However, to our surprise, overexpression of Mint3 failed to modulate HRE reporter under hypoxia. Similar to full-length Mint3, when the N-terminal of protein, which is shown to bind and sequester FIH-1, was transfected into nucleus pulpous cells, HRE-Luc reporter activity was significantly induced only under normoxia (Fig. 4, *C* and *D*). In contrast, overexpression of the C-terminal portion of Mint3 failed to induce HRE reporter activity; rather, a small but significant decrease in activity irrespective of oxemic tension
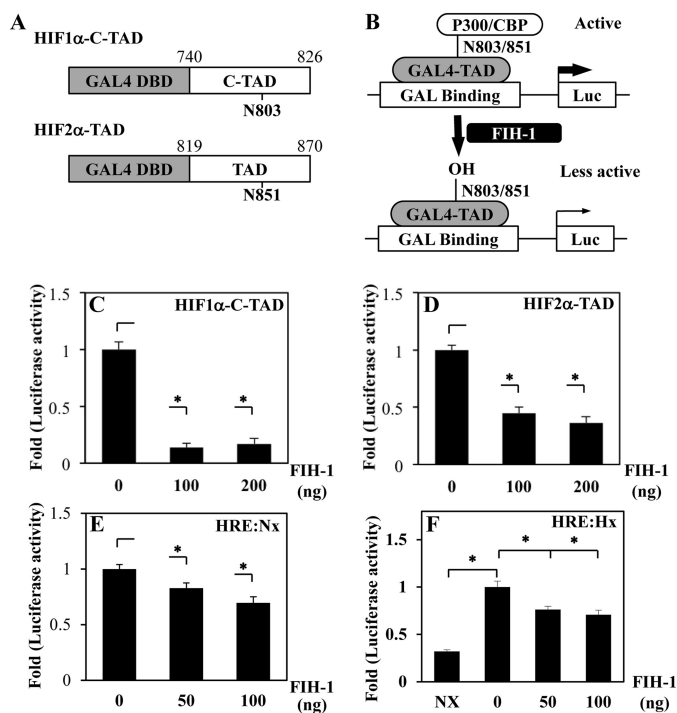
FIGURE 3. HIF transcriptional activity is suppressed by forced expression of FIH-1 in NP cells. *A*, schematic of Gal4 constructs of HIF-1α- and HIF-2α-TAD. *B*, schematic summarizing the known function of FIH-1 in controlling HIF-1 transcriptional activity. Hydroxylation of Asn[803] in HIF-1α-C-TAD catalyzed by FIH-1 blocks interaction with transcriptional co-activators p300/CBP, leading to diminished HIF-1 transcriptional activity. *C* and *D*, overexpression of FIH-1 significantly decreased the HIF-1α- and HIF-2α-TAD activity in NP cells. *E* and *F*, overexpression of FIH-1 resulted in decreased HRE-Luc reporter activity under both normoxia and hypoxia in NP cells. As expected, in hypoxia the activity of HRE reporter was significantly higher than basal activity in normoxia (*NX*). Data represent the mean ± S.E. (*error bars*) of three independent experiments, each performed in triplicate. *, $p < 0.05$.

was seen. To delineate whether Mint3 mediated its action on HIF function through controlling FIH-1, we overexpressed FIH-1 in the presence or absence of exogenous Mint3 and measured HRE reporter activity. Fig. 4, *E* and *F*, clearly shows that, irrespective of pO$_2$, exogenous Mint3 can restore the decrease in HRE reporter activity mediated by exogenous FIH-1, suggesting interaction between these two proteins (Fig. 4, *E* and *F*). To confirm whether FIH-1 binds to Mint3 in NP cells, we performed immunoprecipitation using FIH-1 antibody. Fig. 4*G* shows that FIH-1 interacts with Mint3 under both normoxia and hypoxia with similar affinity (Fig. 4*G*).

Ability of the FIH-1/Mint3 System to Regulate HIF Activity Depends on Subcellular Localization—To further clarify the mechanism of FIH-1/Mint3 action in NP cells, we first examined cellular localization of these two proteins. Immunofluorescence of human NP cells shows that both FIH-1 and Mint3 are localized in both the nucleus and cytoplasm (Fig. 5*A*). Next, using Western blot analysis, we evaluated the expression of FIH-1 and Mint3 in cytoplasmic and nuclear fractions of NP cells following 72 h of culture under normoxia or hypoxia (Fig. 5*B*). Densitometric analysis of multiple Western blots revealed that whereas FIH-1 and Mint3 levels in the cytoplasmic fraction remained unchanged, hypoxia decreased their levels in the nuclear fraction (Fig. 5, *C* and *D*). This clearly suggests that the

hypoxic decrease seen in the levels of both of these proteins in total cell lysates (Fig. 2, *C–E*) is attributable to their reduction in the nuclear fraction. To investigate the importance of intracellular localization of FIH-1 on HIF-1 activity, we performed transfection experiments in the presence of ivermectin, a nuclear import inhibitor, or leptomycin B, a nuclear export inhibitor. As shown in Fig. 5, *E* and *F*, FIH-1-mediated suppression of HRE-Luc reporter activity was abolished by ivermectin treatment. In contrast, the reporter activity further decreased by leptomycin B under both normoxia and hypoxia, suggesting that in NP cells, FIH-1 is only able to regulate HIF activity in the nuclear compartment.

