

## Transcription Factor YY1 Contributes to Tumor Growth by Stabilizing Hypoxia Factor HIF-1 $\alpha$ in a p53-Independent Manner

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### Abstract

In response to hypoxic stress, hypoxia-inducible factor (HIF)-1 $\alpha$  is a critical transcription factor regulating fundamental cellular processes, and its elevated expression level and activity are associated with poor outcomes in most malignancies. The transcription factor Yin Yang 1 (YY1) is an important negative regulator of the tumor suppressor factor p53. However, the role of YY1 under tumor hypoxic condition is poorly understood. Herein, we show that inhibition of YY1 reduced the accumulation of HIF-1 $\alpha$  and its activity under hypoxic condition, and consequently downregulated the expression of HIF-1 $\alpha$  target genes. Interestingly, our results revealed that the downregulation of HIF-1 $\alpha$  by inhibiting YY1 is p53-independent. Functionally, the *in vivo* experiments revealed that inhibition of YY1 significantly suppressed growth of metastatic cancer cells and lung colonization and also attenuated angiogenesis in a p53-null tumor. Collectively, our findings unraveled a novel mechanism by which YY1 inhibition disrupts hypoxia-stimulated HIF-1 $\alpha$  stabilization in a p53-independent manner. Therefore, YY1 inhibition could be considered as a potential tumor therapeutic strategy to give consistent clinical outcomes independent of p53 status. *Cancer Res*; 73(6): 1787–99. ©2012 AACR.

### Introduction

It has been known that Yin Yang 1 (YY1) has a fundamental role in biologic and physiologic processes such as embryogenesis, differentiation, DNA replication, and cellular proliferation

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(1, 2). YY1-deficient embryos die at the time of implantation, and its heterozygote knockout mice display significant growth retardation and neurologic defects (3, 4). YY1 was first discovered as a multifunctional transcription factor that regulates target genes responsible for various biologic processes. YY1 can selectively initiate, activate, or repress transcription depending upon the context in which it binds to recruited cofactors (5). Besides as a transcription factor or cofactor, recent studies have shown that YY1 plays a potential role in cancer biology via its putative interactions with cell-cycle regulators and death genes (5). For example, tumor suppressor gene p53 is significantly induced by the loss of YY1 (6). Meanwhile, overexpression of YY1 is observed in human cancers, such as colon cancer, prostate cancer, and osteosarcoma, and its expression correlates with immune-mediated apoptosis (5, 7, 8).

On the other hand, it is well known that hypoxia is a powerful force driving tumor growth, enhancing tumor metastasis and cell survival, and maintaining cancer stem cells (9–11). The best-characterized hypoxia response is mediated by hypoxia-inducible factor-1 (HIF-1; ref. 12). HIF-1 regulates cell survival, metabolism, tumor angiogenesis, invasion, and metastasis by activating target genes (10, 13). HIF-1 is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$ . HIF-1 $\alpha$  is regulated in an oxygen-dependent manner, whereas HIF-1 $\beta$  is expressed constitutively (14). Under normoxia, HIF-1 $\alpha$  is hydroxylated on Pro-402 and Pro-564 residues by prolyl hydroxylases (PHD). Hydroxylated HIF-1 $\alpha$  binds to the von Hippel-Lindau (VHL) protein, a component of an E3 ubiquitin ligase complex targeting the factor for proteasomal degradation. Under hypoxia, HIF-1 $\alpha$  is stabilized, then dimerizes with HIF-1 $\beta$  to bind the hypoxia

response element (HRE) of target genes (15–17). It has also been shown that HIF-1 $\alpha$  is overexpressed in many cancers and associated with poor prognosis (12).

Although recent studies have shown that YY1 and hypoxia individually play important roles in tumorigenesis, the relationship between them remains unknown. In this study, we showed that YY1 silencing could significantly suppress cellular proliferation under hypoxia and eliminate hypoxia-mediated HIF-1 $\alpha$  accumulation in a p53-independent manner, and it is involved in the ubiquitin proteasome degradation of HIF-1 $\alpha$ . Moreover, we revealed that YY1 inhibition significantly decreases the colonization and proliferation potential of cancer cells, reduces tumor angiogenesis, and suppresses tumor growth in p53-deficient cells.

## Materials and Methods

### Cell lines, treatments, and animals

HeLa and A549 cell lines were obtained from the American Type Culture Collection. The A549-luc-C8 Bioware Cell Line (P/N 119266) that stably expresses the American firefly luciferase gene was from Caliper LifeSciences; Saos-2 human osteosarcoma cells were kindly supplied by Riken Bioresource Center. The wild-type human colon carcinoma HCT116 cells (HCT116 p53<sup>+/+</sup>) and p53-null human colon carcinoma HCT116 cells (HCT116 p53<sup>-/-</sup>) were kindly provided by Dr. B. Vogelstein at The Johns Hopkins University Medicine School (Baltimore, MD). HeLa, A549, and A549-luc-C8 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% FBS (Invitrogen). HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> were maintained in McCoy's 5A medium (Invitrogen) containing 10% FBS, and Saos-2 cells were maintained in McCoy's 5A medium (Invitrogen) containing 15% FBS.

For the *in vivo* tumor study, CB17/Icr-SCID mice (male, body weight: 20–25 g, 6 weeks old) and BALB/c-nu/nu mice (male, body weight: 18–22 g, 6 weeks old) were purchased from Charles River Japan and kept in a laminar air-flow cabinet maintained at 24°C  $\pm$  2°C with 40% to 70% humidity under a 12-hour light/dark cycle and specific pathogen-free conditions. All animal studies were approved by the Animal Ethics Committee of The University of Tokyo (Tokyo, Japan). Animal care and experimental procedures for all experimental animal studies were conducted under a protocol approved by the Animal Ethics Committee of The University of Tokyo.

### Plasmids and constructs

For the YY1 expression vector, the coding region of human YY1 (NM\_003403) was inserted into a pcDNA3 vector (Invitrogen). To construct the Flag-YY1 expression vector, the Flag-YY1 coding region fragment was generated by PCR using pcDNA3-YY1 as a template, and the PCR product was again inserted into a pcDNA3 vector predigested with *Bam*HI and *Eco*RI. Luciferase reporter gene assays were conducted using plasmid DNA encoding luciferase driven by the 5 $\times$  hypoxia-responsive element (5 $\times$ HRE) promoter as reported in our previous study (17). To construct siRNA expression vectors, oligonucleotides with a hairpin structure, overhanging sequences, and terminator were synthesized, annealed, and then inserted into the *Bsp*MI sites of a pcENTRhuU6 vector (18). On the basis of the

results from the application of our algorithm (19), we used the target sequences specific for YY1 genes: siYY1-1 (GCAAGAA-GAGTTACCTCAG) and siYY1-2 (GGCAGAATTGCTAGAA-TG) as used in our previous study (20). We used a T7 siRNA expression vector as a control, which contains a stretch of 7 thymines (T) terminator sequences exactly downstream of the U6 promoter.

### Generation of experimental metastasis models and IVIS *in vivo* imaging

CB17/Icr-SCID mice (male, 6 weeks old) were randomly divided into 3 groups ( $n = 6-7$ ), and each group was injected intravenously with  $1 \times 10^6$  cells of A549-luc cells, A549-luc/shCon cells, or A549-luc/shYY1 cells. We confirmed metastatic nodule formation in the mice lungs immediately after the injection, and 1, 3, 5, and 7 weeks postinjection. The mice were injected with 150 mg/kg body weight D-luciferin (Promega) intravenously and 20 seconds later subjected to image analysis using IVIS imaging system (Xenogen) according to the manufacturer's instructions to count the photons from whole bodies. One minute later, photons from firefly luciferase were counted. Data were analyzed using Living Image software (version 2.5; Xenogen). For *ex vivo* IVIS imaging of the lungs at the end of the experiment, the mice were injected with 150 mg/kg body weight D-luciferin intravenously, immediately anatomized, and the lungs were removed and subjected to IVIS imaging system analysis.

### Micro-computed tomographic analysis

At the end of the lung metastasis model experiments, the images of the mice lungs in each group were acquired with an *in vivo* micro X-ray CT system R\_mCT2 (Rigaku; Scan conditions: Voltage: 90 kV, current: 80  $\mu$ A, FOV: 60 mm). Acquisition time was 17 seconds. Tomographic image datasets were reconstructed using the 3-dimensional ordered subsets-expectation maximum algorithm with 3 iterations and were analyzed by R\_mCT2 Viewer Package software (Rigaku).

