

Mint3 Enhances the Activity of Hypoxia-inducible Factor-1 (HIF-1) in Macrophages by Suppressing the Activity of Factor Inhibiting HIF-1*

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Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor regulating cellular responses to hypoxia and is composed of α and β subunits. During normoxia, factor inhibiting HIF-1 (FIH-1) inhibits the activity of HIF-1 by preventing HIF-1 α binding to p300/CBP via modification of the Asn⁸⁰³ residue. However, it is not known whether FIH-1 activity can be regulated in an oxygen-independent manner. In this study, we survey possible binding proteins to FIH-1 and identify Mint3/APBA3, which has been reported to bind Alzheimer β -amyloid precursor protein. Purified Mint3 binds FIH-1 and inhibits the ability of FIH-1 to modify HIF-1 α *in vitro*. In a reporter assay, the activity of HIF-1 α is suppressed because of endogenous FIH-1 in HEK293 cells, and expression of Mint3 antagonizes this suppression. Macrophages are known to depend on glycolysis for ATP production because of elevated HIF-1 activity. FIH-1 activity is suppressed in macrophages by Mint3 so as to maintain HIF-1 activity. FIH-1 forms a complex with Mint3, and these two factors co-localize within the perinuclear region. Knock-down of Mint3 expression in macrophages leads to redistribution of FIH-1 to the cytoplasm and decreases glycolysis and ATP production. Thus, Mint3 regulates the FIH-1-HIF-1 pathway, which controls ATP production in macrophages and therefore represents a potential new therapeutic target to regulate macrophage-mediated inflammation.

ATP is a vital source of energy for cells to carry out a variety of energy-dependent cellular functions, and it is generated mostly via oxidative phosphorylation carried out in the mitochondria. However, cells are sometimes exposed to hypoxic conditions accidentally or during pathological conditions such as cancer and inflammation, which renders it difficult to produce ATP in mitochondria. During hypoxia, cells strive to maintain ATP production by activating anaerobic glycolysis, which can generate ATP in the absence of oxygen (1). The glycolytic activity of cells is regulated by expression of key enzymes

and transporters in the glycolytic pathway, whose expression is regulated in an oxygen-sensitive manner. These proteins include glucose transporter 1 (GLUT1),² phosphofructokinase, and phosphoglycerate kinase 1 (PGK1), etc. (1), and other genes whose products are necessarily to respond to hypoxic stress. The HIF transcription factor is a key regulator of the expression of genes necessary for adaptation to hypoxic conditions (1, 2). HIF comprises one of three α subunits (HIF-1 α , -2 α , and -3 α) bound to a common HIF-1 β subunit. HIF proteins regulate the expression of hypoxia response genes by binding to the hypoxia response element in the regulatory regions of these target genes. HIF-1 α is expressed nearly ubiquitously, whereas HIF-2 α and HIF-3 α are expressed in a tissue-specific manner (2).

HIF is regulated by modulation of the activity of the α subunits, and the mechanism of this regulation has been studied most extensively for HIF-1 α (see Fig. 1A). During oxygen-rich conditions, prolyl hydroxylases (PHDs) modify proline residues in the oxygen-dependent degradation domain of HIF-1 α , resulting in recognition of the modified HIF-1 α by Von Hippel-Lindau (VHL) protein (3, 4). VHL protein exhibits E3 ubiquitin ligase activity that leads to degradation of the modified HIF-1 α by the proteasome system (5). During hypoxia, the activity of the PHDs is suppressed, which stabilizes HIF-1 α and increases its activity. The activation of transcription by HIF-1 α is mediated by the C-terminal activation domain (CAD) that binds p300/CBP (6, 7). An asparagine residue (Asn⁸⁰³) within the CAD is a target for hydroxylation by FIH-1 during oxygen-rich conditions, and this modification prevents binding to p300/CBP (8, 9). Thus, the activity of HIF-1 α is inhibited by two independent mechanisms, both of which function in an oxygen-sensitive manner. In most cells, HIF activity is strongly suppressed by the action of PHDs and FIH-1 during normoxia.

Although the oxygen-dependent regulation of the activity of the PHDs and FIH-1 has been studied extensively, it is unclear whether oxygen is the sole factor regulating these HIF-1 suppressors. To study further the regulation of HIF-1, we performed a yeast two-hybrid screen using FIH-1 as the "bait" and

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² The abbreviations used are: GLUT, glucose transporter; HIF-1, hypoxia-inducible factor-1; FIH-1, factor inhibiting HIF-1; IB, immunoblotting; APP, amyloid- β precursor protein; PGK, phosphoglycerate kinase; PHD, prolyl hydroxylase; VHL, Von Hippel-Lindau; E3, ubiquitin-protein isopeptide ligase; CAD, C-terminal activation domain; shRNA, small hairpin RNA; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry.

identified several candidate binding partners. After testing these candidate proteins, we demonstrated that only Mint3/APBA3 exhibits strong binding activity toward FIH-1 *in vitro*. We further show that Mint3 regulates HIF-1 activity by modulating FIH-1 and that constitutive suppression of FIH-1 by Mint3 plays a pivotal role in the activation of HIF-1, glycolytic activity, and ATP production in macrophages.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—The yeast two-hybrid screens were performed by Hybrigenics (Paris, France) using FIH-1 as a bait to screen a human placenta random-primed cDNA library, and each protein interaction was assigned with a statistical confidence score as described previously (10).

Cell Culture—HEK293 were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium high glucose (Sigma) supplemented with 10% fetal bovine serum. Bone marrow-derived macrophages were obtained from 6-week-old C57BL/6 mice (Clea Japan, Tokyo, Japan) as described previously (11, 12). The macrophages were rendered quiescent by culturing them overnight in medium lacking the L929-conditioned medium.

Plasmids—Expression constructs for FIH-1NT (encoding amino acids 1–302), Mint3-NT (encoding amino acids 1–214), and Mint3-CT (encoding amino acids 215–575) were prepared using a PCR-based method. Sequences encoding the FLAG or Myc peptide tags were inserted immediately downstream of the furin cleavage site, because it is exposed at the N terminus following proteolytic processing. These proteins were expressed in cells using lentivirus (pLenti6) vectors (Invitrogen). To express proteins for immunoprecipitation and reporter assays, the mammalian expression vector pcDNA3.1 (Invitrogen) was used.

Immunoprecipitation—HEK293 cells were co-transfected with expression plasmids encoding Myc-FIH-1 and a C-terminal FLAG-tagged construct. Transfection was performed with LipofectamineTM 2000 (Invitrogen). Twenty-four h after transfection, the cells were lysed with lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl) and centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatants were collected and incubated with anti-FLAG M2 antibody-conjugated beads (Sigma). The beads were washed, and bound proteins were eluted with FLAG peptides and detected by immunoblot analysis. To detect interaction between FIH-1 and Mint3 in macrophages, the cells were disrupted with lysis buffer and centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatants were collected and incubated with rabbit anti-FIH-1 polyclonal antibody (Novus Biologicals), rabbit anti-PHD2 polyclonal antibody (Novus Biologicals), rabbit anti-Mint3 polyclonal antibody (Affinity BioReagents, Rockford, IL), or the corresponding control IgG (Sigma) overnight at 4 °C. Then protein G-Sepharose (GE Healthcare) was added to the lysate for 30 min at 4 °C. The beads were washed four times with lysis buffer, and then the proteins were eluted with Laemmli sample buffer and analyzed by immunoblot analysis.