Endogenous FIH-1/Mint3 Has Minimal Effect on HIF Transcriptional Activity—To assess the contribution of endogenous FIH-1 and Mint3 on HIF-1 activity in NP cells, we transduced cells with lentivirus expressing Sh-FIH-1, Sh-Mint3, and full-length Mint3 under normoxia. Because FIH-1 and Mint3 levels are comparatively higher in normoxia, and given the constitutive presence of HIF-1 protein in NP cells, silencing of FIH-1 and Mint3 in normoxia was more likely to evince changes in HIF-1 transcriptional activity, if any. Real-time RT-PCR analysis indicates that shRNA selectively suppressed the expression of either *FIH-1* or *MINT3* without affecting the expression of the other (Fig. 6, *A* and *B*). Western blot and densitometric analysis confirmed that shRNAs and overexpression of Mint3 significantly affected expression of the respective protein levels (Fig. 6, *C* and *D*). Next, to determine the changes in global gene expression profile following FIH-1 silencing, we performed microarray analysis on RNA isolated from cells transduced with Sh-FIH-1. The values were plotted in a volcano plot analyzing expression patterns in FIH-1-silenced NP cells *versus* control using the $-\log_{10}(p$ value) and $-\log_2$(-fold change) (Fig. 6*E*). Knockdown of FIH-1 in NP cells induced 1.5-fold or greater change in expression of 535 gene transcripts ($p < 0.1$), of which 289 were up-regulated and 246 were down-regulated. Fig. 6*F* shows the heat map and dendrogram of the 535 gene transcripts. Surprisingly, as shown in Table 1, the expression of several known HIF-1 target genes, including those regulating glycolytic metabolism (*PGK-1*, *PFKFB2*, *ENO1*, *SLC2A1*, and *SLC2A3*), HIF-1 stability and function (*EGLN1*, *EGLN3*, and *HSPA1A*), and NP cell physiology (*CTGF*, *LGALS3*, *B3GAT3*, and *VEGFA*), were not affected by FIH-1 silencing in NP cells. A partial list of the maximally up-regulated and down-regulated transcripts following FIH-1 silencing is given in Table 2. Finally, to validate microarray results, we performed real-time RT-PCR analysis of known HIF target genes in FIH-1-silenced cells. Fig. 7 clearly shows that mRNA expression of HIF target genes in NP cells, such as *EGLN1* (PHD2) (Fig. 7*A*), *EGLN3* (Fig. 7*B*), *VEGFA* (Fig. 7*C*), and *ENO1* (Fig. 7*D*), remained unaffected by silencing of either FIH-1 or Mint3. Likewise, overexpression of Mint3 had no effect on any of these target genes.