### Generation of experimental subcutaneous tumor models

BALB/c-nu/nu mice were randomly divided into 3 groups (male,  $n = 6-7$ ), and each group was injected subcutaneously with  $5 \times 10^6$  HCT116 p53<sup>-/-</sup> mock cells, HCT116 p53<sup>-/-</sup>/shCon cells, or HCT116 p53<sup>-/-</sup>/shYY1 cells, respectively. Tumor size ( $V$ ) was evaluated by caliper every 2 days with reference to the following equation:  $V = a \times b^2/2$ , where,  $a$  and  $b$  are the major and minor axes of the tumor, respectively.

### Statistical analysis

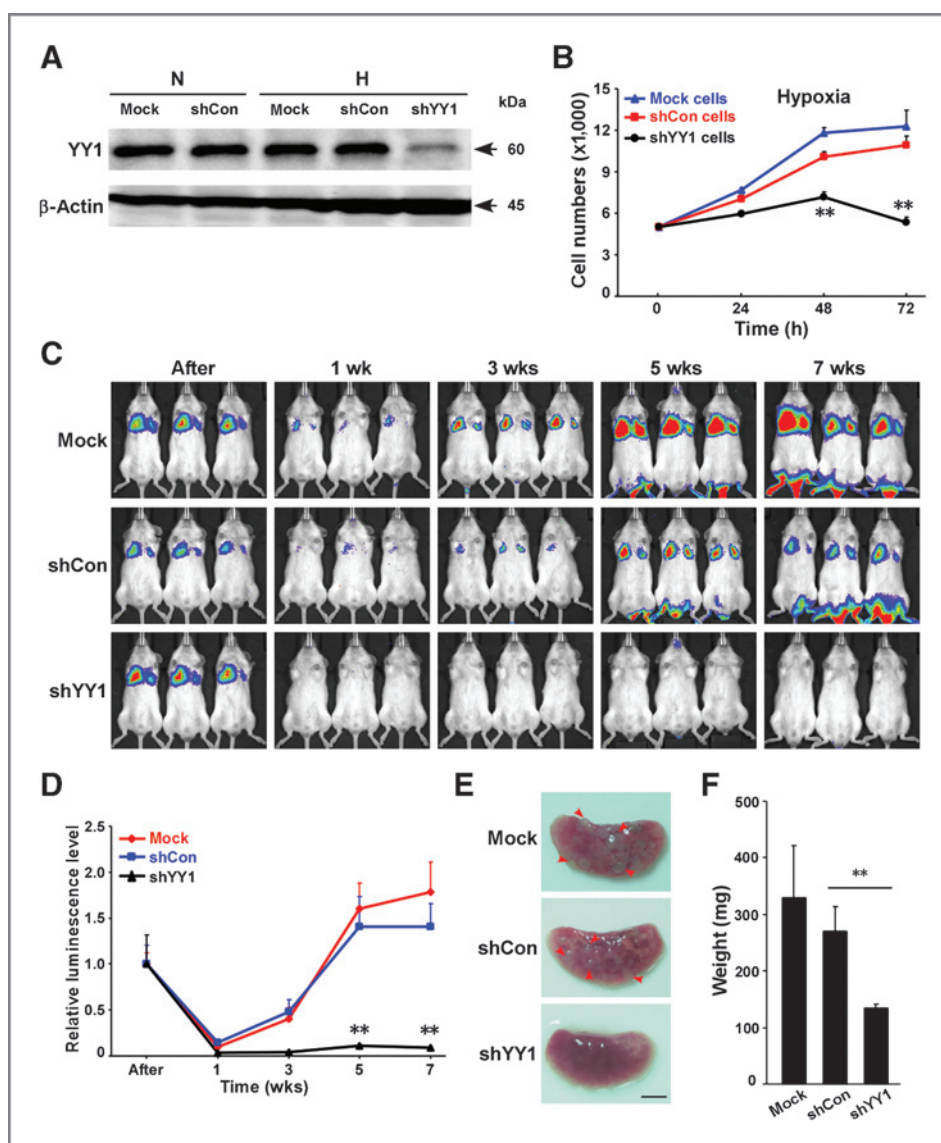
Results are presented as mean and SEM. Statistical comparisons of values were made using the 2-tailed Student  $t$  tests. Statistical significance was defined as  $P < 0.05$  (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

## Results

### YY1 silencing suppressed the colonization and survival of tumor cells in the lungs

To elucidate the relationship between YY1 and hypoxic microenvironment and its biologic effect on cancer cells

**Figure 1.** YY1 silencing suppresses the colonization of A549 cells in mice lung. **A**, Western blotting of YY1 and  $\beta$ -actin in A549-luc mock, A549-luc/shCon, or A549-luc/shYY1 cells cultured under normoxia (N) or hypoxia (H; 1% O<sub>2</sub>) for 6 hours. **B**, proliferation curves of A549-luc mock, A549-luc/shCon, or A549-luc/shYY1 cells cultured under hypoxia for 72 hours. Data show mean and SEM ( $n = 6$ ). \*\*,  $P < 0.01$  compared with A549-luc/shCon cells. **C**, representative luminescence images of the lungs of SCID mice injected intravenously with A549-luc mock, A549-luc/shCon, or A549-luc/shYY1 cells (top, middle, and bottom, respectively) immediately, 1, 3, 5, and 7 weeks after injection. **D**, quantification of IVIS images shown in **C** ( $n = 6-7$ ). \*\*,  $P < 0.01$  compared with mice implanted with A549-luc mock cells or A549-luc/shCon cells. **E** and **F**, representative outlook (**E**) and average wet weights (**F**) of the lungs 7 weeks after transplantation ( $n = 6$ ). Tumors were indicated with red arrowheads. Scale bars, 500  $\mu$ m. \*\*,  $P < 0.01$  compared with mice implanted with A549-luc/shCon cells.



under hypoxia, we investigated the effect of YY1 silencing on the survival and growth of cancer cells under hypoxic condition. We firstly generated short hairpin RNA (shRNA) vectors (shYY1-1 and shYY1-2) for 2 different target sites of YY1 mRNA, which effectively and specifically suppress the expression of YY1 (Supplementary Fig. S1A and S1B). Then, we established 13 A549-luc cell lines stably transfected with shYY1 vector and 3 cell lines stably transfected with shCon vector. The mRNA and protein expression levels of YY1 in these stable cells were confirmed by real-time PCR and Western blotting (Supplementary Fig. S1C and S1D). Among them, A549-luc/shYY1-1 stable cell line showed the lowest expression level of YY1, whereas A549-luc/shCon-1 cell line have no difference in YY1 expression level compared with A549-luc mock cells. On the basis of these results, A549-luc/shCon-1 and A549-luc/shYY1-1 cell lines (referred as A549-luc/shCon and A549-luc/shYY1 cells, respectively) were used for further studies in which A549-luc/shYY1 cells showed the

silencing of endogenous YY1 compared with A549-luc/shCon (Fig. 1A).

We first confirmed whether the transfection of shRNA vectors affects the luminescence intensities of A549-luc cells. As shown in Supplementary Fig. S2A–S2C, the luminescence intensities in both A549-luc/shYY1 and A549-luc/shCon stable cells were not altered compared with that of A549-luc mock cells, indicating that the luminescence activities of the stable cell lines were not affected by the transfection of the shYY1 and shCon vectors. We subsequently analyzed the proliferation potential and cell viability of A549-luc/shYY1 cells under hypoxic condition. As shown in Fig. 1B, after 48-hour exposure to hypoxia, the proliferation rate of A549-luc/shYY1 cells was significantly lower than that of A549-luc/shCon cells and A549-luc mock cells, and after 72 hours, the cell number of A549-luc/shYY1 further decreased. Overexpression of HIF-1 $\alpha$  partly restored the cell numbers (Supplementary Fig. S2D). Of note, the effect of YY1 silencing on the proliferation rate of A549-luc



cells in normoxia was not as significant as that in hypoxia (Supplementary Fig. S2E). On the other hand, relatively short-time exposure to hypoxia, that is, 6 to 24 hours, did not result in significant difference on the proliferation rate and viability of YY1-silenced A549 cells (Supplementary Fig. S2F). Similar results also were obtained from other cell types: HeLa, wild-type HCT116, and p53-null HCT116 cells (Supplementary Fig. S3A–S3C). Together, these results suggest that YY1 plays an important role on cell proliferation and survival on long exposure to hypoxia.