Reporter Assay—The reporter plasmid contains the firefly luciferase gene under control of a transcriptional regulatory unit comprising 4× Gal4-binding elements and the thymidine

kinase-derived TATA box. A pRL vector expressing *Renilla* luciferase (Promega) served as an internal control. HEK293 cells (5 × 10⁴/well) were seeded onto 24-well plates and co-transfected with reporter plasmid (100 ng), internal control vector (10 ng), Gal4BD-CAD plasmid (50 ng), and other plasmids (200 ng) that expressed either full-length or truncated mutants of Mint3. Transfection was performed using LipofectamineTM 2000 (Invitrogen). Luciferase activity was measured using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luminescence was measured in a TD20/20 luminometer (Promega).

Knockdown Experiments Using shRNAs—shRNA sequences used for knockdown of target proteins were as follows: mouse FIH-1 #1, 5'-caccggacctgaatacctgaagcaatcttcgaggtattcggagtcctttt-3' and #2, 5'-caccggaagattgtcatggattctcgaagaagtcacatgacaatcttcc-3'; human FIH-1 #1, 5'-caccgctgaccgacacaaatcttgcgaaacaagattgtgtcggcagctttt-3' and #2, 5'-caccggaagattgtcatggattctcgaagaagtcacatgacaatcttcc-3'; and mouse Mint3 #1, 5'-caccgttctctgttaggaactggctgcgaacagccagttctacaggagaa-3' and #2, 5'-caccgccagttctacaggagaacagcaatgttctctgttaggaactggc-3'. These sequences were subcloned into pENTR/U6 TOPO (Invitrogen) and then transferred via recombination into the lentivirus vector pLenti6 BLOCKiT. shRNA-expressing lentiviral vectors were generated and used according to the manufacturer's instructions.

Recombinant Proteins—Recombinant proteins such as GST, GST-FIH-1, and GST-FIH-1NT were expressed in *Escherichia coli* BL21 Gold (DE3) pLysS (Stratagene) using the pDEST15 expression vector (Invitrogen). Expression was induced at mid-log phase with 0.5 mM isopropyl-*D*-thiogalactoside, and the cells were incubated for a further 3 h at 37 °C. The cells were harvested, then resuspended in phosphate-buffered saline containing 1% Triton X-100 and protease inhibitor mixture III (Merck), and sonicated. Following centrifugation, the supernatants were applied to a glutathione-Sepharose 4B (GE Healthcare) column and washed three times with phosphate-buffered saline, after which recombinant proteins were eluted in TBS buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl).

FIH-1, Mint3, Mint3-NT, HIF-1α CAD (600–826 amino acids), and VHL were expressed in *E. coli* BL21 Gold (DE3) pLysS as His₆-tagged fusion proteins. Construction of the expression plasmids, induction of expression, and preparation of cell lysates were performed as described above. Imidazole (10 mM) was added to the supernatant fractions, and then the samples were loaded onto a Ni²⁺-conjugated chelation Sepharose column (GE Healthcare) and incubated for 1 h at 4 °C. The beads were washed five times with 50 mM imidazole/TBS, and then bound proteins were eluted with 500 mM imidazole and dialyzed against TBS. Protein purity was assessed by SDS-PAGE.

GST Pull-down Assay—Glutathione-Sepharose 4B (GE Healthcare)-conjugated GST fusion proteins (~10 μg) were preincubated with 0.5 mg ml⁻¹ bovine serum albumin in lysis buffer for 30 min at 4 °C. Thereafter, 2 μg of His₆-tagged FIH-1 was resuspended in lysis buffer containing 0.5 mg ml⁻¹ bovine serum albumin and added to the beads. After mixing on a rotator for 2 h, the beads were washed four times with lysis buffer. The proteins were eluted with Laemmli sample buffer and analyzed

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by SDS-PAGE, followed by immunoblot analysis using anti-His₆ antibody (Roche Applied Science) or anti-GST antibody (GeneTex, Irvine, CA).

Immunoblot Analysis—The cells were lysed with lysis buffer and centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatants were collected, and protein content was measured using the Bradford assay (Bio-Rad). To detect HIF-1 α and lamin A/C, nuclear proteins were collected using a nuclear extract kit (ActiveMotif). The lysates were separated by SDS-PAGE, transferred to membrane filters, and subjected to immunoblot analysis using anti-lamin A/C mouse antibody (BD Biosciences), using anti-HIF-1 α mouse antibody (EXALPHA, Shirley, MA), anti-Myc mouse antibody (Roche Applied Science), anti-V5 mouse antibody (Invitrogen), anti-Mint3 mouse antibody (BD Biosciences), anti-FIH-1 goat antibody (Santa Cruz Biotechnology), anti-actin mouse antibody (Millipore), or anti-FLAG epitope M2 antibody (Sigma).

Immunostaining—The cells were fixed with 4% paraformaldehyde and permeabilized using 0.01% Triton X-100 for 10 min. After blocking in phosphate-buffered saline containing 5% goat serum and 3% bovine serum albumin, the cells were incubated with mouse anti-FLAG M2 antibody (Sigma), mouse anti-Mint3 antibody (BD Biosciences), mouse anti-GM130 (BD Biosciences), rabbit anti-GM130 (Novus Biologicals), or rabbit anti-FIH-1 antibody (Novus Biologicals) for 1 h and then washed three times and incubated for 1 h with anti-mouse IgG Alexa488 conjugate or anti-rabbit Alexa594 (Invitrogen). The cells were counter-stained with Hoechst33342, washed five times with phosphate-buffered saline, mounted, and observed under CCD microscopy. To analyze the accumulation of FIH-1 in the Golgi body, FIH-1 signal intensities from whole cells were quantified by ImageJ software (National Institutes of Health). FIH-1 signals exceeding twice the average signal intensity in each cell were extracted and merged with GM130 signals.

Mass Spectrometry Analysis of Products of *in Vitro* Hydroxylation—For mass spectrometry, 2 μ g of N-terminal biotinylated peptide from HIF-1 α (788–822 amino acids) and 5 μ g of GST-FIH-1 were incubated in 100 μ l of hydroxylation buffer (40 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM dithiothreitol, 3 mM MgCl₂, 4 mM ascorbic acid, 1.5 mM FeSO₄, and 4 mM 2-oxoglutarate) with or without His₆-tagged Mint3NT or Mint3CT, as indicated. After incubation at 30 °C for 2 h, the biotinylated peptide was purified with a C18 Zip Tip (Millipore), analyzed by MALDI-TOF-MS and subjected to partial sequence analysis by MS/MS using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems).

RNA Isolation, Reverse Transcription, and Real Time PCR—Total RNA was isolated from macrophages using TRIzol (Invitrogen) and subjected to reverse transcription using SuperscriptII (Invitrogen) and random primers. Then the reverse transcription products were subjected to real time PCR in a Smart Cycler II System (Cepheid, Sunnyvale, CA) using SYBR GREEN I (TaKaRa, Shiga, Japan) and the following specific primers: β -actin sense, 5'-gccaacacagtctgtctgg-3' and antisense, 5'-atctgctggaaggtggacag-3'; GLUT1 sense, 5'-gggcagtgtctccagtatgt-3' and antisense, 5'-acgaggagcaccgtgaagat-3'; PGK1 sense, 5'-tgtcgtttccaacaagctg-3' and antisense, 5'-ggtgctcataaggacaacg-3'; and F4/80 sense, 5'-cctgtctgcatgatcat-

cac-3' and antisense, 5'-caatggccttgaaggtcagc-3'. PCR products were sequenced, and their homogeneity was confirmed by dissociation temperature monitoring of SYBR GREEN I fluorescence.

