The transcription factor ZFHX3 is crucial for the angiogenic function of hypoxia-inducible factor 1α in liver cancer cells

Changying Fu1,2, Na An1,2, Jinming Liu1, Jun A1,2, Baotong Zhang3, Mingcheng Liu1,2, Zhiqian Zhang2, Liya Fu1, Xinxin Tian2, Dan Wang2, Jin-Tang Dong2,*

1 Department of Genetics and Cell Biology, College of Life Sciences, Nankai University, 94 Weijin Road, Tianjin 300071, China
2 Southern University of Science and Technology, School of Medicine, 1088 Xueyuan Road, Shenzhen, Guangdong 518055, China
3 Winship Cancer Institute, Department of Hematology and Medical Oncology, Emory University School of Medicine, 1365C Clifton Road, Atlanta, Georgia 30322, USA

* To whom correspondence should be addressed: Jin-Tang Dong; Southern University of Science and Technology, School of Medicine, Shenzhen, Guangdong 518055, China; dongjt@sustech.edu.cn; Tel: 86-755-88018032

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ABSTRACT

Angiogenesis is a hallmark of tumorigenesis, and hepatocellular carcinoma (HCC) is hypervascular and therefore very dependent on angiogenesis for tumor development and progression. Findings from previous studies suggest that in HCC cells, hypoxia-induced factor 1 alpha (HIF1A) and zinc finger homeobox 3 (ZFHX3) transcription factors functionally interact in the regulation of genes in HCC cells. Here, we report that hypoxia increases the transcription of the ZFHX3 gene and enhances the binding of HIF1A to the ZFHX3 promoter in the HCC cell lines HepG2 and Huh-7. Moreover, ZFHX3, in turn, physically associated with and was functionally indispensable for HIF1A to exert its angiogenic activity, as indicated by in vitro migration and tube formation assays of human umbilical vein endothelial cells (HUVECs) and microvessel formation in xenograft tumors of HCC cells. Mechanistically, ZFHX3 was required for HIF1A to transcriptionally activate the vascular endothelial growth factor A (VEGF A) gene by binding to its promoter. Functionally, down-regulation of ZFHX3 in HCC cells slowed their tumor growth, and addition of VEGFA to conditioned medium from ZFHX3-silenced HCC cells partially rescued the inhibitory effect of this medium on HUVEC tube formation. In human HCC, ZFHX3 expression was up-regulated, and this up-regulation correlated with both HIF1A up-regulation and worse patient survival, confirming a functional association between ZFHX3 and HIF1A in human HCC. We conclude that ZFHX3 is an angiogenic transcription factor that is integral to the HIF1A–VEGFA signaling axis in HCC cells.

ZFHX3 is frequently mutated in advanced...
ZFHX3 is integral to HIF1A/VEGFA function

prostate cancer (13,14), and deletion of Zfhx3 in mouse prostates induces intraepithelial neoplasia and promotes tumorigenesis induced by the loss of Pten (15), indicating a tumor suppressor activity of ZFHX3 in prostate cancer. In HCC, ZFHX3 is infrequently altered (16), while its mRNA expression has been inconsistently reported in published studies (17,18).

In this study, we examined whether ZFHX3 and HIF1A functionally interact with each other using in vitro and in vivo models of HCC angiogenesis. We found that the expression of ZFHX3 was significantly increased by hypoxia via the binding of HIF1A to ZFHX3’s promoter in HCC cells, and ZFHX3 then became necessary for the angiogenic activity of HIF1A via transcriptional activation of the VEGFA angiogenic effector. ZFHX3 silencing attenuated HCC angiogenesis and inhibited tumor growth in nude mice. In human HCCs, higher levels of ZFHX3 expression correlated with higher HIF1A expression and worse disease-free survival. These findings indicate that ZFHX3 is integral to HIF1A function in HCC angiogenesis.