It is well known that hypoxia is a common characteristic of a wide range of solid tumors and is an important driving force of tumor growth. To further elucidate the effect of YY1 silencing on the *in vivo* proliferation rate of cancer cells, we transplanted A549-luc/shYY1 cells by intravenously injecting them into severe-combined immunodeficient (SCID) mice and analyzed their proliferation *in vivo* by monitoring the luminescence intensity in the murine lungs using an IVIS imaging system. As shown in Fig. 1C, luminescence was observed in the lungs of all mice immediately after the injection of A549-luc cells, indicating that the cancer cells were deposited in the lungs tissues after injection. However, 1 week after transplantation, luminescence in the lungs became weaker, especially in the mice transplanted with A549-luc/shYY1 cells, where luminescence was barely detectable. The decrease of luminescence in the mice transplanted with A549-luc/shYY1 cells continued until the end of the experimental period (7 weeks). In contrast, 3 weeks after transplantation, luminescence in the mice transplanted with A549-luc mock or A549-luc/shCon cells was enhanced and continued to increase. We quantified the luminescence intensities of the implanted mice and analyzed them statistically. These quantitative results showed that YY1 silencing significantly decreased the luminescence intensity in the murine lungs (Fig. 1D). IVIS imaging of the excised lungs and their quantitative analysis further confirmed these results, suggesting that YY1 silencing significantly suppressed the colonization and proliferation of cancer cells in murine lungs (Supplementary Fig. S4A and S4B). Thus, in concurrent with the *in vitro* results, the *in vivo* results showed that YY1 silencing inhibited the proliferation rate and cell viability when exposed to hypoxia for a relatively longer time.

Next, we observed the morphology of the excised lung. As shown in Fig. 1E, the outlooks of the excised lungs showed colonization of cancer cells in the lungs of the mice transplanted with A549-luc mock or A549-luc/shCon cells, whereas cancer cells almost could not be observed in mice transplanted with A549-luc/shYY1 cells. The weights of the lungs of the mice transplanted with A549-luc/shYY1 cells were also significantly lower than those of the mice transplanted with A549-luc mock or A549-luc/shCon cells (Fig. 1F).

These results were further confirmed by micro-computed tomographic (CT) scanning. The lungs were almost intact in mice transplanted with A549-luc/shYY1 cells, although they were destroyed in those transplanted with A549-luc mock or A549-luc/shCon cells (Fig. 2A). The results of hematoxylin and eosin (H&E) staining also showed that cancer cells (indicated by red arrowheads) colonized in the lungs of mice transplanted

with A549-luc mock or A549-luc/shCon cells but not in those of the mice transplanted with A549-luc/shYY1 cells (Fig. 2B).

Next, we conducted double immunohistochemical staining against Ki67, a marker of cell proliferation and luciferase. Consistent with the results of IVIS imaging, we found that the number of Ki67/luciferase -positive cells was significantly higher in the mice transplanted with A549-luc mock and A549-luc/shCon cells than in the mice transplanted with A549-luc/shYY1 cells (Fig. 2C). In addition, the results of immunohistochemical staining against CD31, a vascular endothelial marker, also revealed that Ki67-positive cells mainly located in areas with a CD31-positive vascular network in the mice transplanted with A549-luc mock or A549-luc/shCon cells, whereas in the mice transplanted with A549-luc/shYY1 cells, Ki67-positive cells almost could not be observed in those areas (Fig. 2D). Collectively, these results elucidated that YY1-silenced A549-luc cells were not able to colonize and proliferate in the lungs, although they did reach there.

### The effect of YY1 on HIF-1 $\alpha$ expression under hypoxia

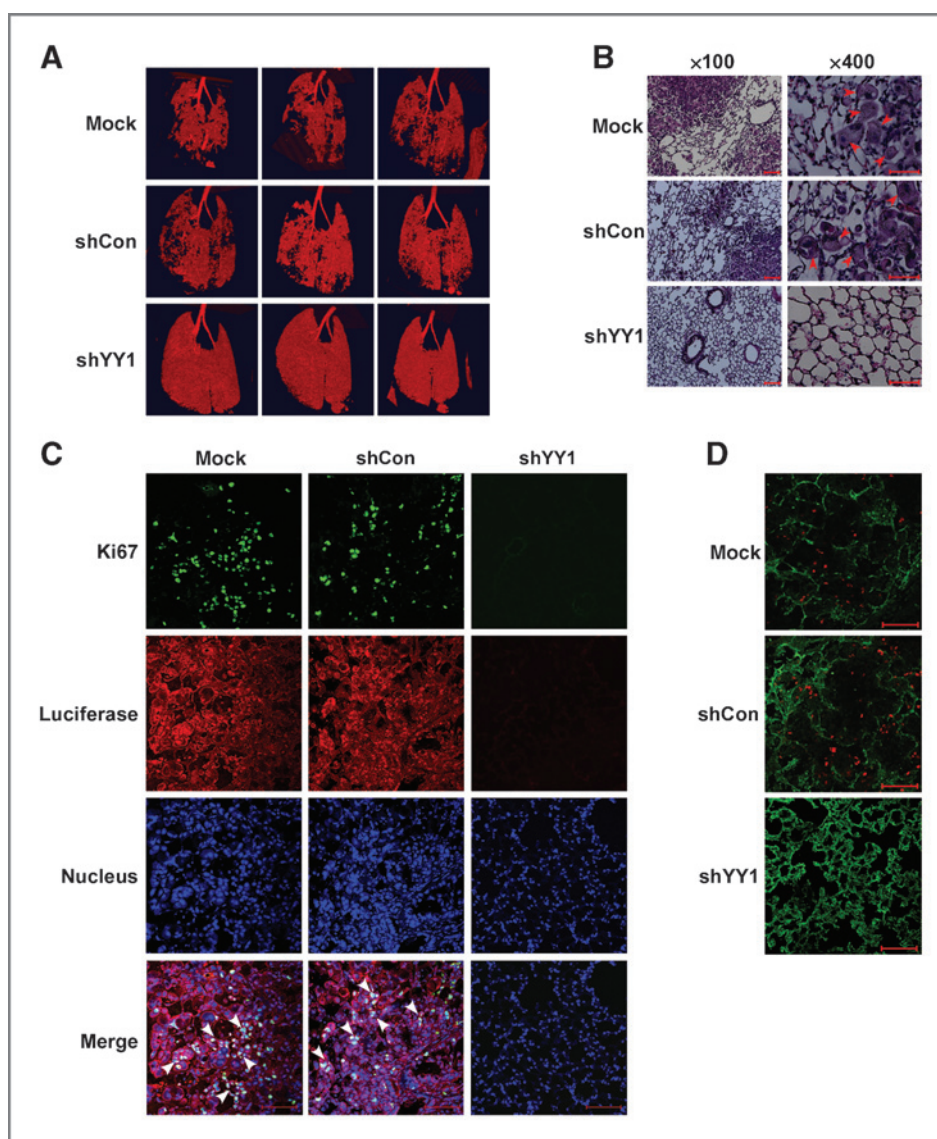
As it is well-known that HIF-1 $\alpha$  is the key response factor that regulates a number of target genes in response to hypoxic stress and plays a crucial role in tumor growth (12), we next investigated the effect of YY1 silencing on the expression of HIF-1 $\alpha$  protein under hypoxia. As shown in Fig. 3A, we found that while the accumulation of HIF-1 $\alpha$  protein in HeLa cells was induced by hypoxia, this accumulation was significantly eliminated by YY1 silencing. We also confirmed that YY1 silencing significantly increased the expression of tumor suppressor factor p53, which has been reported to be negatively regulated by YY1 (ref. 6; Fig. 3A). The results of immunocytochemical staining against HIF-1 $\alpha$  further showed that the accumulation of HIF-1 $\alpha$  in the nuclei in response to hypoxic stress was significantly decreased by YY1 silencing (Fig. 3B). Similar results were also observed in A549 cells, where YY1 silencing also resulted in the suppression of HIF-1 $\alpha$  protein, which was upregulated upon exposure to hypoxic stress or CoCl<sub>2</sub>, a chemical mimic of hypoxia (Fig. 3C and D).

Next, we tried to confirm whether YY1 silencing affects the transcriptional activity of HIF-1 $\alpha$ . We used firefly luciferase reporter gene with 5 HREs (5 $\times$ HRE reporter gene), whose activity increased HIF-1 $\alpha$  dependently. In concurrent with the results of the Western blotting, we observed that under hypoxia, the relative luciferase activity of the 5 $\times$ HRE reporter was reduced to more than 50% when YY1 was silenced (Fig. 3E). Taken together, these results indicate that YY1 is involved in the regulation of HIF-1 $\alpha$  protein under hypoxia.