To ascertain whether the seeming inability of FIH-1 to regulate HIF is a trait shared by other physiologically hypoxic tissues, we investigated the effect of FIH-1 and Mint3 knockdown in T/C-28 cells, a human chondrocyte line. Interestingly, following transfection with shRNA targeting *FIH-1* as well as *MINT3*, HRE reporter activity did not change in the chondro-
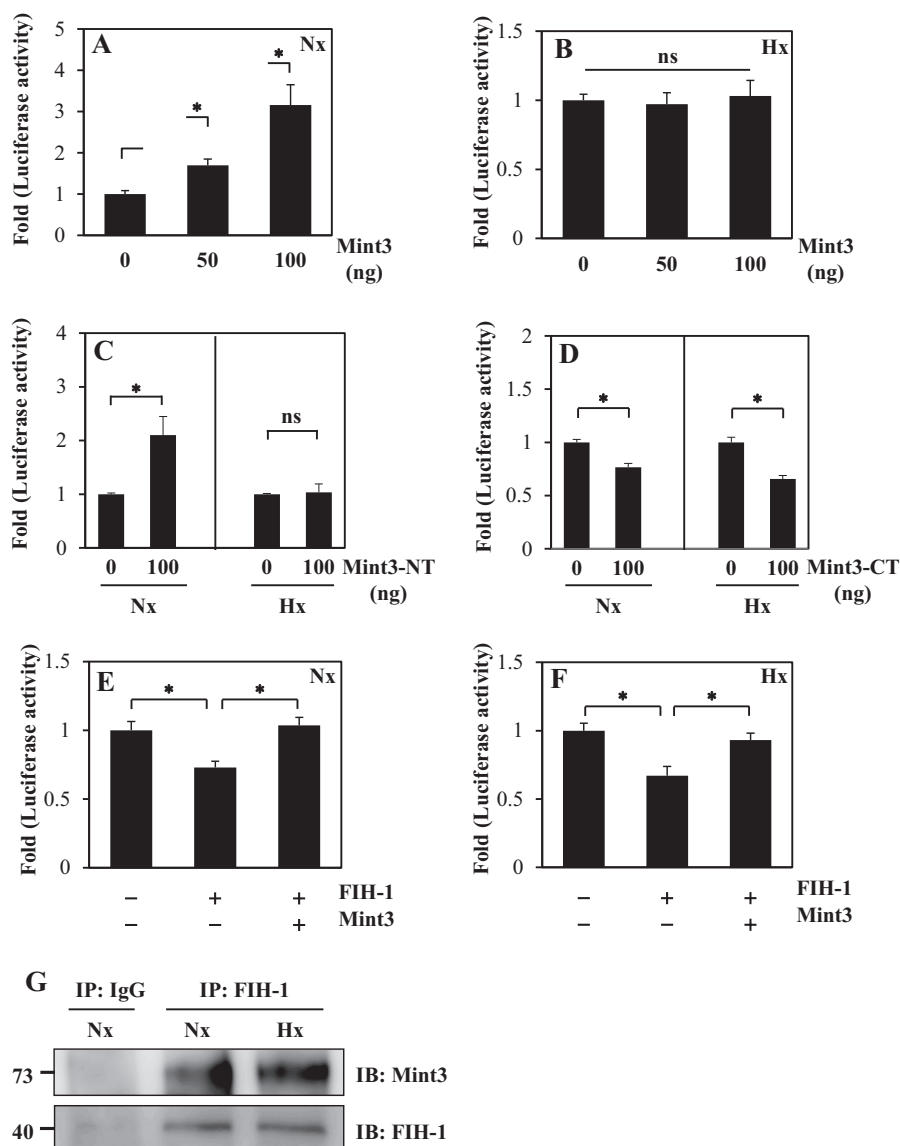
FIGURE 4. **Decrease in HIF transcriptional activity by exogenous FIH-1 is rescued by Mint3 overexpression.** *A* and *B*, overexpression of Mint3 in NP cells increased HRE reporter activity in normoxia (*Nx*) (*A*) but not in hypoxia (*Hx*) (*B*). *C* and *D*, the overexpression of the N terminus (*Mint3-NT*) (*C*) but not the C terminus (*Mint3-CT*) (*D*) of Mint3 is necessary and sufficient for increasing HRE reporter activity in normoxia. No increase in HRE activity was seen by either of the shorter Mint3 constructs under hypoxia. HRE activity is represented as relative change compared with the respective controls in normoxia or hypoxia. *E* and *F*, overexpression of Mint3 can rescue loss of HRE reporter activity induced by exogenous FIH-1 irrespective of pO_2, suggesting that the level of endogenous FIH-1 in hypoxia is low and thus has little effect on HIF activity. Data represent mean ± S.E. of three independent experiments, each performed in triplicate (*, $p < 0.05$). *G*, immunoprecipitation (*IP*) of FIH-1 from rat NP cells in normoxia or hypoxia followed by Western blot analysis (*IB*) using Mint3 antibody indicated association between FIH-1 and Mint3. When an isotype IgG was used in place of FIH-1 antibody in normoxia, precipitation of either FIH-1 or Mint3 was absent. *ns*, not significant.

cytes at 3% oxygen, which is known to stabilize HIF-1α (data not shown), suggesting that, similar to NP cells, HIF activity in chondrocytes is not regulated by endogenous FIH-1. Furthermore, we examined the expression of HIF target genes in T/C-28 cells transduced with shRNA targeting *FIH-1* and *MINT3*. Results clearly show that *VEGFA*, *PHD2*, and *ENO1* expression was unaltered in FIH-1- or Mint3-silenced T/C28 cells (data not shown).

Notch1-ICD and Notch2-ICD Restores Suppression of HIF-α-TAD Activity Mediated by Exogenous FIH-1—Due to known functional interactions between Notch receptors and FIH-1, we first determined whether FIH-1 knockdown in NP cells affects the expression of genes concerned with the Notch