Measurement of ATP Concentrations—The cells were cultured, and their ATP levels were determined using the ATP Bioluminescence assay kit CLS II (Roche Applied Science). ATP levels were normalized according to the total protein concentration, determined using a Bradford assay kit (Bio-Rad).

Measurement of Lactic Acid—Macrophages were seeded at 1 × 10⁶ cells/well in triplicate six-well plates. Conditioned medium was collected after 6 h, and lactic acid production was determined using a L-lactic acid kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

Statistical Analysis—The data are represented as the means \pm S.D. The unpaired Student's *t* test was used for analyzing differences between experimental groups.

RESULTS

Identification of Mint3 as a New Binding Partner of FIH-1—Yeast two-hybrid screening of a human placental cDNA library identified many potential binding partners for FIH-1 with varying degree of reliability scores (see "Experimental Procedures"). We chose eight proteins with higher scores and tried to confirm their binding to FIH-1 using an immunoprecipitation assay. Each candidate protein was expressed as a FLAG-tagged fusion protein, and we examined the ability of these fusions to bind Myc-tagged FIH-1 that was co-expressed in HEK293 cells with the FLAG-tagged fusions (Fig. 1B). FLAG-tagged FIH-1 was used as a positive control for binding to Myc-tagged FIH-1 in the following immunoprecipitation studies, because FIH-1 is known to form a homodimer (8, 13). The test fusion proteins were immunoprecipitated from transfected cell lysates using an anti-FLAG antibody (Fig. 1B, upper two panels) and the precipitates were subjected to immunoblot (IB) analysis to confirm presence of the test fusions (Fig. 1B, top panel). Myc-tagged FIH-1 in the precipitates (IP) or in the whole cell lysates was also detected by immunoblot analysis, using anti-Myc antibody (IB, lower two panels). Among the FLAG-tagged fusions examined, only the FLAG-tagged FIH-1 and FLAG-tagged Mint3 precipitated co-expressed Myc-tagged FIH-1. Conversely, immunoprecipitation of FLAG-tagged FIH-1 also resulted in detection of V5-tagged Mint3 in the immunoprecipitates when both proteins were expressed in HEK293 cells (Fig. 1C). Thus, Mint3 and FIH-1 expressed in transfected cells can form a stable complex. FLAG-tagged I κ B α protein, which has been reported recently to bind FIH-1 via its ankyrin repeats (14), was detected only weakly in our experiments with longer exposure of the blot (data not shown).

Mint3 Competes with HIF-1 α for Binding to FIH-1—Mint3 is a member of the X11 protein family (15, 16). The C-terminal half of Mint3 contains one phosphotyrosine-binding and two PDZ domains (Fig. 2A), which are common to other family members, and is important for binding membrane proteins such as amyloid- β precursor protein (17). The N-terminal half of each X11 protein family member is unique, and that of Mint3 does not contain any known protein motifs. Mint3 is also

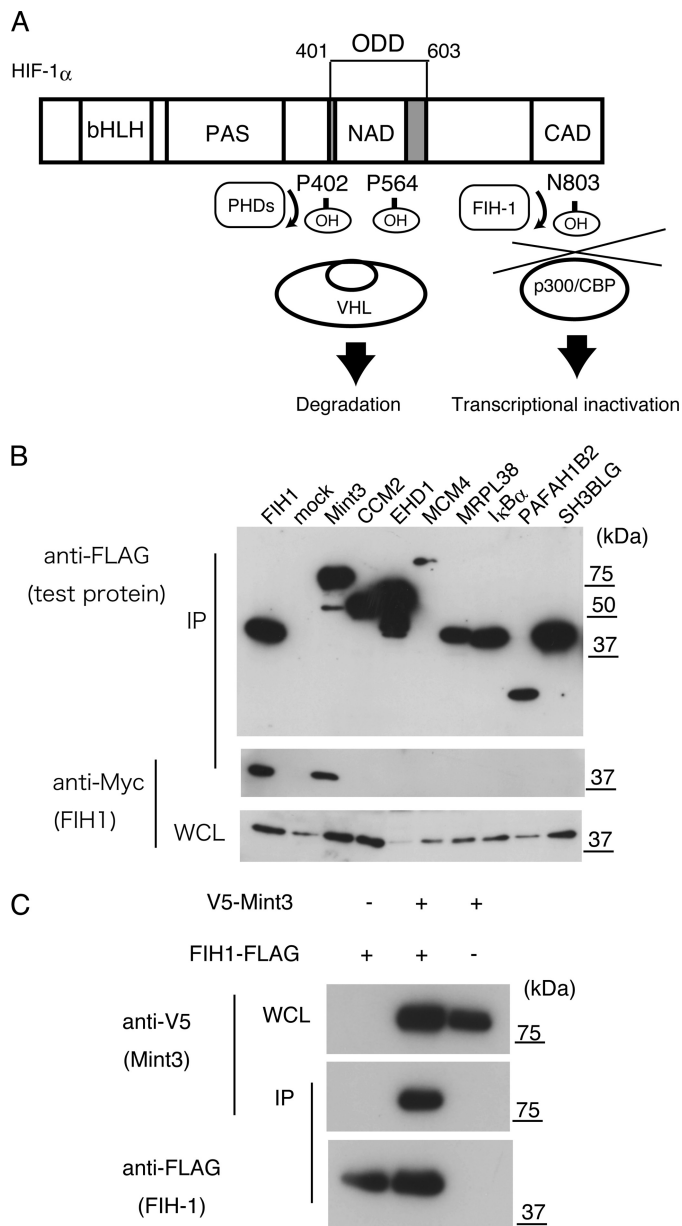


FIGURE 1. Identification of Mint3 as a binding partner of FIH-1. *A*, schematic depiction showing how HIF-1 α is regulated by PHDs and FIH-1. *bHLH*, basic helix-loop-helix; *PAS*, Per-Arnt-Sim; *NAD*, N-terminal transactivation domain; *ODD*, oxygen-dependent degradation domain. *B*, Myc-tagged FIH-1 was co-expressed with FLAG-tagged test proteins in HEK293 cells. Immunoprecipitation (IP) of FLAG-tagged proteins was performed and detected by immunoblot analysis using indicated the antibodies (top two panels). FIH-1 in the precipitates and whole cell lysate (WCL) was detected by IB analysis as in the bottom two panels. *C*, V5-tagged Mint3 was co-expressed with FLAG-tagged FIH-1 in HEK293 cells. Immunoprecipitation (IP) of FLAG-tagged FIH-1 was performed, and the immunoprecipitates were subsequently analyzed by immunoblot analysis using the indicated antibodies (bottom two panels). Mint3 in the immunoprecipitates and whole cell lysate was detected by IB analysis as in the top two panels.

reported to regulate transport of vesicles containing bound proteins from the trans-Golgi network to the cell surface (18).

To identify the domains of Mint3 that mediate binding to FIH-1, we co-expressed FLAG-tagged variants of full-length Mint3 (Mint3(full)) or deletion mutants of Mint3 lacking either the conserved C-terminal domain (Mint3(NT)) or the unique N-terminal domain (Mint3(CT)), together with a Myc-tagged

FIH-1 in HEK293 cells. The FLAG-tagged proteins were immunoprecipitated and analyzed by immunoblot analysis using anti-Myc-antibody (Fig. 2*B*). Both Mint3(full) and Mint3(NT) precipitated FIH-1, whereas Mint3(CT) did not, suggesting that a region within the N-terminal domain of Mint3 mediates binding to FIH-1.