RESULTS

Hypoxia increases the expression of ZFHX3 at both mRNA and protein levels

To explore whether ZFHX3 is functionally associated with HIF1A, we first determined whether ZFHX3 expression is affected by hypoxia, which induces the accumulation of HIF1A. The HCC cell lines HepG2 and Huh-7 were exposed to hypoxia (1% O2) for different times, and expression of ZFHX3 and HIF1A was analyzed. Consistent with previous studies (19), the HIF1A protein level was elevated after 6 hours of hypoxia treatment, reached peak at 12 hours, and then dropped at 24 hours (Fig. 1A). Interestingly, the ZFHX3 protein level also increased after 6 hours of hypoxia treatment, and continued to increase at both 12 and 24 hours of treatment (Fig. 1A, S1K). At the mRNA level, HIF1A was not increased by hypoxia, as hypoxia stabilizes the HIF1A protein mainly by post-translational modification (20,21). ZFHX3 mRNA levels, however, were increased after 6 and 24 hours of hypoxia treatment (Fig. 1B), which is consistent with changes in ZFHX3 protein level and suggests that hypoxia induces the upregulation of ZFHX3 mRNA. As expected, VEGFA, a canonical downstream effector of HIF1A, was also upregulated at the mRNA level by hypoxia (Fig. 1B).

HCC cell lines HepG2 and Huh-7 were also treated with deferoxamine (DFO), a chemical that has hypoxia-mimetic effects, and the same patterns of expression were detected for ZFHX3, HIF1A and VEGFA in both a time- and dose-dependent manner (Fig. 1C-1F, S1L-S1M). Similar results were also obtained in the BEL-7402 cell line, which was originally reported to originate from a 53-year old male patient with HCC but later was confirmed to be a HeLa derivative (22), and HeLa cells (Fig. S1A-S1G). Therefore, hypoxia increases both the protein and mRNA levels of ZFHX3 in HCC cells.

Upregulation of ZFHX3 by hypoxia depends on HIF1A

Hypoxia induced factor-1A (HIF1A) is the key transcription factor that is stabilized by hypoxia to regulate the expression of hypoxia responsive genes (3). We thus examined whether the upregulation of ZFHX3 by hypoxia involves HIF1A. We silenced HIF1A by transfecting two siRNAs against HIF1A in HCC cell lines HepG2 and Huh-7. Interestingly, the upregulation of ZFHX3 protein and mRNA expression by hypoxia and DFO was dramatically inhibited after HIF1A silencing (Fig. 2A-2D). Considering that HIF2A has significant overlapping functions with HIF1A, we also knocked down HIF2A in HepG2 and Huh-7 cells and analyzed whether HIF2A is involved in ZFHX3 expression. Unlike HIF1A, silencing HIF2A did not prevent the induction of ZFHX3 by hypoxia (Fig. S2A-S2D). In

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addition, when HepG2 cells with HIF1A knockdown were transfected with HIF1A plasmid to restore HIF1A protein level, hypoxia-induced ZFHX3 expression was partly restored (Fig. 2E-2F).

Binding of HIF1A to ZFHX3 promoter is required for hypoxia to induce ZFHX3 transcription

As a transcription factor, HIF1A binds to hypoxia response elements (HREs) in the promoters of hypoxia-responsive genes to induce their transcription (23), and the core HRE sequence is RCGTG (R = A/G) (24). To determine whether ZFHX3 is a direct transcriptional target gene of HIF1A, we first analyzed ZFHX3’s promoter, and found 6 putative HREs (Fig. 3A). We then cloned the ZFHX3 promoter into the pGL3 luciferase reporter plasmid, and analyzed ZFHX3’s promoter activities. As expected, the ZFHX3 promoter displayed significant activity, and the activity was significantly increased by hypoxia (Fig. 3B, WT). Each of the 6 HREs in the ZFHX3 promoter was mutated, and the effect of promoter mutations on luciferase activity was analyzed. Mutation of HRE 2 (H2) did not affect promoter activity at all, suggesting that HRE 2 is not involved in ZFHX3 transactivation (Fig. 3B). Mutations in HREs 1 and 3 (H1 and H3) significantly reduced ZFHX3 promoter activity, but they did not eliminate the promoter’s response to hypoxia (Fig. 3B). Mutations in HREs H4-H6 not only reduced ZFHX3 promoter activities but also eliminated the promoter’s response to hypoxia (Fig. 3B). It is thus likely that, while HREs 1 and 3-6 are all involved in the maintenance of ZFHX3 transcription in HCC cells, only HREs 4-6 are responsible for the effect of hypoxia on ZFHX3 transcription.