### The downregulation of HIF-1 $\alpha$ downstream genes by YY1 silencing under hypoxia

To further explore the functional effects of YY1 silencing, we examined the effect of YY1 silencing on the expression of HIF-1 $\alpha$  target genes, including angiogenic factors, such as VEGF and TGF- $\alpha$  (Fig. 4A and B), and genes involved in cell survival, such as phosphoglycerate kinase (PGK), carbonic anhydrase 9 (CA9), and BCL2/adenovirus E1B 19 kD-interacting protein 3 (BNIP3; Fig. 4C–E). In addition, we also analyzed the expression

**Figure 2.** YY1 silencing represses tumor formation of A549 cells in mice lung. **A**, micro-CT analysis of the lungs of SCID mice injected intravenously with A549-luc mock cells (top), A549-luc/shCon cells (middle), or A549-luc/shYY1 cells (bottom) 7 weeks after transplantation. Black parts of the lungs indicated the formed tumor tissues; red parts of the lungs indicated the intact tissues. **B**, H&E staining images of the lungs 7 weeks after transplantation. Tumor cells were indicated with arrowheads. Scale bars, 200  $\mu$ m in  $\times 100$  photos and 50  $\mu$ m in  $\times 400$  photos. **C**, immunohistochemical staining of the lungs 7 weeks after transplantation for Ki67 (green) and luciferase (red). Nuclei were counterstained with ToTo-3 iodide (blue). Ki67/luciferase double-positive cells are indicated with arrowheads. Scale bars, 100  $\mu$ m. **D**, immunohistochemical staining of the lungs for CD31 (green) and Ki67 (red). Scale bars, 100  $\mu$ m.



of a HIF-1 target gene involved in metabolism, glucose transporter 1 (Glut1; Fig. 4F). The induction of these genes by hypoxia was significantly reduced by YY1 silencing.

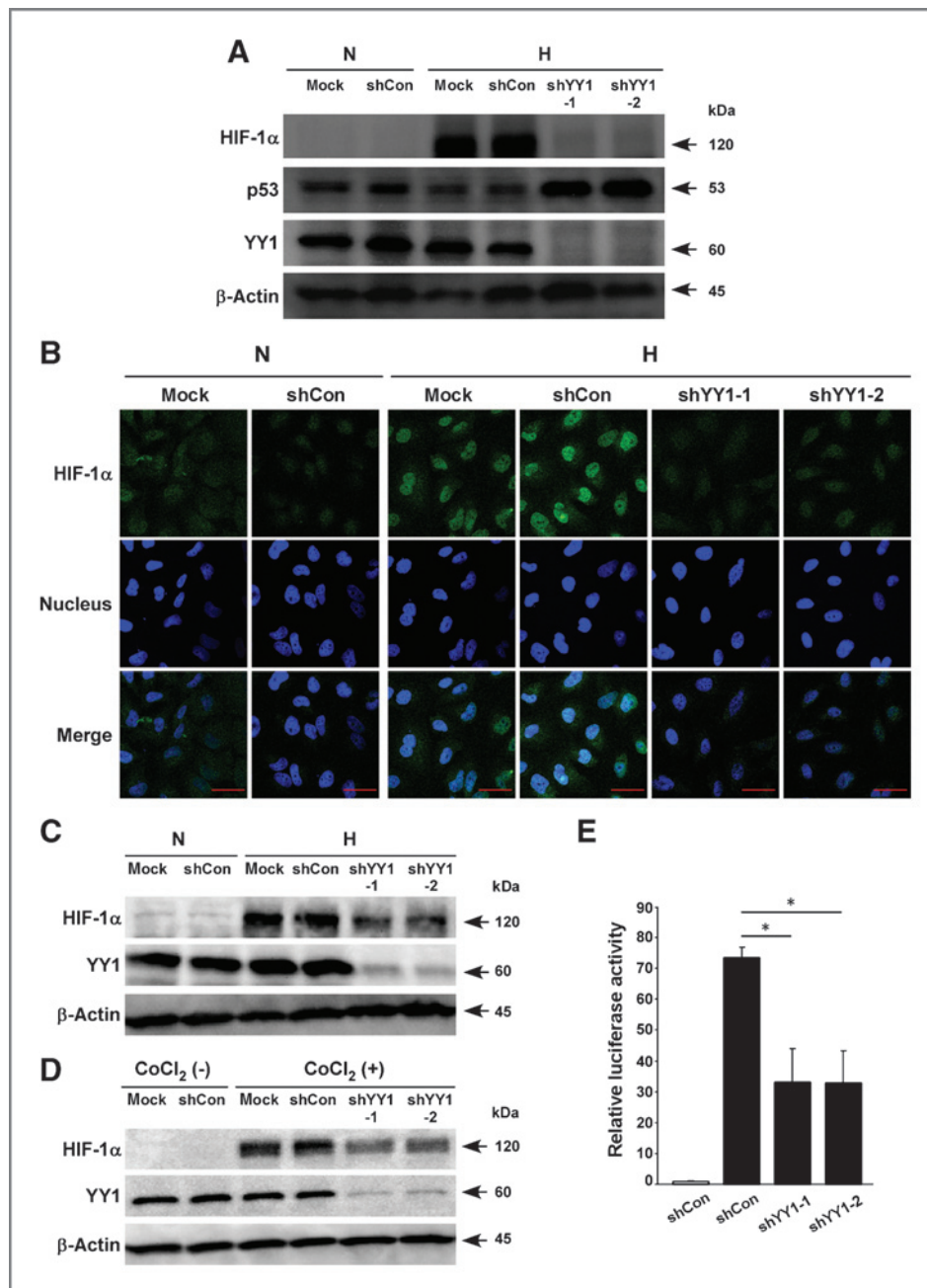
Furthermore, we conducted Western blotting and ELISA (Fig. 4G and H) for investigating the expression level of VEGF protein. Consistent with the results relating to its mRNA levels, the total protein level of VEGF and the amount of secreted VEGF under hypoxia were also significantly reduced by YY1 silencing.

Together with the fact that YY1 silencing reduced the levels of protein amount and transcriptional factor activities of HIF-1 $\alpha$ , these results indicated that YY1 contributes to the regulation of HIF-1 $\alpha$  pathways under hypoxic condition. Furthermore, as HIF-1 and some of its target genes, that is, VEGF, TGF- $\alpha$ , CA9, and PGK, are known to be associated with tumorigenesis (12), these results elucidated that the contribution of YY1 to tumorigenesis is, at least in part, via HIF-1 and its target genes pathways.

#### p53-independent YY1/HIF-1 $\alpha$ pathway

Our results shown above revealed the possibility that YY1 silencing inhibits tumorigenesis by downregulating HIF-1 $\alpha$  protein and its target genes under hypoxia. On the other hand, recent studies have revealed a relationship between tumor suppressor factor p53, which is also known to be negatively regulated by YY1, and HIF-1 in tumor progression. To elucidate the dependency of the YY1/HIF-1 $\alpha$  pathway on p53, we further investigated the effect of YY1 silencing on HIF-1 $\alpha$  under hypoxia by using p53-null HCT116 p53<sup>-/-</sup> cells.

First, we confirmed the effect of YY1 silencing on HIF-1 $\alpha$  in wild-type HCT116 cells (HCT116 p53<sup>+/+</sup> cells). As shown in Fig. 5A, YY1 silencing eliminated the increase of HIF-1 $\alpha$  protein amount in HCT116 p53<sup>+/+</sup> cells upon exposure to hypoxic stress, and at the same time, increased the amount of p53 protein. Furthermore, the tendency that YY1 silencing significantly decreases the accumulation of HIF-1 $\alpha$  protein under hypoxia was also observed in HCT116 p53<sup>-/-</sup> cells (Fig. 5B). It



**Figure 3.** YY1 silencing suppresses hypoxia-induced HIF-1 $\alpha$  protein accumulation. **A**, Western blotting of HIF-1 $\alpha$ , p53, YY1, and  $\beta$ -actin in HeLa cells transiently transfected with the indicated shRNA expression vectors and then cultured under normoxia or hypoxia for 6 hours. **B**, confocal microphotographs showing HIF-1 $\alpha$  and nucleus staining in HeLa cells transfected with the shCon, shYY1-1, and shYY1-2 vectors and cultured under normoxia or hypoxia for 6 hours. Scale bars, 20  $\mu$ m. **C**, Western blotting of HIF-1 $\alpha$ , YY1, and  $\beta$ -actin in A549 cells transiently transfected with the indicated shRNA expression vectors and then cultured under normoxia or hypoxia for 6 hours. **D**, Western blotting of HIF-1 $\alpha$ , YY1, and  $\beta$ -actin in A549 cells transiently transfected with the indicated shRNA expression vectors and then cultured with or without CoCl<sub>2</sub> (100  $\mu$ mol/L, a mimic of hypoxia condition) for 6 hours. **E**, luciferase activities in HeLa cells transiently cotransfected with 5 $\times$ HRE luciferase reporter gene, pRL-SV40, and the indicated shRNA expression vectors, and cultured under normoxia or hypoxia for 6 hours. White bar (left lane), normoxia; black bars, hypoxia. \*,  $P < 0.05$ . N, normoxia; H, hypoxia.

is noteworthy that YY1 silencing did not affect the protein amount of HIF-1 $\alpha$ , another subunit of HIF-1 complex, in both p53<sup>+/+</sup> and p53<sup>-/-</sup> cells. These results suggested that the downregulation of HIF-1 $\alpha$  by YY1 silencing occurs via p53-independent mechanism.