signaling pathway. Fig. 8*A* shows that FIH-1 silencing results in induction of several known Notch signaling pathway genes, including *HELT* (HES/HEY-like transcription factor), a basic helix-loop-helix-containing transcriptional repressor, and neuralized homolog (*Drosophila*) (*NEURL*), an activator of JAG1 signaling, whereas expression of reelin (*RELN*), a large secreted extracellular matrix protein involved in controlling cell-cell interactions, was suppressed. Next, we investigated whether co-expression of Notch1-ICD or Notch2-ICD can rescue FIH-1 mediated inhibition of HIF-α transcriptional activity. Fig. 8, *B–E*, clearly shows that a decrease in HIF-1α-C-TAD (Fig. 8, *B* and *D*) and HIF-2α-TAD (Fig. 8, *C* and *E*) activity by exoge-
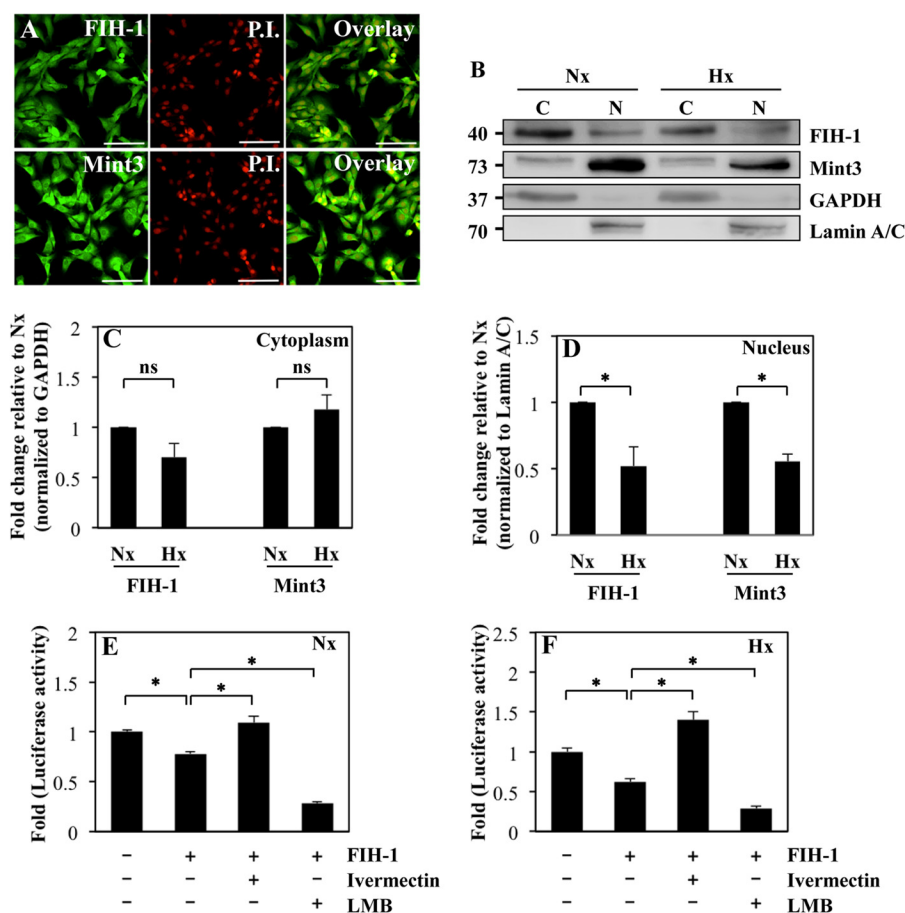
FIGURE 5. **Ability of the FIH-1/Mint3 system to regulate HIF activity in NP cells depends on subcellular localization.** *A,* immunofluorescence analysis of cellular localization of FIH-1 and Mint3 in rat NP cells. Both FIH-1 and Mint3 are present in both the nucleus and cytoplasm. Propidium iodide (*P.I.*) was used to label cell nuclei. *Scale bars,* 100 μm. *B,* Western blot analysis of cellular localization of FIH-1 and Mint3 in NP cells. FIH-1 is localized in the cell nucleus and cytoplasm. The Mint3 level is higher in the nucleus than in the cytoplasm. Both FIH-1 and Mint3 are decreased by hypoxia in the nuclear fraction. *C,* cytoplasm. *N,* nucleus. *C* and *D,* multiple blots were quantified by densitometric analysis. Expression of GAPDH for cytoplasmic and Lamin A/C for nuclear protein was used as a loading control and to calculate relative expression levels. Hypoxia decreased nuclear levels of both FIH-1 and Mint3. *E* and *F,* reduction of HRE-Luc activity mediated by exogenous FIH-1 was abolished by treatment with ivermectin, a nuclear import inhibitor. In contrast, treatment with leptomycin B, a nuclear export inhibitor, further reduced FIH-1-dependent HRE reporter activity under both normoxia (*Nx*) and hypoxia (*Hx*). *LMB,* leptomycin B. Data represent mean $\pm$ S.E. (*error bars*) of three independent experiments. *, $p < 0.05$.

nous FIH-1 can be restored by co-expression of either Notch1-ICD (Fig. 8, *B* and *C*) or Notch2-ICD (Fig. 8, *D* and *E*).

DISCUSSION

The cells of the NP reside in a physiologically hypoxic niche. The cellular processes through which the cells survive in hypoxia are not wholly understood. We have previously shown that the cells of the NP robustly express HIF-1α and HIF-2α, and these isoforms play an important role in regulating glycolytic metabolism, survival, and synthesis of extracellular matrix (4–9). It is noteworthy that in NP cells, HIF-1 activity is uniquely regulated in that PHDs play a limited role in its proteasomal degradation. Relevant to this study, we have also shown that PHD2 is capable of modulating HIF-1α levels in NP cells even under hypoxia, indicating that even at low oxemic tension, molecular oxygen is not a limiting substrate for PHD2-mediated hydroxylation of HIF-1α (10). In light of these observations, it was important to investigate the role of FIH-1, an oxygen-dependent asparaginyl hydroxylase in the control of HIF-1 activity in the NP. This is the first study to our knowledge

that investigates the expression and role of the FIH-1/Mint3 system in the intervertebral disc.