ChIP-PCR assay was performed in HCC cell lines HepG2 and Huh-7 cells with HIF1A antibody to evaluate whether HIF1A physically binds to the promoter of ZFHX3 (Fig. 3C, 3D). According to the 6 consensus HREs, 12 pairs of PCR primers were designed to amplify fragments that span different regions of the ZFHX3 promoter. While no binding was detected under normoxia conditions, binding of HIF1A occurred in the proximal region (–367 to –168) of the ZFHX3 promoter under hypoxia, which contained HREs 5 and 6 (Fig. 3A). Binding was detectable for the fragment flanked by P6/7F and P6/7R, which spans HRE 4 (Fig. 3A). No binding to other fragments was detectable. Binding of HIF1A to the promoter of ZFHX3 was also detected by CHIP-PCR in the BEL-7402 cell line, a HeLa derivative (Fig. S3). Collectively, these findings suggest that under hypoxia, HIF1A directly binds to proximal HREs of the ZFHX3 promoter in HCC cells.

ZFHX3 activates the transactivation of VEGFA in coordination with HIF1A under hypoxia

Considering that ZFHX3 is a transcription factor and that VEGFA is the most potent functional effector and a direct transcriptional target gene of HIF1A in hypoxia-induced tumor angiogenesis, it is likely that upregulation of ZFHX3 by hypoxia also plays a role in the transcription of VEGFA. To test this notion, we knocked down ZFHX3 and detected VEGFA expression under hypoxia by real time PCR and ELISA assay in HepG2 cells (Fig. 4A, 4B), and found that ZFHX3 knockdown greatly reduced hypoxia-induced VEGFA expression. Similar effects of ZFHX3 on VEGFA expression were also observed in Huh-7 cells under hypoxia and DFO treatment (Fig. 4C, 4D). To further determine whether ZFHX3 plays a role in VEGFA transcription, we performed a promoter luciferase reporter assay using the HRE-luciferase reporter under different ZFHX3 conditions. As expected, RNAi-mediated ZFHX3 silencing significantly reduced hypoxia-induced HRE promoter activity, while ectopic expression of ZFHX3 partially rescued the effect (Fig. 4E). The functional necessity of ZFHX3 for
ZFHX3 is integral to HIF1A/VEGFA function

VEGFA expression under hypoxia was also confirmed in the HeLa-derived BEL-7402 cells (Fig. S4A, S4B). While ZFHX3 was required for hypoxia to induce VEGFA expression (Fig. 4A, 4B), knockdown of ZFHX3 did not completely eliminate the induction, as detected by real-time PCR (Fig. 4F). On the other hand, knockdown of both ZFHX3 and HIF1A completely eliminated the effect (Fig. 4F).

To further test the effect of ZFHX3 on VEGFA transcription and whether it is related to the effect of HIF1A, we performed ChIP-PCR with ZFHX3 antibody in HepG2 cells under hypoxia, and found that ZFHX3 bound to the promoter of VEGFA (Fig. 4G, left). Interestingly, knockdown of HIF1A decreased the binding of ZFHX3 to VEGFA promoter, as no ZFHX3-bound VEGFA promoter DNA was detectable in the ChIP-PCR assay after HIF1A knockdown (Fig. 4G, right). Conversely, while HIF1A bound to the promoter of VEGFA under hypoxia as expected (Fig. 4H, 4I), knockdown of ZFHX3 decreased (Fig. 4H) while ectopic expression of ZFHX3 increased (Fig. 4I) the amount of HIF1A-bound VEGFA promoter DNA. Therefore, ZFHX3 is also required for hypoxia to upregulate VEGFA and ZFHX3 appears to coordinate VEGFA’s transcriptional activation under hypoxia.