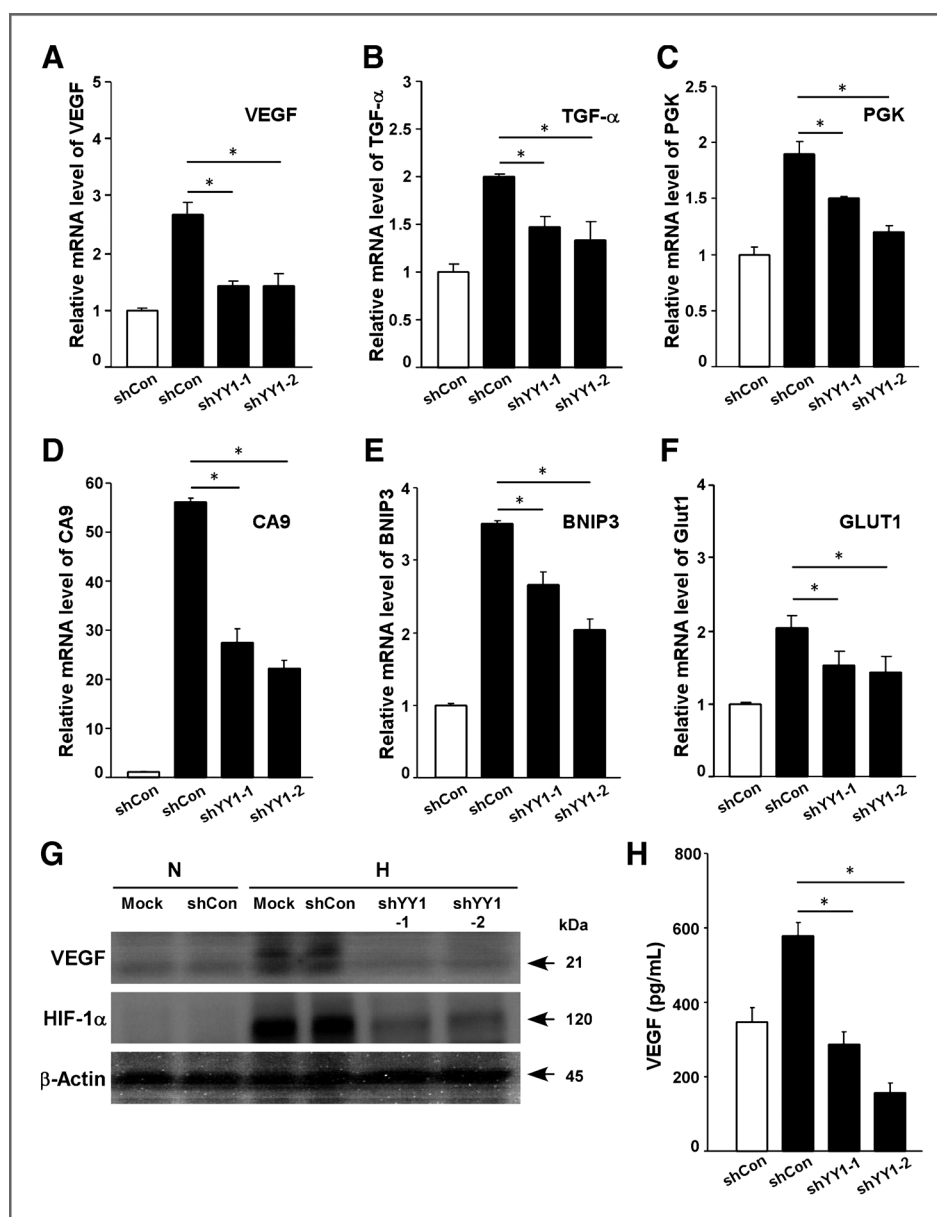
To further confirm the functional effect of YY1 silencing in HCT116 p53<sup>-/-</sup> cells, we investigated the effect of YY1 silencing on HIF-1 target genes and found that the mRNA levels of VEGF, TGF- $\alpha$ , PGK, CA9, BNIP3, and GLUT1 were significantly decreased by YY1 silencing under hypoxia (Fig. 5C–H, respectively). Similar tendency was also observed for the amount of VEGF protein secreted from the YY1-silenced HCT116 p53<sup>-/-</sup>

cells under hypoxia (Fig. 5I). Together, these results showed that the downregulation of HIF-1 $\alpha$  and its target genes by YY1 silencing occurred even in the absence of p53 expression.

To further confirm the generality of this phenomenon, we also analyzed the effect of YY1 silencing on HIF-1 $\alpha$  expression in Saos-2 cells, a human osteosarcoma cell line defects in p53 expression (Supplementary Fig. S5A). Under hypoxia, HIF-1 $\alpha$  protein in Saos-2 cells was induced in a time-dependent manner (Supplementary Fig. S5B); however, this induction was significantly reduced by YY1 silencing (Supplementary Fig. S5C). Furthermore, the luciferase activity of the 5 $\times$ HRE reporter gene and the amount of secreted VEGF in hypoxia



**Figure 4.** YY1 silencing decreases the expression of the HIF-1 $\alpha$  target genes under hypoxic condition. A–F, mRNA levels of VEGF (A), TGF- $\alpha$  (B), PGK (C), CA9 (D), BNIP3 (E), and GLUT1 (F) in YY1-silenced and control HeLa cells cultured under hypoxia for 6 hours. White bar (left lane), normoxia; black bars, hypoxia. Data show mean and SEM of 3 independent experiments. \*,  $P < 0.05$  compared with control under hypoxia. G, Western blotting of VEGF, HIF-1 $\alpha$ , and  $\beta$ -actin in HeLa cells transiently transfected with the indicated vectors and cultured under normoxia or hypoxia for 12 hours. H, hypoxia; N, normoxia. H, the levels of secreted VEGF in the culture mediums of YY1-silenced and control HeLa cells under hypoxia. Data show mean and SEM of 3 independent experiments. \*,  $P < 0.05$  compared with control under hypoxia. White bar (left lane), normoxia; black bars, hypoxia.