FIH-1 contains a cupin-like domain responsible for dioxygenase activity that is located N-terminal to the C-terminal dimerization domain (Fig. 2*A*, FIH-1). The latter domain allows FIH-1 to bind HIF-1 α as a dimer, and the former domain modifies Asn⁸⁰³ within the HIF-1 α CAD upon binding (8, 19). We expressed GST-fused FIH-1 proteins and His₆-tagged Mint3 or VHL proteins in *E. coli* and used the purified proteins for pull-down assays. VHL protein, which binds to the N-terminal portion of FIH-1 (20), was used as a control. Using this assay, we observed that FIH-1 bound to the CAD fragment of HIF-1 α , Mint3(NT), and VHL protein (Fig. 3*A*, GST-FIH-1). However, truncation of the C-terminal domain of FIH-1 (GST-FIH-1NT) abrogated the binding to both the HIF-1 α CAD and Mint3(NT) (Fig. 3*A*, GST-FIH-1NT), whereas VHL protein still bound GST-FIH-1NT (Fig. 3*A*). Thus, the C-terminal dimerization domain of FIH-1 is necessary to bind both Mint3 and HIF-1 α . Furthermore, we observed that Mint3 competed with the HIF-1 α CAD for binding to FIH-1. Mint3(NT) inhibited pull-down of the HIF-1 α CAD with GST-FIH-1 in a dose-dependent manner (Fig. 3*B*), whereas it did not affect pull-down of VHL (Fig. 3*C*).

Mint3 Abrogates the Ability of FIH-1 to Modify HIF-1 α in Vitro—FIH-1 has been reported to bind HIF-1 α and to catalyze the hydroxylation of Asn⁸⁰³ within the HIF-1 α CAD in vivo and in vitro (8, 9). Because Mint3 competed with HIF-1 α for binding to FIH-1, we examined whether Mint3 also inhibited the ability of FIH-1 to modify HIF-1 α in vitro, according to methods described previously (9, 20). For this assay, we used a biotinylated peptide (Fig. 4*A*, 788–822 amino acids) derived from HIF-1 α as a substrate for FIH-1 by incubating the former with GST-FIH-1 in vitro. Using mass spectrometry, we measured the mass of the unmodified peptide to be 4055.10 Daltons (Fig. 4*B*, peptide alone). Incubation of the peptide with FIH-1 increased its molecular weight by 16 Daltons (Fig. 4*B*, peptide + FIH-1). This increase in mass corresponds exactly to what would be expected following hydroxylation at a single amino acid residue in the peptide. The addition of Mint3(NT) to the reaction mixture inhibited this hydroxylation in a dose-dependent manner (Fig. 4*B*, peptide + FIH-1 + Mint3NT). Further analysis to identify the modified amino acid residue by sequential mass spectrometry analysis revealed that the asparagine residue modified by FIH-1 corresponded to Asn⁸⁰³ (Fig. 4*C*).

Functional Analysis of FIH-1 and Mint3 in HEK293 Cells—Mint3 inhibited the ability of FIH-1 to modify HIF-1 α in vitro. We next examined whether Mint3 inhibited FIH-1 activity in cells and whether such inhibition in turn affected the transcriptional activity of HIF-1 α . To monitor the transcriptional activity of HIF-1 α , we employed a reporter assay system composed of a fusion protein comprising the Gal4 DNA-binding domain fused to the CAD of HIF-1 α and a luciferase gene whose expression is controlled by Gal4-binding elements (Fig. 5*A*). Reporter expression was monitored in HEK293 cells. Importantly, the

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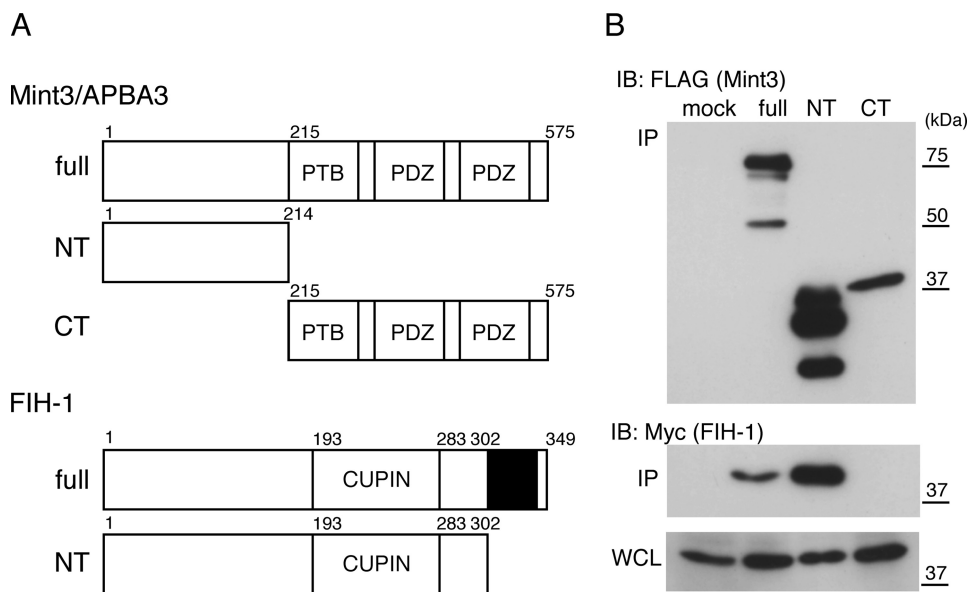


FIGURE 2. An N-terminal domain of Mint3 mediates binding to FIH-1. *A*, domain structure of Mint3 and FIH-1. Deletion mutants of Mint3 are illustrated: full-length (*full*), N-terminal (*NT*), and C-terminal (*CT*) portions. The C-terminal portion contains one phosphotyrosine-binding (*PTB*) and two PDZ domains. The amino acid positions are indicated. The domain structure of FIH-1 is shown in the lower panel. *CUPIN*, cupin-like domain responsible for catalytic activity; *black box*, dimerization domain; *NT*, C-terminally truncated mutant. *B*, Mint3 binds FIH-1. Myc-tagged FIH-1 was co-expressed in HEK293 cells together with each of the FLAG-tagged Mint3 derivatives. FLAG-tagged proteins were immunoprecipitated (*IP*) and detected by IB analysis using anti-FLAG antibody (*top panel*). FIH-1 in the precipitates and whole cell lysate (*WCL*) was detected by immunoblot analysis using anti-Myc antibody (*bottom two panels*).