**ZFHX3 physically associates with HIF1A**

The findings that ZFHX3 protein level affected the binding of HIF1A to VEGFA promoter (Fig. 4H, 4I) and that HIF1A is required for ZFHX3 to bind VEGFA promoter (Fig. 4G) suggest that ZFHX3 and HIF1A associate with each other in VEGFA transcription. To test this notion, we performed co-IP and western blotting in HepG2 cells treated with hypoxia to detect the association between the two. HIF1A dimerizes with HIF1B to drive gene transcription, so both HIF1A and HIF1B were analyzed. Interestingly, both ZFHX3 and HIF1B were detected in the HIF1A protein precipitates (Fig. 5A, left), HIF1A and ZFHX3 detected in the HIF1B protein precipitates (Fig. 5A, middle), and HIF1A and HIF1B detected in the ZFHX3 protein precipitates (Fig. 5A, right), indicating an interaction between ZFHX3 and the HIF1A/HIF1B complex under hypoxia.

We also expressed six HA-tagged overlapping fragments of ZFHX3 (Fig. 5B) and FLAG-tagged HIF1A (FLAG-HIF1A) in 293T cells and performed IP and IB with FLAG antibody. Fragments A, C and E of the 6 interacted with HIF1A (Fig. 5C). Different fragments of ZFHX3 may have different cellular localizations, which could affect their interactions with HIF1A, so we separated the nucleus and the cytosol from hypoxia-treated cells and performed IP and IB. While all fragments were primarily located in the nucleus, fragments A, B, C and F were also detectable at varying levels in the cytoplasm while fragments D and E were not (Fig. 5D). Again, HIF1A was detected in the precipitates of fragments A, C and E from the nucleus but not in those of B, D and E (Fig. 5D). A weak but detectable signal was also present in the cytoplasmic fraction for C and E (Fig. 5D). Therefore, ZFHX3 and HIF1A physically interact with each other in the nucleus involving multiple regions in fragments A, C and E of ZFHX3.

**ZFHX3 is crucial for hypoxia to promote tube formation and migration of endothelial cells via VEGFA**

Hypoxia promotes angiogenesis by activating multiple pro-angiogenic pathways, particularly the HIF1A pathway, and VEGFA is an essential functional mediator of HIF1A. Based on the findings that ZFHX3 was necessary for VEGFA transcription (Fig. 4), ZFHX3 and HIF1A coordinated with each other in VEGFA transcription, and ZFHX3 was upregulated by hypoxia via HIF1A, it is reasonable to propose a functional significance of ZFHX3 in angiogenesis. In testing this idea, we performed tube formation and migration assays using human umbilical vein endothelial cells (HUVECs), which
ZFHX3 is integral to HIF1A/VEGFA function

are in vitro indicators of hypoxia-induced angiogenesis. Conditioned media (CM) from HepG2 cells with or without ZFHX3 knockdown and hypoxia treatment were used to treat HUVECs. While CM of hypoxia-treated tumor cells significantly increased the total length of tubes and the number of tube nodes as expected, knockdown of ZFHX3 almost eliminated the increases (Fig. 6A-6C), indicating a crucial role of ZFHX3 in HUVECs tube formation. In a transwell assay, CM from hypoxia-treated HepG2 and Huh-7 cells promoted the migration of HUVECs as expected, but again hypoxia’s promoting effect was eliminated by ZFHX3 knockdown (Fig. 6D-6F). Collectively, these findings indicate that attenuation of ZFHX3 upregulation in HCC cells prevents hypoxia from promoting tube formation and migration of HUVECs, in vitro indicators of angiogenesis.