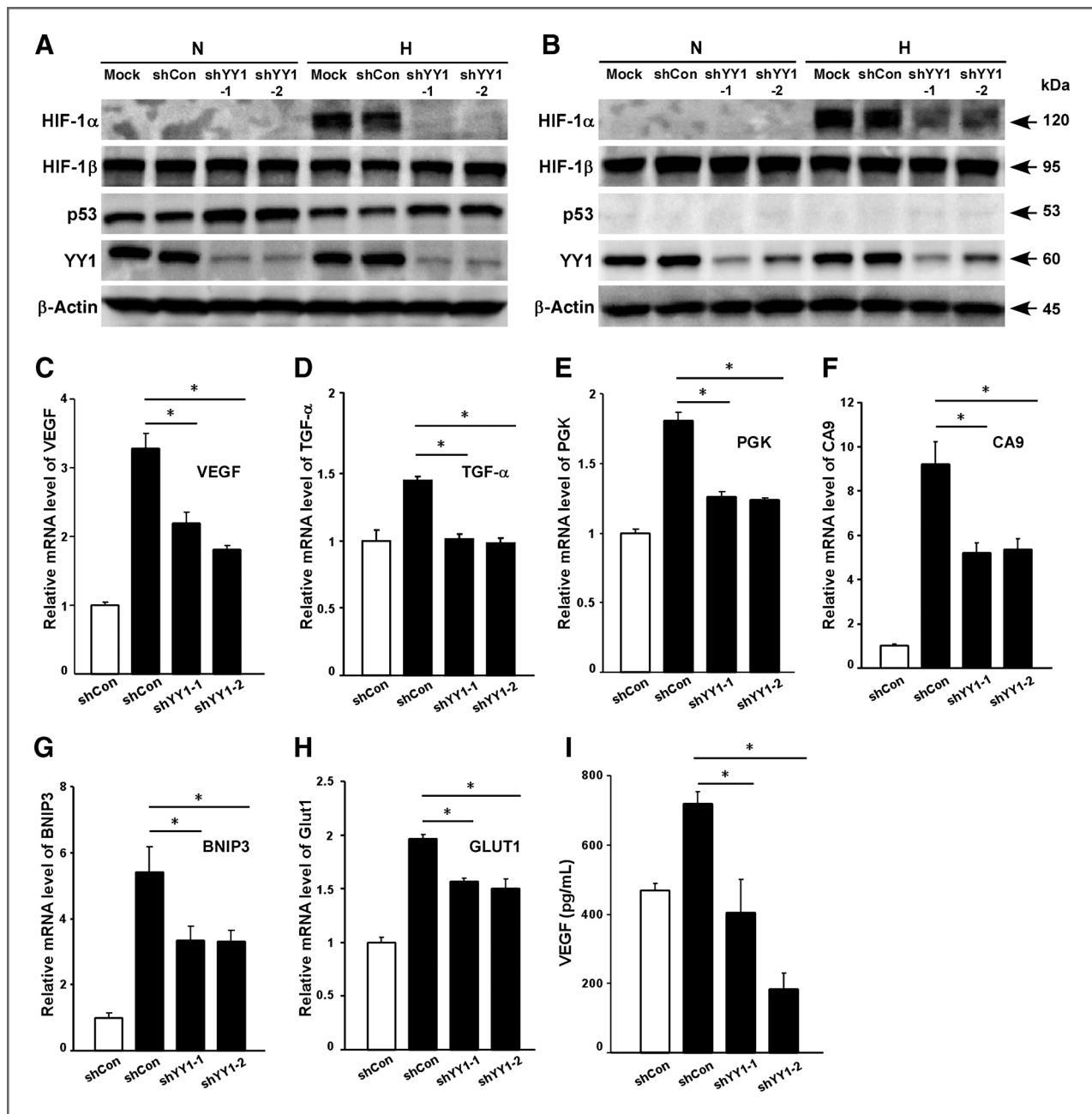


were also significantly reduced when YY1 was silenced (Supplementary Fig. S5D and S5E). The fact that similar results were also observed in Saos-2 cells suggested that p53-independent HIF-1 $\alpha$  downregulation by YY1 silencing might be a robust mechanism.

#### Effect of YY1 silencing on tumorigenesis of p53-null tumor

It has been known that p53 mutation is an obstacle in clinical tumor treatment, and thus, a therapeutic strategy overcoming p53 mutation is strongly needed. As our studies revealed that YY1 silencing could suppress the expressions of HIF-1 target genes even in the absence of p53, we raised the question whether the contribution of YY1 inhibition to tumor suppression depends on the p53 status.

To elucidate the role of the YY1 inhibition on tumorigenesis of p53-null tumor, we established stable YY1-silenced HCT116 p53<sup>-/-</sup> cell lines (HCT116 p53<sup>-/-</sup>/shYY1) by introducing shYY1 vectors. We firstly determined the mRNA level of YY1 in the established 17 HCT116 p53<sup>-/-</sup>/shYY1 and 4 HCT116 p53<sup>-/-</sup>/shCon cell lines (Supplementary Fig. S6A), and on the basis of these results, we further confirmed the YY1 protein levels in 5 stable HCT116 p53<sup>-/-</sup>/shYY1 cell lines (Supplementary Fig. S6B). Among them, HCT116 p53<sup>-/-</sup>/shYY1-2 showed the lowest YY1 protein level, whereas HCT116 p53<sup>-/-</sup>/shCon-1 showed no difference compared with HCT116 p53<sup>-/-</sup> mock cells (Supplementary Fig. S6C). Thus, we chose HCT116 p53<sup>-/-</sup>/shYY1-2 and HCT116 p53<sup>-/-</sup>/shCon-1 for further *in vivo* experiments (referred to as HCT116 p53<sup>-/-</sup>/shYY1 and HCT116 p53<sup>-/-</sup>/shCon, respectively). As shown in Fig. 6A,

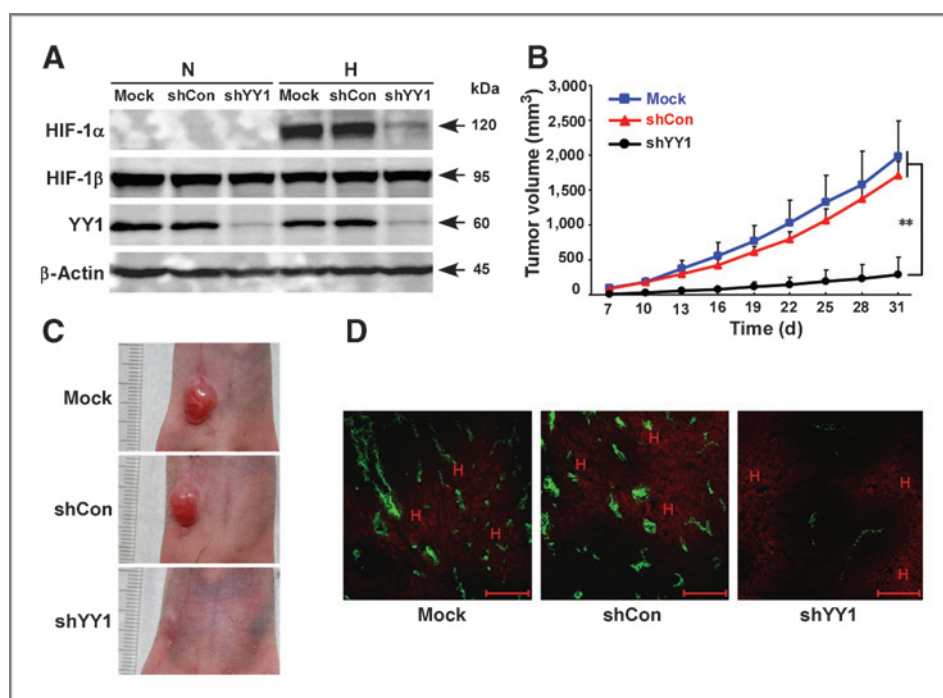


**Figure 5.** YY1 silencing eliminates hypoxia-induced HIF-1 $\alpha$  protein accumulation p53-independently. **A**, Western blotting of HIF-1 $\alpha$ , HIF-1 $\beta$ , p53, YY1, and  $\beta$ -actin in wild-type HCT116 p53<sup>+/+</sup> cells transiently transfected with the indicated shRNA expression vectors and then cultured under normoxia or hypoxia for 6 hours. **B**, Western blotting of HIF-1 $\alpha$ , HIF-1 $\beta$ , p53, YY1, and  $\beta$ -actin in HCT116 p53<sup>-/-</sup> cells transiently transfected with the indicated shRNA expression vectors and then cultured under normoxia or hypoxia for 6 hours. **C–H**, the mRNA levels of VEGF (**C**), TGF- $\alpha$  (**D**), PGK (**E**), CA9 (**F**), BNIP3 (**G**), and GLUT1 (**H**) in YY1-silenced and control HCT116 p53<sup>-/-</sup> cells cultured under hypoxia for 6 hours. Data show mean and SEM of 3 independent experiments. **I**, the amount of secreted VEGF in YY1-silenced and control HCT116 p53<sup>-/-</sup> cells cultured under hypoxia for 12 hours. Data show mean and SEM of 3 independent experiments. H, hypoxia; N, normoxia. White bar, normoxia; black bars, hypoxia. \*,  $P < 0.05$ .

YY1 silencing decreased HIF-1 $\alpha$  expression, but not HIF-1 $\beta$ , in stable HCT116 p53<sup>-/-</sup>/shYY1 cells under hypoxia. Then, we transplanted HCT116 p53<sup>-/-</sup> mock, HCT116 p53<sup>-/-</sup>/shCon, and HCT116 p53<sup>-/-</sup>/shYY1 cells subcutaneously into nude mice and observed the tumor formations. The volume of the tumors formed in the mice transplanted with HCT116 p53<sup>-/-</sup>

mock or HCT116 p53<sup>-/-</sup>/shCon cells was about 5 times greater than that of those in the mice transplanted with HCT116 p53<sup>-/-</sup>/shYY1 cells (Fig. 6B). The representative appearances of the tumors in each group are shown in Fig. 6C. These results indicate that tumor suppression by YY1 inhibition could occur independent of p53 status.





**Figure 6.** YY1 silencing suppresses the tumor-initiating potential of HCT116 p53<sup>-/-</sup> cells. **A**, Western blot analysis of HIF-1 $\alpha$ , HIF-1 $\beta$ , YY1, and  $\beta$ -actin in HCT116 p53<sup>-/-</sup> mock cells, HCT116 p53<sup>-/-</sup>/shCon cells, and HCT116 p53<sup>-/-</sup>/shYY1 cells. Cells were cultured under normoxia or hypoxia for 6 hours. H, hypoxia; N, normoxia. **B**, HCT116 p53<sup>-/-</sup> mock cells, HCT116 p53<sup>-/-</sup>/shCon stable cells, and HCT116 p53<sup>-/-</sup>/shYY1 stable cells were injected subcutaneously into nude mice. Tumor volume was measured at the indicated time points and the results presented as mean and SEM ( $n = 6-7$ ). \*\*,  $P < 0.01$  compared with control group. **C**, representative images showing tumor in mice injected with HCT116 p53<sup>-/-</sup> mock cells, HCT116 p53<sup>-/-</sup>/shCon cells, and HCT116 p53<sup>-/-</sup>/shYY1 cells. Ruler shows centimeters. **D**, immunohistochemical staining for CD31 (green) and tissue hypoxia area (red) in tissue sections of xenografted tumors in mice injected subcutaneously with HCT116 p53<sup>-/-</sup> mock cells (left), HCT116 p53<sup>-/-</sup>/shCon cells (middle), or HCT116 p53<sup>-/-</sup>/shYY1 cells (right). H, hypoxia area in tumor tissue. Scale bars, 100  $\mu$ m.