Results of our studies show that NP cells express both FIH-1 and Mint3. Interestingly, although some localization of Mint3 was seen in Golgi, consistent with other reports (25), unlike other cell types, Mint3 showed a strong nuclear localization in NP cells. Furthermore, in contrast to previous findings in macrophages that showed a prominent perinuclear co-localization between Mint3 and FIH-1 (23), these proteins showed a strong nuclear staining and co-localization in NP cells. This raised a possibility that in NP, they interact in this subcellular compartment, a notion further confirmed by their co-immunoprecipitation. These results also suggest that the subcellular location of interaction between FIH-1 and Mint3 is cell type-specific. This hypothesis is further validated by our finding that in NP cells, the ability of exogenously expressed FIH-1 to repress HIF-1 activity was dependent on its nuclear localization. Thus, when exogenous FIH-1 was restricted to the cytoplasm by ivermectin, which targets importin α/β, its ability to modulate HIF activity was abrogated, whereas when it was retained in the
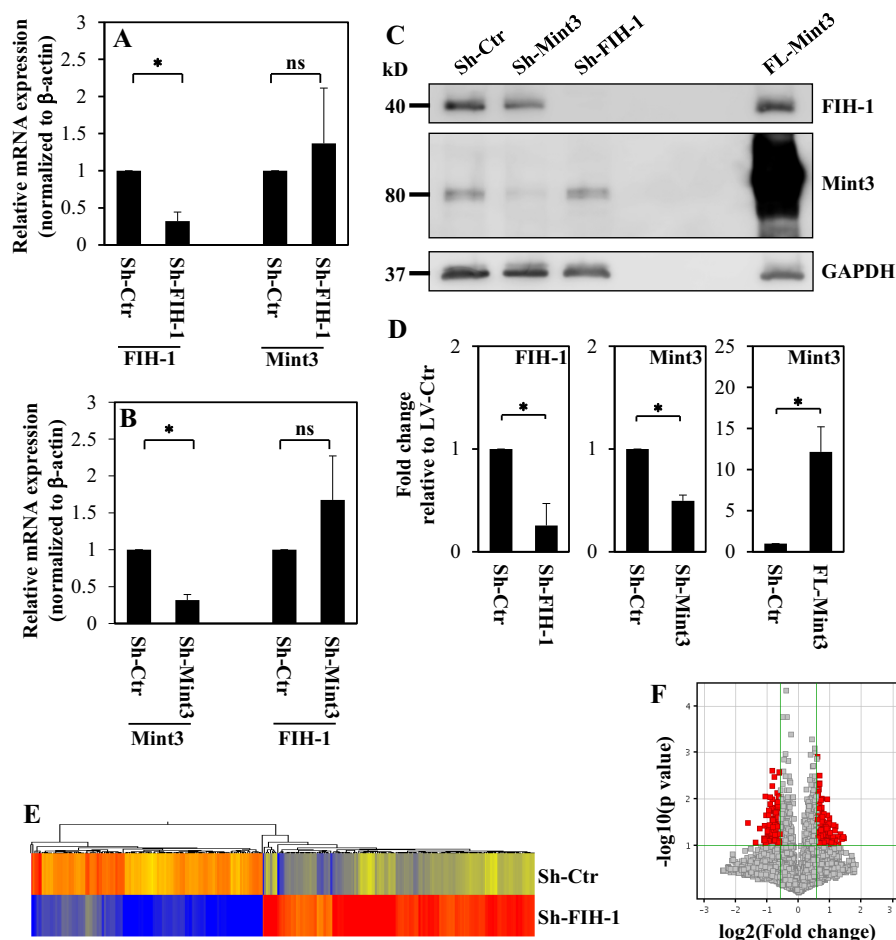
FIGURE 6. **FIH-1 silencing has no effect on HIF transcriptional activity under normoxia.** *A* and *B*, real-time RT-PCR analysis shows that lentiviral (*LV*) transduction of NP cells with Sh-FIH-1 (*A*) and Sh-Mint3 (*B*) selectively suppressed the mRNA expression of either FIH-1 or Mint3 without affecting the expression of the other. *C*, Western blot analysis of cells transduced with Sh-Control, Sh-Mint3, Sh-FIH-1, or FL-Mint3. The expression of FIH-1 or Mint3 was suppressed by respective shRNA compared with cells transduced with lentivirus expressing Sh-control. *D*, densitometric analysis of three independent experiments described in *C* shows that transduction of cells with Sh-FIH-1 or Sh-Mint3 significantly decreases respective expression, whereas transduction with FL-Mint3 significantly overexpressed Mint3 protein in cells. Data represent mean ± S.E. (*error bars*) of three independent experiments. *, $p < 0.05$. *E*, volcano plot of differentially expressed transcripts between cells transduced with Sh-control and Sh-FIH-1. The *x* axis represents the $\log_2$ values of the -fold change observed for each transcript, whereas the *y* axis depicts the $\log_{10}$ values of the *p* value of the significance tests between triplicates for each transcript. Genes that demonstrate a 1.5-fold or greater difference in expression at $p < 0.1$ in FIH-1-silenced cells compared with control cells are displayed in *red*. *F*, heat map and dendrogram of differentially expressed transcripts. Dendrograms are reflective of the genes with a differential expression of >1.5-fold from three independent experiments ($p < 0.1$). In the dendrogram, a *shorter arm* indicates higher similarity, whereas a *longer arm* indicates lower similarity. The resulting heat map of the dendrogram tree reveals groups of genes with high expression levels (*red*), low expression levels (*blue*), or background expression levels (*yellow*).