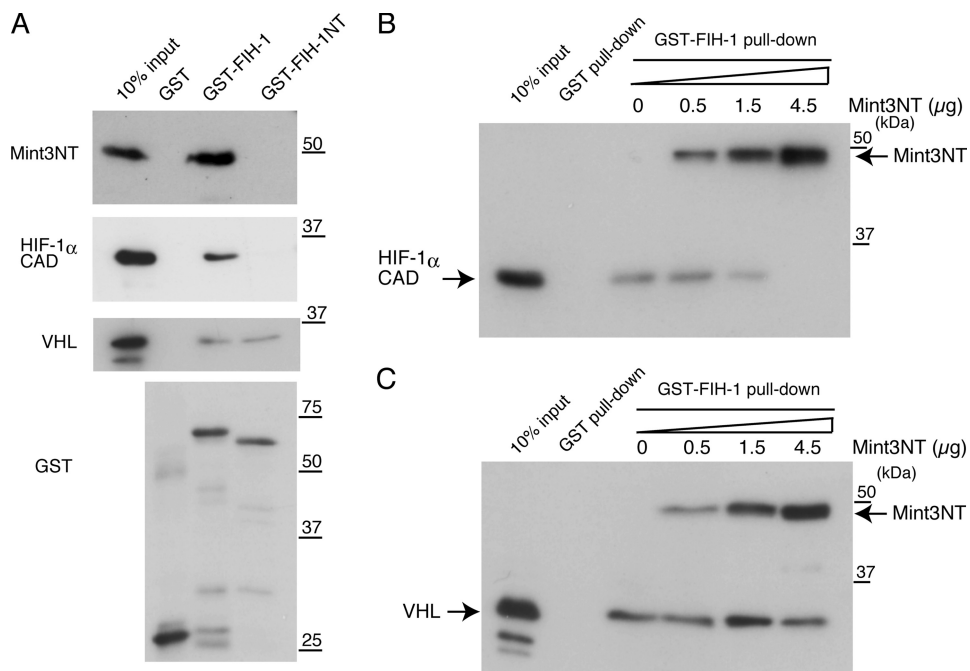


FIGURE 3. Mint3 competes with HIF-1 α for binding to FIH-1. *A*, the C-terminal dimerization domain of FIH-1 is important for binding to Mint3 and HIF-1 α . A pull-down assay was performed using agarose beads conjugated to GST, GST-FIH-1, or GST-FIH-1NT. The His₆-tagged Mint3NT fragment, HIF-1 α CAD and VHL were tested, and bound proteins were detected by immunoblot analysis using specific antibodies. *B* and *C*, Mint3 competes with HIF-1 α for binding to FIH-1. Pull-down of His₆-tagged HIF-1 α CAD (*B*) or VHL (*C*) with GST-FIH-1-conjugated beads was performed in the presence of increasing amounts of His₆-tagged Mint3NT.

Gal4-CAD fusion protein is not sensitive to the endogenous PHDs in HEK293 cells because the CAD fragment lacks the oxygen-dependent degradation domain. Thus, we can monitor only FIH-1 activity through expression of the lucifer-

ase. First, we examined whether endogenous FIH-1 inhibited the effect of HIF-1 α CAD on reporter gene expression in these cells. Expression of FIH-1 was knocked down using different shRNA sequences (Fig. 5*B*, lanes #1 and #2). Knockdown of FIH-1 expression increased CAD activity 4–5-fold compared with that observed in cells transfected with a control shRNA (Fig. 5*C*, shLacZ). The enhancement in CAD activity was proportional to the knockdown efficiency. Thus, endogenous FIH-1 in HEK293 cells can inhibit the activity of HIF-1 α CAD following forced expression of the latter. Expression of a control shLacZ construct itself did not affect reporter gene expression (data not shown).

As expected from the *in vitro* study, forced expression of Mint3 in the cells increased CAD activity (Fig. 5*D*, Mint3 full and NT). Expression of Mint3(NT), which binds FIH-1, was sufficient to enhance the CAD activity, whereas expression of Mint3(CT), which fails to bind FIH-1, was not (Fig. 5*D*). The effect of Mint3 expression on CAD activity required the presence of FIH-1 in the cells, because Mint3 did not augment CAD activity beyond the levels observed following release from suppression by FIH-1 (Fig. 5*E*, shFIH-1 + Mint3).

Endogenous Mint3 Enhances HIF-1 Activity in Macrophages—Whereas most cells show low levels of HIF-1 activity, constitutive HIF-1 activity is important for macrophages to maintain ATP production via glycolysis even during normoxia (21, 22). Three known PHDs were expressed in macrophages isolated from mouse bone marrow (not shown). Indeed, we observed a low level of HIF-1 α protein expression during normoxia and increased expression following exposure of the cells to hypoxia (Fig. 6*A*). To identify a role for Mint3 in regulating HIF-1 activity in macrophages

during normoxia, we knocked down Mint3 expression using two different shRNA expression vectors (Fig. 6*B*) by monitoring expression of HIF-1 target genes, GLUT1 and PGK1 mRNAs (Fig. 6*C*). Knockdown of Mint3 expression in the cells

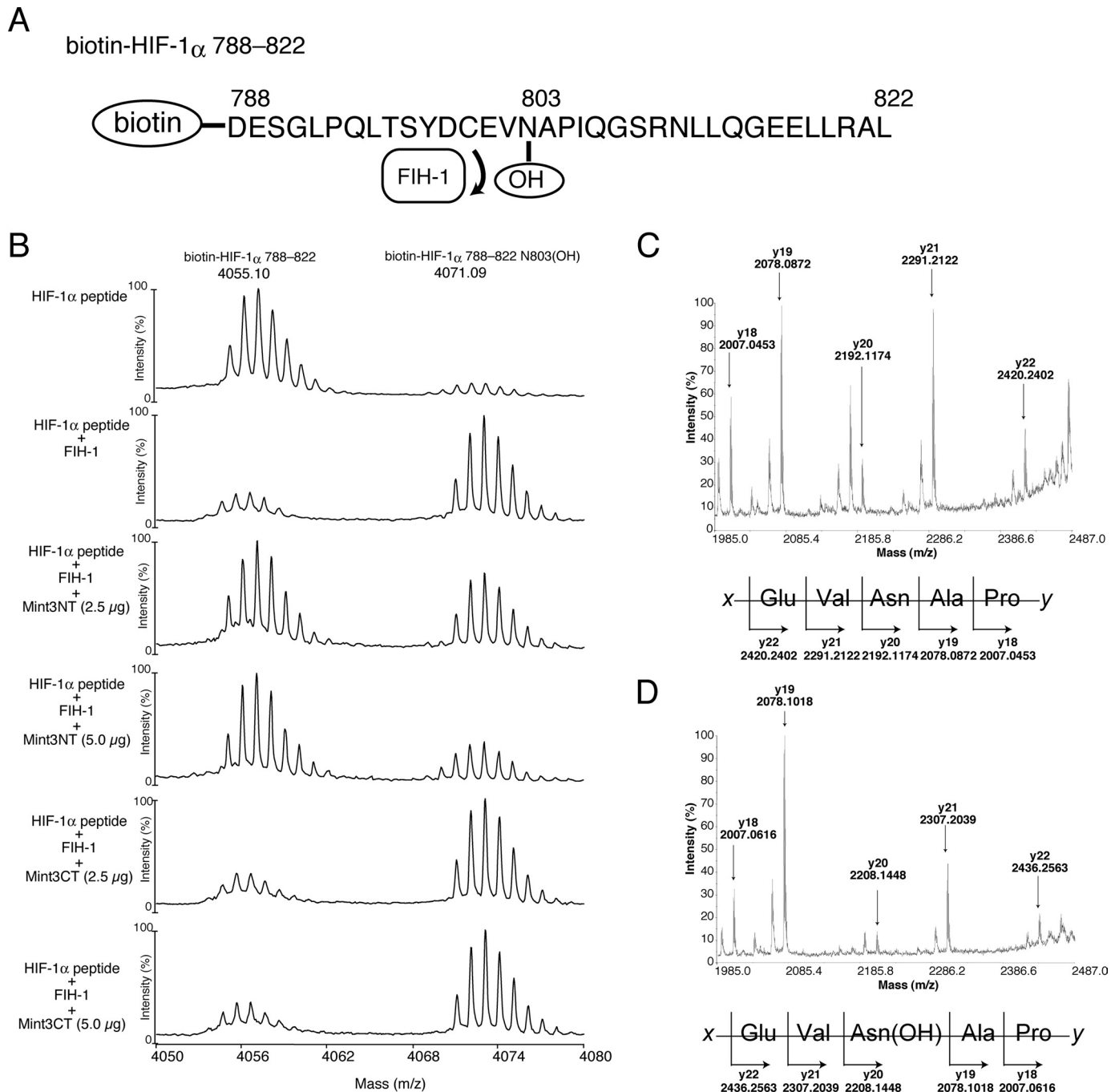


FIGURE 4. Mint3 inhibits the hydroxylation of the HIF-1 α peptide by FIH-1 *in vitro*. *A*, the N-terminal biotin-labeled peptide derived from HIF-1 α (amino acids 788–822) and the target asparagine residue for hydroxylation are shown. *B*, hydroxylation of the substrate peptide by FIH-1. A MALDI-TOF spectrum of the peptide was obtained after incubation of the peptide with FIH-1 *in vitro*. Peaks at $m/z = 4055.10$ and 4071.09 correspond to monoisotopic unhydroxylated and hydroxylated peptides, respectively. *C* and *D*, MS/MS spectra of the m/z 4055.10 (*C*) and m/z 4071.09 (*D*). Parent ions indicate that Asn⁸⁰³ is the site of hydroxylation in the HIF-1 α peptide. A 16-Da shift in the y -ion series appears at y_{20} , corresponding to the fragment containing Asn⁸⁰³.