One key aspect of tumorigenesis is the formation of new blood vessels driven by hypoxia microenvironment, which occurs mainly via HIF-1 pathway. The newly formed blood vessels effectively provide nutrition to support tumor growth (12, 21). Thus, we further analyzed whether the formation of blood vessels in the HCT116 p53<sup>-/-</sup> tumors was affected by YY1 silencing. The results of immunohistochemical staining against CD31 revealed that in tumor hypoxic areas (marked with a hypoxyprobe; red areas), the number of CD31-positive cells in the tumors of mice transplanted with HCT116 p53<sup>-/-</sup>/shYY1 cells (Fig. 6D, right) was lower than that in mice transplanted with HCT116 p53<sup>-/-</sup> mock cells or HCT116 p53<sup>-/-</sup>/shCon cells (Fig. 6D, left and middle). These results suggested that the number of blood vessels in tumor formed in mice transplanted with YY1-silenced cells was significantly suppressed and that YY1 contributes to hypoxia-driven tumor angiogenesis in a p53-independent manner.

#### YY1 silencing affects HIF-1 $\alpha$ stabilization under hypoxia

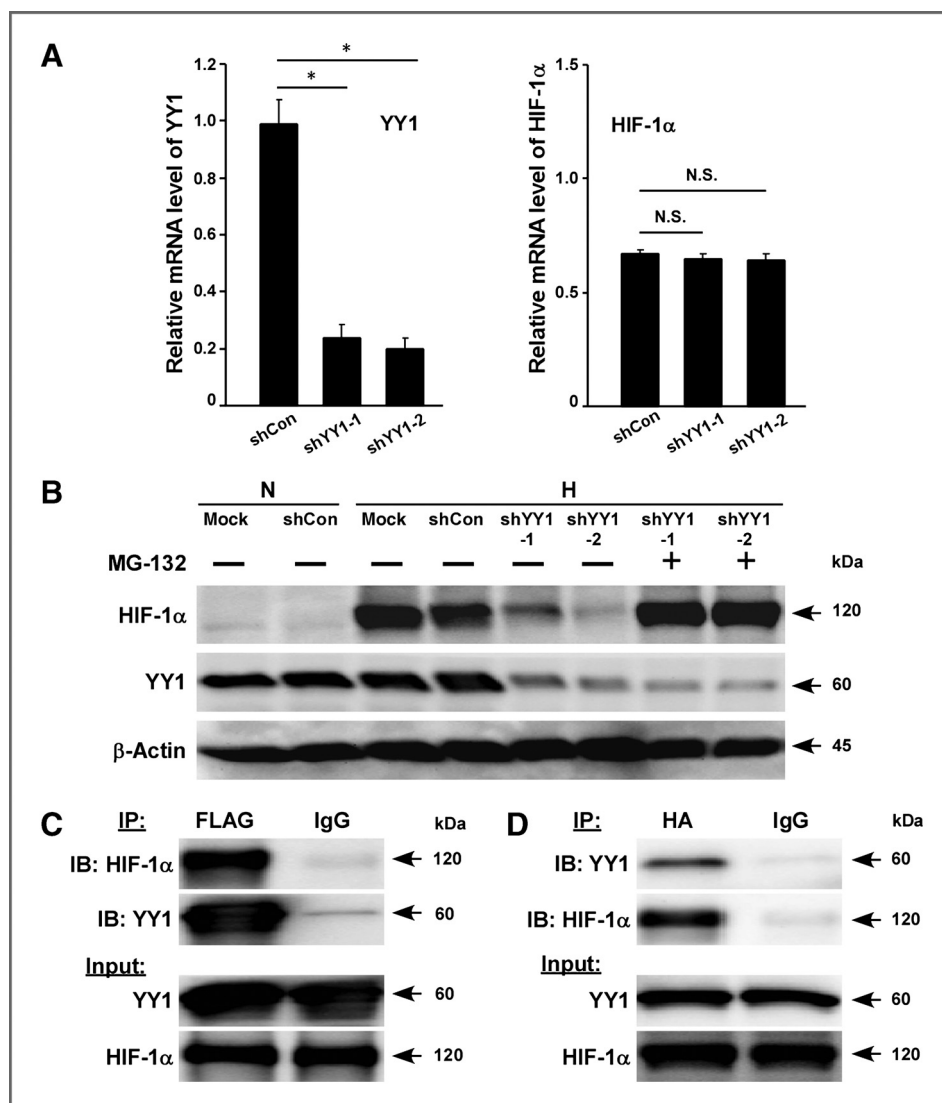
It is well known that HIF-1 $\alpha$  protein is degraded via an ubiquitin-proteasome degradation system under normoxia, whereas it is stabilized in hypoxic condition. On the other hand, our results showed that under hypoxic condition, YY1 silencing affects only the protein level of HIF-1 $\alpha$  in HCT116 p53<sup>-/-</sup> cells, and not its mRNA level (Figs. 5B and 7A). These

facts suggested that the downregulation of HIF-1 $\alpha$  by YY1 silencing was mediated through a posttranslational mechanism. Next, we investigated whether YY1 is involved in the stabilization of HIF-1 $\alpha$  protein. We found that under hypoxia, addition of MG-132, a proteasome inhibitor that blocks HIF-1 $\alpha$  degradation (22), rescued the decrease of HIF-1 $\alpha$  protein accumulation in YY1-silenced cells, whereas the expression levels of YY1 remained unchanged (Fig. 7B). It is noteworthy that overexpression of YY1 did not induce significant changes in the levels of HIF-1 $\alpha$  and its target genes under hypoxic condition (Supplementary Fig. S7A-S7D), and it is probably due to the saturation of HIF-1 $\alpha$  protein under hypoxia.

Next, we conducted an immunoprecipitation assay to investigate the physical interaction between YY1 and HIF-1 $\alpha$  under hypoxia. As shown in Fig. 7C and D, under hypoxia, YY1 could bind to HIF-1 $\alpha$ . Taken together, these results suggest that YY1 is able to interact physically with HIF-1 $\alpha$  and is involved in the stabilization of the HIF-1 $\alpha$  protein in response to hypoxia stress.

#### Discussion

Hypoxia is a well-recognized powerful driver of malignant progression (21, 23, 24). Stabilization of HIF-1 $\alpha$  in response to tumor hypoxia alters the expressions of many tumor-related genes (12, 25), whereas overexpression of HIF-1 $\alpha$  has been



**Figure 7.** YY1 contributes to the hypoxia-induced HIF-1 $\alpha$  stabilization. **A**, the mRNA levels of YY1 (left) and HIF-1 $\alpha$  (right) in YY1-silenced and control HCT116 p53<sup>-/-</sup> cells cultured under hypoxia for 6 hours. Data show mean and SEM of 3 independent experiments. \*,  $P < 0.05$  compared with control HCT116 p53<sup>-/-</sup> cells under hypoxia. N.S., no significant difference. **B**, Western blot analysis for HIF-1 $\alpha$  and YY1 in HCT116 p53<sup>-/-</sup> cells transfected with shCon, shYY1-1, and shYY1-2 vectors. Cells were cultured under normoxia or hypoxia for 8 hours in the presence (+) or absence (-) of ubiquitin-proteasome inhibitor MG-132 (10  $\mu$ mol/L). H, hypoxia; N, normoxia. **C** and **D**, co-immunoprecipitation using the whole protein extracted from the HCT116 p53<sup>-/-</sup> cells transfected with FLAG-pcDNA3-YY1 vector (**C**) and HA-pcDNA3-HIF-1 $\alpha$  vector (**D**). Cells were cultured under hypoxia for 24 hours and subjected to coimmunoprecipitation. Lysates were immunoprecipitated with anti-FLAG or anti-IgG antibodies and blotted with anti-HIF-1 $\alpha$  or anti-YY1 antibodies (**C**). Lysates were immunoprecipitated with anti-HA or anti-IgG antibodies and blotted with anti-YY1 or anti-HIF-1 $\alpha$  antibodies (**D**).

shown to be closely associated with increased tumor growth, vascularization, and metastasis (10, 23, 26, 27). These facts show the key role of HIF-1 $\alpha$  in tumor biology.