TABLE 1
Expression of known HIF-1 target genes is not affected by FIH-1 silencing in NP cells

Gene symbol	Description	*p* value
PGK1	Phosphoglycerate kinase 1	0.32
PFK2 (PFKFB3)	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	0.34
Galectin-3 (LGALS3)	Lectin, galactoside-binding, soluble, 3	0.36
GLUT1 (SLC2A1)	Solute carrier family 2 (facilitated glucose transporter), member 1	0.47
GLUT3 (SLC2A3)	Solute carrier family 2 (facilitated glucose transporter), member 3	0.47
ENOLASE1 (ENO1)	Enolase1 (α)	0.37
PHD2 (EGLN1)	Egl nine homolog 1 (*Caenorhabditis elegans*)	0.29
PHD3(EGLN3)	Egl nine homolog3 (*C. elegans*)	0.69
VEGFA	Vascular endothelial growth factor A	0.28
B3GAT3	β-1,3-Glucuronyltransferase 3 (glucuronosyltransferase I)	0.25
HSP-70 (HSPA1A)	HSPA1A/HSPA1B	0.83
CCN2 (CTGF)	Connective tissue growth factor	0.62

nucleus by the CRM1 inhibitor, leptomycin B, a further decrease in FIH-1-mediated HIF-1 function was seen. These results are consistent with those seen in breast cancer (26), pancreatic endocrine tumors (27), and clear cell renal cell carcinoma (28), where low nuclear FIH-1 expression is correlated with poor prognosis, suggesting that although FIH-1 expression may be predominantly cytoplasmic (29), it is only the nuclear fraction that has an active role in the regulation of HIF activity.

Because NP is an avascular and hypoxic tissue, it was important to determine the effect of hypoxia on FIH-1 and Mint3
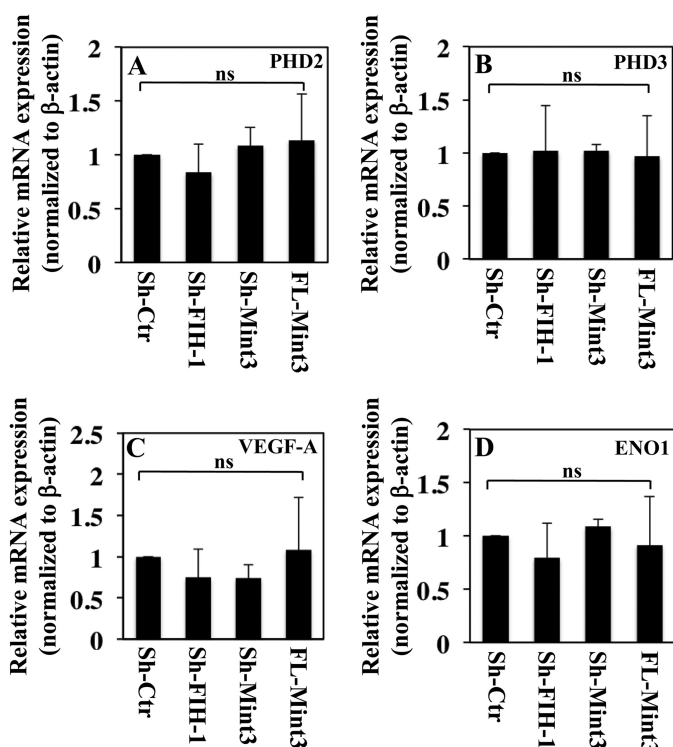
TABLE 2
Transcripts significantly affected by FIH-1 silencing in NP cells

Gene symbol	Description	-Fold change
CBFA2T	Core-binding factor, runt domain, a subunit 2; translocated to, 3	+2.5
MMP21	Matrix metallopeptidase 21	+2.4
MIR219-1	MicroRNA 219-1	+2.3
SOWAHB	Sosondowah ankyrin repeat domain family member B	+2.2
MIR132	MicroRNA 132	+2.2
NKX2−4	NK2 homeobox 4	+2.2
CCL26	Chemokine (C-C motif) ligand 26	+2.2
CPN2	Carboxypeptidase N, polypeptide 2	+2.1
IGFBP1	Insulin-like growth factor-binding protein 1	+2.1
FAM3A	Family with sequence similarity 3, member A	+2.1
HAPLN1	Hyaluronan and proteoglycan link protein 1	−3.1
SPTCL2	Serine palmitoyltransferase, long chain base subunit 2	−2.6
WBP4	WW domain binding protein 4	−2.4
ITGB1BP1	Integrin β1-binding protein 1	−2.1
FUNDC2	FUN14 domain containing 2	−2.1
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	−2.1
HIF1AN	Hypoxia-inducible factor 1, α subunit inhibitor	−2.1
RNF2	Ring finger protein 2	−2.1
GALNT7	N-Acetylgalactosaminyltransferase 7	−2.1
CDC23	Cell division cycle 23 homolog (*Saccharomyces cerevisiae*)	−2.1