VEGFA was transcriptionally upregulated by ZFHX3 and HIF1A under hypoxia, and ZFHX3 silencing downregulated VEGFA, which suggests that downregulation of VEGFA by ZFHX3 silencing has functional significance. We thus added VEGFA to the CM from cells with ZFHX3 knockdown rescued tube formation of HUVECs (Fig. 6G-6I). Therefore, ZFHX3 plays a crucial role in hypoxia-induced angiogenesis. Given that both HIF1A and ZFHX3 play important roles in hypoxia-induced angiogenesis, we also performed migration assays with CM from HepG2 and Huh-7 cells. The results show that ZFHX3 or HIF1A was sufficient to prevent the promotion of endothelial cell migration by hypoxia, and simultaneous knockdown of both ZFHX3 and HIF1A did not show an additive effect (Fig. S5).

ZFHX3 promotes xenograft tumor growth likely due to its angiogenic activity

To test whether the necessity of ZFHX3 for hypoxia-HIF1A-VEGFA signaling to promote angiogenic activity affects tumor growth, we knocked down ZFHX3 using lentiviruses expressing ZFHX3 shRNAs or knocked out ZFHX3 using the CRISPR/Cas9 system in HepG2 cells, and injected cells into nude mice subcutaneously for tumor growth analysis. Both knockdown and knockout of ZFHX3 significantly reduced tumor growth, as indicated by tumor images and weights at excision and tumor volume-based growth curve (Fig. 7A-7G). The inhibitory effect of knockout was more potent than that of knockdown as expected. Deletion of ZFHX3 in isolated clones of HepG2 cells was confirmed by Western blotting (Fig. 7H) and sequencing (Fig. 7I). Tumor tissue sections were immunohistochemically stained with anti-CD31 to detect microvessels and with VEGFA antibody to detect its expression (Fig. 7J, 7K). Knockout or knockdown of ZFHX3 significantly decreased the number of microvessels in xenograft tumors, as indicated by IHC staining of CD31 to detect microvessels and with VEGFA antibody to detect its expression (Fig. 7J, 7K). Knockout or knockdown of ZFHX3 also reduced VEGFA protein expression (Fig. 7J, 7K, 7N, 7O), which is consistent with in vitro findings. IHC staining also detected the hypoxia marker CA9 in foci within xenograft tumors (Fig. S6), a typical pattern of CA9 expression in hypoxic areas. ZFHX3 thus has a promoting effect on both tumor growth and angiogenesis of HCC cells.

Upregulation of ZFHX3 and its correlation with both HIF1A upregulation and worse patient survival in HCC

To determine the clinical relevance of ZFHX3-promoted angiogenesis in HCC, we collected HCC samples in the GEO and TCGA databases that had genome-wide expression data, and analyzed ZFHX3 expression. The ZFHX3 mRNA level was clearly higher in HCC samples than in normal liver tissues based on the GEO and RNA-Seq data (Fig. 8A). We then tested whether ZFHX3 expression correlates with HIF1A expression in HCC specimens. Spearman correlation analysis demonstrated that
expression levels of ZFHX3 and HIF1A positively correlated with each other in the TCGA database (Fig. 8B).

In the TCGA database, some HCC samples have both patient survival data and ZFHX3 expression information. Kaplan-Meier analysis of such cases demonstrated that patients with higher ZFHX3 expression levels had poorer disease-free survival (Fig. 8C), further implicating higher ZFHX3 expression in HCC development.

DISCUSSION

ZFHX3 is an angiogenic transcription factor in HCC cells

Angiogenesis is crucial for the adaptation of tumor cells to hypoxic stress by providing oxygen and nutrients for the growth and progression of tumors. Angiogenesis occurs at a high level in HCC because the median O$_2$ partial pressure in human HCC (6 mm Hg) is about one fifth of that in normal liver tissue (30 mm Hg) (25), and thus HCC has greater vascularization and is more dependent on angiogenesis for growth (5). Angiogenesis is regulated by several signaling pathways via key transcription factors and downstream effectors. HIF1A is the most potent known transcription factor, and VEGFA is a cytokine that is induced by HIF1A to mediate HIF1A’s function. HIF1A and VEGFA thus form a signaling axis that promotes tumor angiogenesis in various types of cancers including HCC. In addition to hypoxia, various oncogenic signaling pathways promote tumor angiogenesis by activating the HIF1A/VEGF axis (26). For example, the SUMO E3 ligase Cbx4 enhances HIF1A sumoylation, which in turn increases its transcriptional activity and VEGF expression and subsequent angiogenesis and tumor growth in HCC (27). Activation of the PI3K or MAP kinase signaling also upregulates VEGF (28). Accordingly, targeting the HIF1A/VEGF axis has become a meaningful approach for the treatment of HCC (29,30).