led to a decrease in the expression of GLUT1 and PGK1 mRNAs, whereas the expression of the F4/80 mRNA was unaffected (Fig. 6C). To evaluate whether the effect of Mint3 knockdown was mediated by endogenous FIH-1, we next knocked down both the expression of FIH-1 and Mint3 (Fig. 6D). The reduction in expression of GLUT1 and PGK1 mRNAs observed in the Mint3 knockdown cells was antagonized by the additional knockdown of the endogenous FIH-1 (Fig. 6E, GLUT1 and 6F, PGK1). Reflecting the

inhibitory role of Mint3 on FIH-1 activity in macrophages, knockdown of Mint3 decreased the production of lactate, the end product of glycolysis (Fig. 6G), and ATP production (Fig. 6H).

Mint3 Forms a Complex with FIH-1 in Macrophages—Because Mint3 inhibits FIH-1 by binding to it, the complex formed might be detectable in macrophages as a co-localization pattern of the two proteins. Mint3 localized to the perinuclear region where GM130, a component of the Golgi body, also

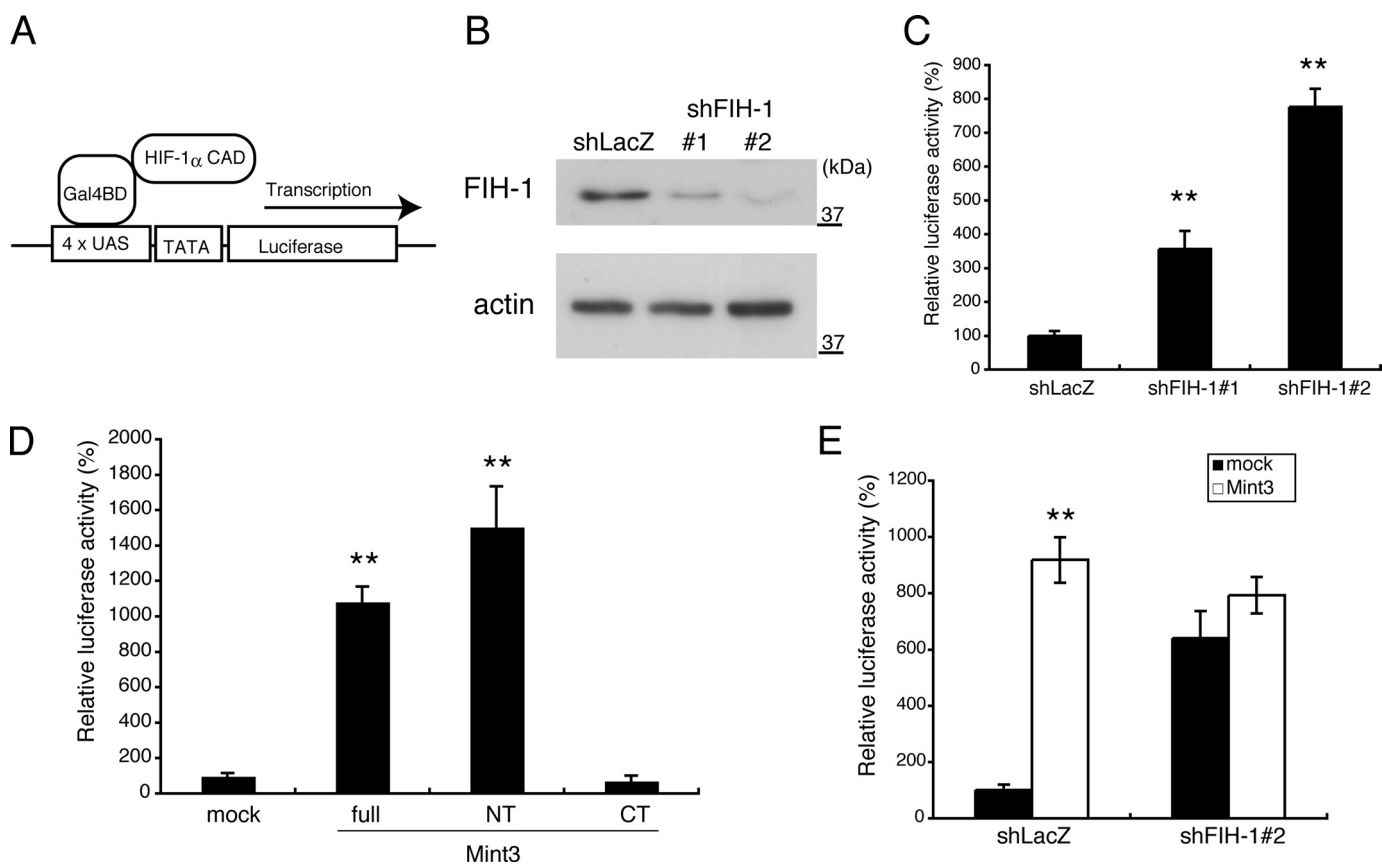


FIGURE 5. Mint3 activates HIF-1 α CAD by suppressing FIH-1 in HEK293 cells. *A*, schematic illustration of the reporter assay to monitor transcriptional activity of HIF-1 α CAD, the HIF-1 α transcriptional activation domain; Gal4BD, Gal4 DNA-binding domain; UAS, Gal4-binding site; TATA, thymidine kinase minimal promoter. *B*, Expression of FIH-1 was reduced in HEK293 cells using two shRNA sequences targeted to FIH-1 (#1 and #2). *C*, Knockdown of FIH-1 enhances HIF-1 α CAD activity. *D*, Mint3 enhances HIF-1 α CAD activity by abrogating FIH-1 activity in HEK293 cells. Note that the Mint3 N-terminal fragment is sufficient to enhance HIF-1 α CAD activity. *E*, Mint3 cannot stimulate HIF-1 α CAD activity further when FIH-1 is absent in the cells. The data in *C–E* were analyzed using Student's *t* test. **, $p < 0.01$ ($n = 3/\text{group}$).

localized (Fig. 7A). Thus, Mint3 localizes within the Golgi body in macrophages as has also been reported for other types of cells, suggesting potential roles for this protein in vesicle transport to the plasma membrane (18, 23, 24).