In the present study, we showed that inhibition of YY1 represses HIF-1 $\alpha$  accumulation and its target genes expressions under hypoxia. YY1 has already been shown to modulate numerous genes, including oncogenes such as *c-myc* and *c-fos*, and tumor suppressor gene *p53* (5). The fact that YY1 inhibition eliminates the accumulation of HIF-1 $\alpha$  protein also reveals another important aspect of YY1 in tumor biology. It is noteworthy that the YY1 shRNA expression vectors used in this study specifically suppresses the expression of YY1 and not YY2, a mammalian YY1 homolog that shares 95% identity in the zinc finger-binding regions. Furthermore, the decrease of HIF-1 $\alpha$  transcriptional activity under hypoxia is a specific effect of YY1 silencing, not an effect of YY2 silencing (Supplementary Fig. S8A–S8F). YY2 silencing has no effect on both HIF-1 $\alpha$  mRNA and hypoxia-induced HIF-1 $\alpha$  accumulation (Supplementary Figs. S8C and S8G).

Our results showed that YY1 might be involved in the stabilization of HIF-1 $\alpha$  under hypoxia, most likely via its contribution to the HIF-1 $\alpha$  ubiquitin-proteasome pathway. However, it seemed that YY1 silencing does not affect the expression of prolyl hydroxylase domain-2 (PHD2), an important regulator of HIF-1 $\alpha$  degradation (Supplementary Fig. S5F). Furthermore, we investigated whether or not HIF-1 $\alpha$  is hydroxylated by YY1 silencing under hypoxic condition. The results showed that the ratio of the amount of hydroxyl-HIF-1 $\alpha$  (Pro564) to the amount of HIF-1 $\alpha$  is not significantly increased by YY1 silencing under hypoxic condition in both HCT116 p53<sup>-/-</sup> and HCT116 p53<sup>+/+</sup> cells (Supplementary Figs. S8H and S8I), indicating that the regulation of HIF-1 $\alpha$  degradation induced by YY1 silencing occurs mainly not through the hydroxylation of proline 564. Indeed, recent reports showed that besides hydroxylation, regulation of HIF-1 $\alpha$  protein is also mediated by multiple modification pathways (5), such as phosphorylation, SUMOylation, and degradation in cytosol (28–31). Whether YY1 silencing mediated HIF-1 $\alpha$  regulation

occurs through one of these pathways, or through other pathways, which has not been reported, requires further detailed investigations.

It is well known that tumors need angiogenesis not only for the provision of nutrition and oxygen but also for translocation to distant organs (32). Our results here showed that YY1 silencing disrupts the stability of HIF-1 $\alpha$  protein under hypoxic condition and downregulates its protein amount. Consequently, this posttranscriptional regulation of HIF-1 $\alpha$  eliminates the hypoxia-induced upregulation of HIF-1 $\alpha$  target angiogenic factors, VEGF and TGF- $\alpha$ , and subsequently, results in a significant decrease in the number of blood vessels formed in tumor hypoxic areas. On the other hand, a recent study has shown that YY1 forms a complex with HIF-1 $\alpha$ , upregulates the transcriptional activity of VEGF promoter, and contributes to neoangiogenesis (33). Thus, it is highly probable that the decrease of VEGF expression we showed here is due to both the contribution of YY1 silencing on its promoter activity as reported previously, and the contribution of YY1 silencing on a more upstream pathway, that is, the downregulation of HIF-1 $\alpha$  protein under hypoxia, which we showed in the present study. Altogether, these results convincingly show that YY1/HIF-1 $\alpha$  pathway plays an essential role in tumor angiogenesis.

YY1 inhibition also leads to the decrease of genes involved in cell survival (PGK and CA9) under hypoxia. Consistently, the cell proliferation rate and viability of YY1-silenced cells under hypoxia are significantly reduced. Notably, in normoxia, the effect of YY1 silencing on cell proliferation rate was not as significant as that observed in hypoxia, suggesting that these phenomena are particularly important in hypoxia. The effect of YY1 silencing on cell proliferation also occurs *in vivo*, as the number of Ki67/luciferase double-positive A549 cells in murine lung tumor model is significantly lower in mice transplanted with YY1-silenced A549-luc cells than in those transplanted with the controls. On the basis of our *in vitro* results and the previous studies showing the involvement of HIF-1 $\alpha$  in cell survival and proliferation (12, 34, 35), it is likely that the decrease of cell colonization and proliferation in murine lungs due to YY1 silencing occurs, at least partly, through the downregulation of HIF-1 $\alpha$ . Cell survival, colonization, and proliferation are important aspects for tumor metastasis. Thus, our results indicate that YY1 might also be involved in tumor metastasis. However, tumor metastasis is a complex process comprising multiple steps such as the acquisition of an invasive phenotype, epithelial-to-mesenchymal transformation (EMT), invasion of the surrounding stroma (local invasion), translocation of cancer cells to a distant organ, extravasations, and invasion of cancer cells into foreign tissue (36, 37). Therefore, further detail investigations are required to clarify whether or not YY1 is involved throughout tumor metastasis process.

Extensive evidences show that YY1 inhibition induces the expression and activation of the tumor suppressor p53 (6, 38), whereas on the other hand, a recent study has revealed that p53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis (39), indicating that YY1 inhibition might reduce HIF-1 activity via increasing p53 expression. Interestingly, our results show that even in p53-null HCT116 p53<sup>-/-</sup> cells and Saos-2 cells, YY1 silencing could also significantly suppress the accu-

mulation and activities of HIF-1 $\alpha$  under hypoxia, suggesting that the downregulation of HIF-1 $\alpha$  by YY1 inhibition could occur in a p53-independent manner. Notably, compared with the cell in which p53 is present, the effect of YY1 silencing on HIF-1 $\alpha$  expression in the p53-null cell is slightly weaker, suggesting that there may be both p53-dependent and -independent pathways in the downregulation of HIF-1 $\alpha$  by YY1 inhibition under hypoxia.

Cancer cells consume large quantities of glucose and use glycolysis for ATP production. This metabolic signature (the Warburg effect) enables cancer cells to direct glucose to biosynthesis, supporting their rapid growth and proliferation (40). Recent study has shown that p53 inhibits the pentose phosphate pathway (PPP), which is a main pathway for glucose catabolism and biosynthesis in cancer cells, and loss of p53 function increases glucose consumption, NADPH production, and biosynthesis via PPP, which leads to the enhancement of cancer cell growth and proliferation (41). Furthermore, the loss of p53 function, which occur in more than half of tumor patients, is one of the major reasons for the failure of clinical therapies (42), and recent studies have also shown that the efficacy of a treatment outcome frequently depends on p53 status (43). Our results show that YY1 silencing could suppress the expressions of genes involved in angiogenesis (VEGF and TGF- $\alpha$ ), survival (PGK and CA9), and metabolism (GLUT1) in a p53-independent manner, and subsequently, inhibition of YY1 also suppresses p53-null tumorigenesis *in vivo*.

In tumor biology, YY1 had recently been found to be associated with the tumor suppressor p53 (6, 38), c-Myc (44), EGF receptor (EGFR; ref. 45), p27 (46), VEGF (33), and others (5, 47). Thus, although the whole p53-dependent and -independent mechanisms of YY1 contribution to HIF-1 $\alpha$  stabilization and tumorigenesis remains to be elucidated, our results showing that YY1 is associated with HIF-1 $\alpha$  regulation under hypoxia further elucidated a novel important role of YY1 in tumor biology, and targeting YY1 might be a potential therapeutic strategy that might give consistent clinical outcomes independent of p53 status in cancer therapy. On the other hand, as YY1 is also involved in other essential physiological process, further studies are needed to reveal whether or not YY1 inhibition results in other severe side effects. Alternatively, appropriate drug delivery system (DDS), such as target-specific tissue technology or localized delivery system, might be chosen instead of systemic treatment to avoid severe side effects (48).

In short, our results show a novel mechanism by which YY1 inhibition disrupts HIF-1 $\alpha$  stabilization under hypoxia and prevents tumor growth regardless of p53 status. Altogether, although further studies may be needed to fully elucidate the responsible mechanism, we believe that our study will provide a promising therapeutic strategy for treating cancers with or without p53 mutation.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Development of methodology:** S. Wu, Y. Miura

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Wu, M.R. Kano, S. Tanaka, Y. Miura, X. Liu, A. Matsuhashi

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Wu, V. Kasim, M.R. Kano, S. Ohba, U.-I. Chung, L. Yang, M. Miyagishi

**Writing, review, and/or revision of the manuscript:** S. Wu, V. Kasim, M.R. Kano, S. Ohba, K. Miyata, U.-I. Chung, K. Kataoka, N. Nishiyama, M. Miyagishi

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**Study supervision:** M.R. Kano, U.-I. Chung, K. Kataoka, N. Nishiyama, M. Miyagishi

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