FIGURE 7. HIF activity in NP cells is refractory to FIH-1/Mint3-mediated regulation. *A–D*, real-time RT-PCR analysis of known HIF-1 target genes in NP cells. Stable suppression of FIH-1 or Mint3 or forced expression of Mint3 in NP cells by lentivirally delivered ShRNA or Mint3 cDNA has no effect on mRNA expression of HIF-1 target gene *ELGN1* (*PHD2*) (*A*), *EGLN3* (*PHD3*) (*B*), *VEGA* (*C*), or *ENO1* (*D*) in normoxia. Data represent the mean ± S.E. (*error bars*) of three independent experiments. *, $p < 0.05$. *ns*, not significant.

expression and activity. Prolonged hypoxia resulted in a decrease in FIH-1 at both the protein and the transcript level and a decrease in Mint3 at the protein level. Because in NP cells, HIF-1 is a major regulator of hypoxia-dependent transcription, and considering that hypoxic expression of PHD1–3 is under the control of HIF-1 (15), it was surprising that the decrease in FIH-1 transcript in hypoxia was independent of HIF-1α. The repression is possibly mediated by HIF-2 but more likely is through a HIF-independent hypoxic pathway (*e.g.* through PKCζ, which is both activated by hypoxia (30) and capable of

modulating FIH-1 expression through Cut-like homeodomain protein (CDP/Cut) (31)).

Concerning FIH-1 function in NP cells, our results show that exogenously overexpressed FIH-1 is capable of repressing the activity of both HIF-1α-C-TAD and HIF-2α-TAD as well as an HRE-luciferase reporter. It is noteworthy that overexpression of FIH-1 results in a decrease in HIF-1α activity even under hypoxic (1% O_2) conditions, similar to what was seen previously for PHD2 activity in NP cells (10). Moreover, these results are consistent with previous findings that the *in vitro* K_m for O_2 of FIH-1 is much smaller (90 μM) than that of PHD2 (250 μM) (32, 33) and that substantial levels of hydroxylated Asn[803] can be detected even at 0.1% O_2 (20) in RCC4 and MCF7 cells, suggesting preservation of FIH-1 enzymatic function under hypoxia. Furthermore, a very recent report that FIH-1 can still suppress HIF-dependent GLUT1 and VEGF-A expression in hypoxic glioblastoma *in vitro* and also *in vivo* in a xenograft model supports this idea (34). Because in NP Mint3 and FIH-1 colocalize in the cell nucleus and interact, overexpression of Mint3 would be expected to increase HIF-1 activity, through inhibition of FIH-1. Of note, forced overexpression of Mint3 was able to increase HRE-luc activity only under normoxic conditions; in hypoxia, overexpression of exogenous Mint3 had no effect on HIF-1 activity. Importantly, consistent with studies of macrophages, the N terminal portion of the protein shown to interact with FIH-1 was important for its action on HIF-1 function (23). Taken together, these results suggest that endogenous FIH-1 is not present in a concentration sufficient to repress HIF-1 activity in the hypoxic NP cells and are consistent with our observation that the level of FIH-1 protein is decreased in hypoxia.