Co-localization of FIH-1 with Mint3 was observed in the perinuclear region (Fig. 7B), suggesting that Mint3 binds FIH-1 mostly in this region. Knockdown of Mint3 expression diminished the Mint3 signal greatly, and enrichment of FIH-1 in the perinuclear area appeared to be reduced in these cells (Fig. 7B). We then examined whether Mint3 regulates accumulation of FIH-1 in the Golgi body using Mint3 knockdown cells (Fig. 7C). To discriminate areas of specific FIH-1 accumulation more clearly, we digitally extracted FIH-1 signals that exceeded twice the average intensity level in each cell, as indicated in Fig. 7C. Finally, the numbers of cells were counted in which extracted FIH-1 signals overlapped with GM130 (Fig. 7D). The number of cells with enriched FIH-1 signals overlapping the Golgi body area was greatly decreased with the knockdown of Mint3 expression.

Interaction between Mint3 and FIH-1 in macrophages was further examined by immunoprecipitation assay. Endogenous FIH-1 was immunoprecipitated, and co-precipitation of endogenous Mint3 was confirmed by immunoblot analysis (Fig. 7E, the band marked by an asterisk in the top panel). Similarly, immunoprecipitation of Mint3 was shown to co-precipitate

FIH-1 (Fig. 7E, the band marked by an asterisk in the second panel). In contrast, PHD2, which hydroxylates the oxygen-dependent degradation domain of HIF-1 α , did not precipitate either Mint3 or FIH-1. Thus, Mint3 and FIH-1 form a specific complex in macrophages.

DISCUSSION

In this study, we identified Mint3 as a new regulator of the FIH-1-HIF-1 pathway. Mint3 is a member of the X11 family of proteins that bind APP via a C-terminal conserved domain (15, 25). It is reported to localize to the Golgi complex and is implicated in vesicle transport (15, 18, 23–26). Whereas Mint3 binds APP via a C-terminal domain, it binds FIH-1 via a domain within the N-terminal portion of the protein. Thus, Mint3 can act as an adaptor to recruit cytoplasmic FIH-1 to sites where Mint3 is localized and can thereby act to mediate an indirect association of FIH-1 with Mint3-binding proteins, such as APP. Because FIH-1 can modify asparagine residues within ankyrin repeats (14, 27, 28), FIH-1 may modify proteins within the Golgi complex following its recruitment to this subcellular region. Although this is an interesting possibility, we did not address this point in this study.

Mint3 and HIF-1 α competed for binding to an identical domain within FIH-1. Mint3 thus inhibited the ability of FIH-1 to modify the asparagine residue within the CAD peptide in

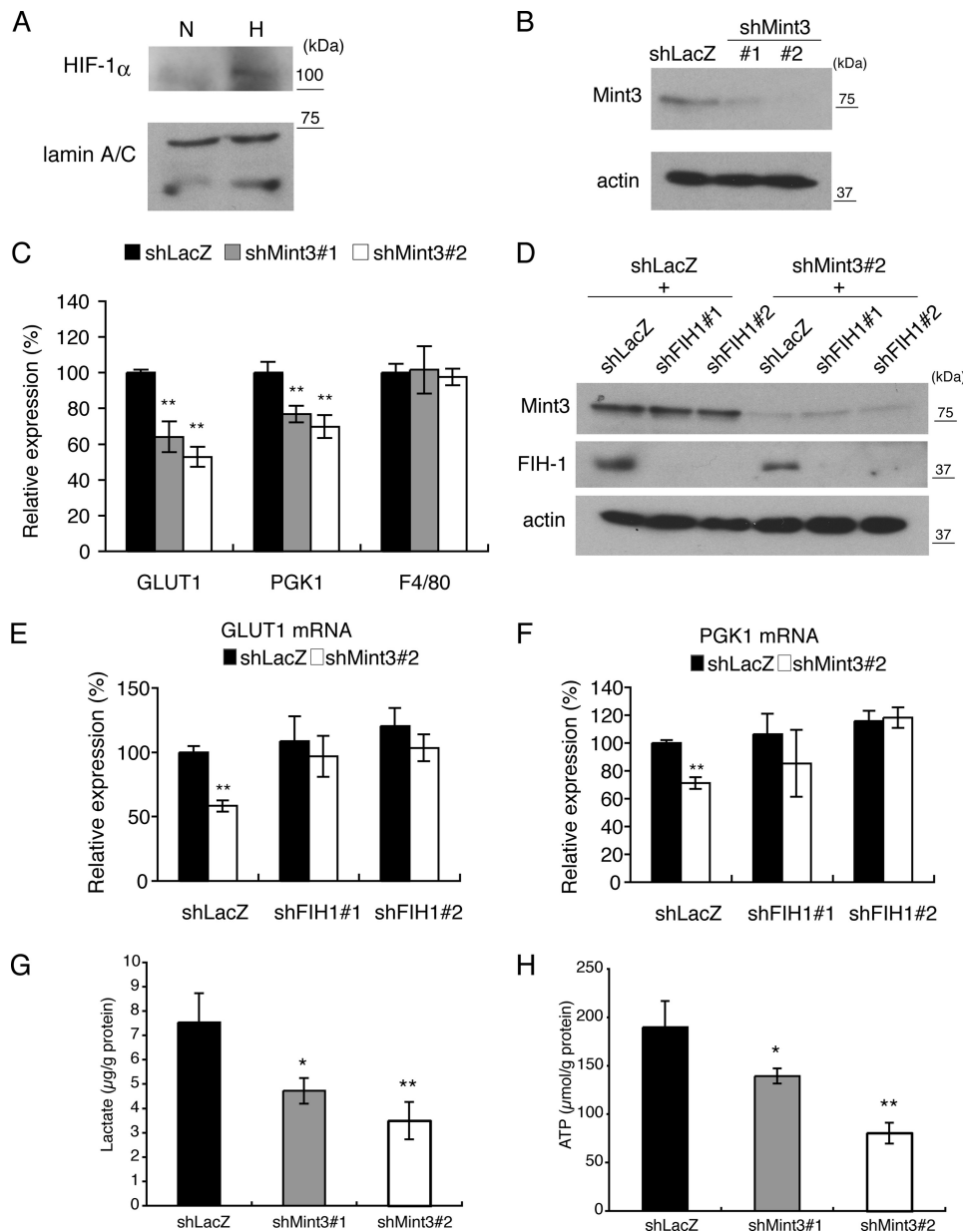


FIGURE 6. Mint3 regulates glycolysis and energy production in macrophages. *A*, expression of HIF-1 α in macrophages. Note that the expression of HIF-1 α is very low in macrophages under normoxia. *N*, normoxia; *H*, hypoxia. *B*, expression of Mint3 was reduced in macrophages following transfection of vectors expressing either of two shRNA sequences targeted to Mint3 (lanes #1 and #2). *C*, levels of mRNA encoding GLUT1, PGK1, and F4/80 in control and Mint3 down-regulated macrophages. Following reverse transcription, we performed real time PCR and then normalized values using β -actin. The values are presented relative to the control macrophage levels, which were set at 100%. *D*, expression of FIH-1 was reduced in control (shLacZ) and Mint3 knocked down (shMint3#2) macrophages following transfection of vectors expressing either of two shRNA sequences targeted to FIH-1 (#1 and #2). *E* and *F*, levels of mRNA encoding GLUT1 (*E*) and PGK1 (*F*) in control and FIH-1 down-regulated macrophages. *G*, Mint3 augments glycolytic activities of macrophages. The cells were cultured, and the amount of lactate accumulated in the culture media was measured. *H*, down-regulation of Mint3 affects ATP levels in macrophages. The data in *C* and *E–H* were analyzed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$ ($n = 3/\text{group}$).

in vitro. During normoxia, FIH-1 suppresses activity of HIF-1 α in an oxygen-dependent manner (2). Indeed, we demonstrated that endogenous FIH-1 suppressed the activity of exogenously expressed HIF-1 α CAD in HEK293 cells. This is consistent with previous studies by others (9, 20). Additional forced expression of Mint3 along with HIF-1 α CAD in the cells increased CAD activity, as expected from the ability of

Mint3 to inhibit FIH-1. Thus, Mint3 can regulate HIF-1 α activity via FIH-1 *in vitro* and *in vivo*.

In HEK293 cells, FIH-1 is reported to localize primarily to the cytoplasm regardless of the oxygen status of the cells (29). In contrast, we detected FIH-1 within the perinuclear region in macrophages, where Mint3 was also localized. Perinuclear localization of FIH-1 was dependent on the presence of Mint3 in macrophages, because knockdown of Mint3 altered the distribution of FIH-1 toward the cytoplasm.

Macrophages are unique in that they use glycolysis for ATP production, and the presence of constitutively active HIF-1 has been shown to be responsible for this characteristic (21, 22). Loss of HIF-1 α in macrophages by targeted disruption of the gene has been reported to reduce glycolytic activity and ATP production to 20% of that observed within the wild-type cells (22). This reduction in ATP levels causes severe defects in macrophage functions, such as recruitment of the cells to acute inflammatory tissues, cytokine production, migration, and invasion. The ability to capture and kill bacteria was also strongly inhibited (22). Thus, HIF-1 activity is critical for the overall regulation of energy-dependent macrophage functions.

We found that HIF-1 activity is maintained by inactivation of FIH-1 in macrophages, as evidenced by the effect of knockdown of Mint3 and FIH-1 in these cells. Knockdown of FIH-1 expression did not affect HIF-1 activity, as evaluated by the expression of GLUT1 and PGK1 mRNAs (Fig. 6, *E* and *F*). In contrast, knockdown of Mint3, which abrogates FIH-1 activity, decreased expression of these mRNAs (GLUT1 and PGK1), glycolytic activity (Fig. 6*G*), and ATP levels to 40% of the control cells (Fig. 6*H*). Therefore, Mint3-mediated inhibition of FIH-1 contributes significantly to the maintenance of cellular ATP levels and therefore to the maintenance of energy-dependent macrophage functions. Thus, Mint3-FIH-1 pathway is a possible new target for the development of therapeutics that can disrupt macrophage functions during pathological situations.

Mint3 Regulates HIF-1

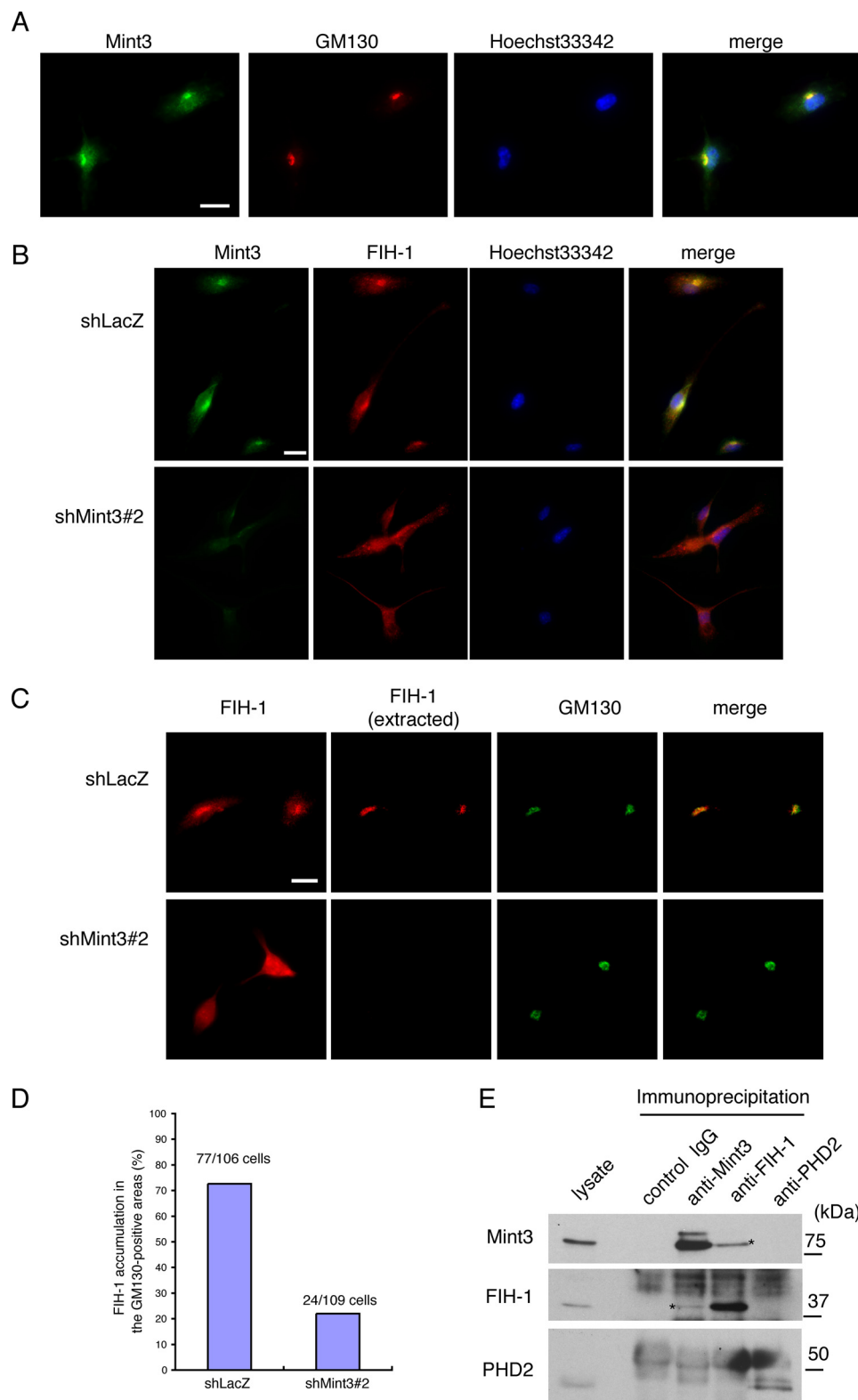


FIGURE 7. Complex formation between FIH-1 and Mint3 in macrophages. *A*, Mint3 localizes to the Golgi body in macrophages. Immunostaining of endogenous Mint3 and GM130 was carried out using specific antibodies. Hoechst33342 is a marker for the nucleus. *Bar*, 25 μ m. *B*, co-localization of FIH-1 and Mint3 in macrophages. Immunostaining of FIH-1 and Mint3 was carried out using specific antibodies. Note that FIH-1 largely co-localizes with Mint3, most prominently in the perinuclear region that corresponds to the Golgi area. Knockdown of Mint3 abolished accumulation of FIH-1 in the perinuclear region. *Bar*, 25 μ m. *C*, Mint3-dependent accumulation of FIH-1 in the Golgi body. GM130 and FIH-1 were visualized in macrophages by immunostaining (*left two panels*, respectively). FIH-1 signals exceeding twice the average signal intensity in each cell were extracted (*extracted*) and merged with GM130 signals (*merge*). *D*, numbers of cells in which FIH-1 signals were clearly enriched in the GM130-positive areas were counted. *E*, Mint3 binds FIH-1 in macrophages. Binding of FIH-1 to Mint3 in macrophages was demonstrated by immunoprecipitation of either FIH-1 or Mint3 followed by detection of the other protein by immunoblot analysis. Co-precipitated Mint3 and FIH-1 are marked by *asterisks*. PHD2 serves as a negative control that does not bind Mint3.

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