Because our initial results suggested a limited role of FIH-1 in regulation of HIF-1 activity, we decided to apply an unbiased approach of microarray analysis after FIH-1 silencing in primary human NP cells. Consistent with our transfection results, knockdown of FIH-1 did not significantly increase expression of any of the classically regulated HIF target genes, which include enzymes of glycolysis (*PFK2*, *PGK1*, and *ENO1*), glucose transporters (*SLC2A1* and *SLC2A3*), and genes important for NP physiology and function (*VEGFA*, *EGLN1*, *EGLN3*, *CTGF*, and *LGALS3*). These results were confirmed by qPCR
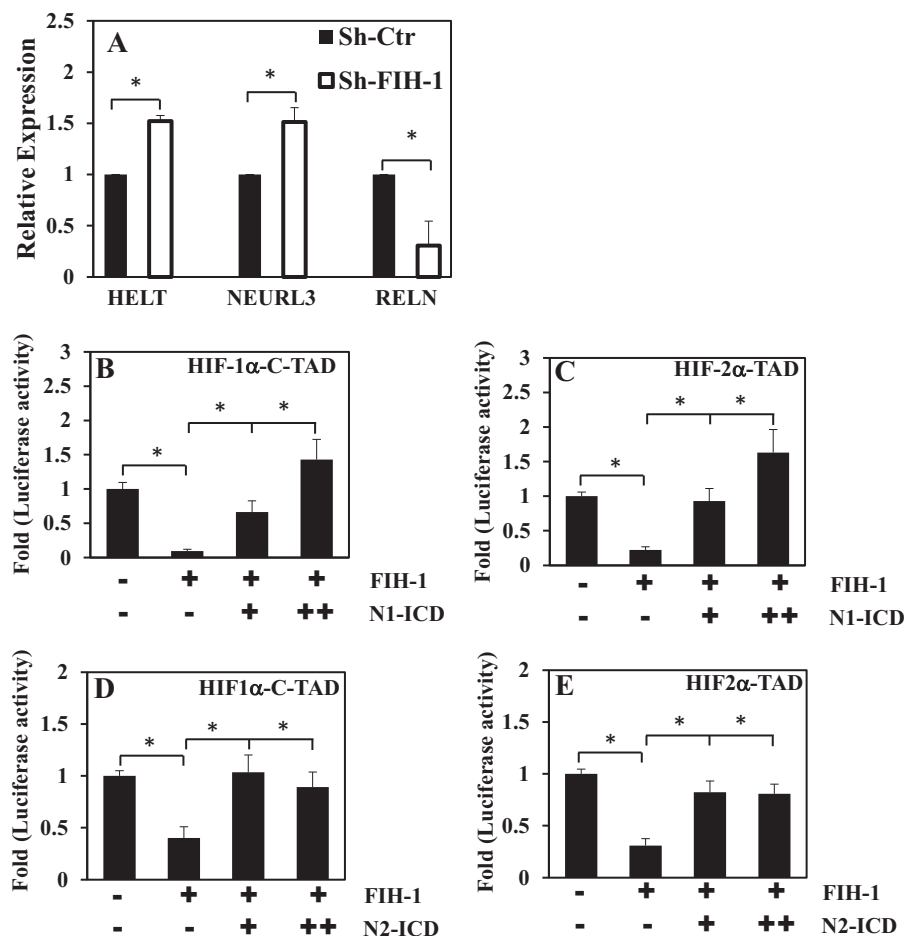
FIGURE 8. **Relationship between HIFs, FIH-1, and Notch signaling pathways in NP cells.** *A*, stable suppression of FIH-1 by Sh-FIH-1 in human NP cells alters the expression of known Notch signaling pathway genes. FIH-1-silenced NP cells show increased basal expression of HELT and NEURL3, whereas expression of RELN mRNA is decreased. *B–D*, Notch-ICD blocks the inhibitory effects of exogenous FIH-1 on HIF-α transactivation. Suppression of HIF-1α-TAD (*B* and *D*) and HIF-2α-TAD (*C* and *E*) activity by exogenous FIH-1 (100 ng) in NP cells is restored by co-expression of either Notch1-ICD (N1-ICD) (*B* and *C*) or Notch2-ICD (N2-ICD) (*D* and *E*) used at concentrations of 100–200 ng. Data represent the mean ± S.E. (*error bars*) of three independent experiments. *, $p < 0.05$.

for validated HIF-1 target genes in NP cells. It is noteworthy that knockdown of FIH-1 via lentivirally delivered shRNA in T/C-28 cells, a human chondrocyte cell line, showed similar results; there was no increase in HRE reporter activity and no increase in HIF-1 target gene expression with FIH-1 knockdown. Together, these results clearly suggest that in physiologically hypoxic tissues, like NP and cartilage, where HIF activity is crucial for cell survival, FIH-1 does not play a major role in regulating HIF activity, possibly due to its low levels. Our results are similar to what was reported in glomerular podocytes of the kidney (35). The fact that podocytes are prone to hypoxic insult during glomerular injury (36) and that activation of HIF target genes provides renal protection in acute and certain chronic conditions (37, 38) underscores a compelling argument that in cell types prone to experience hypoxia, FIH-1 activity does not represent a major mechanism of regulating HIF-1 transcriptional activity.

Based on our findings, we conclude that FIH-1 does not play a major role in regulating HIF-1 activity in physiologically hypoxic NP tissue. This inability to regulate HIF-1α is probably due to stoichiometry and a low level of expression, especially under hypoxic conditions, and not due to substrate limitation of molecular oxygen. Thus, the preservation of enzymatic func-

tion of FIH-1 in hypoxia raises the question of its physiological role in NP tissue. Although apparent deformity of the spinal column has not been reported in FIH-1-null mice, a careful analysis of the intervertebral disc phenotype has not been performed (39). Given the number of genes affected by FIH-1 silencing in NP cells, including those concerned with the Notch pathway, and in recognition of the fact that FIH-1 is known to hydroxylate many proteins with ankyrin repeats, including p105, IκBα (40), and members of the Notch receptor family (41), it is very plausible that morphological and functional deficits may exist in discs of FIH-1 null mice with aging. Of note, the *in vitro* K_m of FIH-1 for Notch1 is much lower (~250-fold) than it is for HIF-1α (42), although *in vivo* analyses suggest that hydroxylation of ankyrin repeat domain proteins and HIF-1α occurs within the same range (43). It is noteworthy that Notch1 and Notch2 are highly expressed in the NP, and their expression is hypoxia-sensitive (44). Importantly, our data suggest that both Notch1- and Notch2-ICD can rescue FIH-1-mediated suppression of HIF-1α and HIF-2α transactivation, raising the possibility that the high Notch expression in the NP may serve as a preferential substrate and outcompete HIF-1α for binding with FIH-1, contributing to the seeming lack of regulation of HIF-1 signaling by FIH-1. Moreover, FIH-1 has also

recently been reported to interact with and inhibit the proapoptotic protein Bax, independent of its hydroxylase activity (45). In light of our recent finding of PHD3 activating p65 signaling in NP cells independent of proline hydroxylation (46), it is important to also consider the potential role of FIH-1 independent of asparagine hydroxylation in NP physiology.

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