Our findings in this study establish the ZFHX3 transcription factor as a novel angiogenic factor in HCC. This conclusion is supported by multiple lines of evidence, including tube formation and migration assays of human umbilical vein endothelial cells (HUVECs) (Fig. 6, S5) and the essential role of ZFHX3 in transcriptional induction of the VEGFA gene (Fig. 4, S4), which encodes a heparin-binding protein that induces proliferation and migration of vascular endothelial cells in both physiological and pathological angiogenesis (31). In addition, silencing ZFHX3 reduced tumor vascularization in the HepG2 xenograft model of HCC (Fig. 7J-7M). These functional studies indicate that, like HIF1A, ZFHX3 is also essential for hypoxia to induce angiogenesis in HCC cells. Nevertheless, while ZFHX3 clearly plays a role in angiogenesis, there is also a possibility that other mechanisms are also involved in ZFHX3-promoted tumor growth.

ZFHX3 is both a transcriptional target and functional partner of HIF1A in HCC cells

HIF1A is the master regulatory transcription factor under hypoxia. It is composed of two subunits: HIF1A and HIF1B. While HIF1B is constitutively expressed, HIF1A is maintained at a low protein level by the ubiquitin proteasome pathway under normoxia; only under hypoxia is HIF1A stabilized and translocated into the nucleus to promote angiogenesis (32). While a large number of genes are regulated by HIF1A, only some are functional effectors of HIF1A. ZFHX3 is clearly a functional effector of HIF1A in HCC, as ZFHX3 is a transcriptional target gene of HIF1A (Fig. 3, S3), and even in HCC specimens, ZFHX3 upregulation significantly correlated with HIF1A upregulation (Fig. 8). More importantly, upregulated ZFHX3 is necessary for hypoxia to induce the expression of VEGFA (Fig. 4, S4), a cytokine essential for tumor angiogenesis, as knockdown of ZFHX3
ZFHX3 is integral to HIF1A/VEGFA function
downregulated VEGFA (Fig. 4, S4) via direct binding to the promoter of VEGFA (Fig. 4, S4). In addition, the angiogenic activity of ZFHX3, as indicated by tube formation and migration of HUVECs, clearly involved VEGFA (Fig. 6, S5). Furthermore, ZFHX3 and HIF1A coordinated to induce VEGFA transcription, as the binding of ZFHX3 to VEGFA promoter depended on HIF1A (Fig. 4, S4), and protein levels of ZFHX3 also affected the binding of HIF1A to the VEGFA promoter (Fig. 4, S4). Interestingly, ZFHX3 and HIF1A proteins indeed associated with each other in hypoxia-treated HCC cells (Fig. 5), and the interaction involved more than one region of ZFHX3 protein (Fig. 5).

Therefore, while induced by hypoxia via HIF1A, ZFHX3 also functions as part of the HIF1A/VEGFA signaling axis in hypoxia-induced angiogenesis in HCC. Nevertheless, several important questions remain to be addressed regarding how ZFHX3 functions in the context of HIF1A/VEGFA signaling. For example, ZFHX3 is quite a large transcription factor with 23 zinc fingers, while HIF-1 functions in a heterodimer. Although certain cofactors of HIF1A have been identified to have a role in the binding of HIF1A to its target gene promoters, including the Tat interactive protein Tip60 (33), whether ZFHX3 is a cofactor of HIF1A and how these two very different transcription factors coordinate to induce VEGFA transcription are interesting but unanswered questions. Furthermore, the SUMOylation status of HIF1A is important for its stability (34), and ZFHX3 SUMOylation could also modulate its function (35). Whether SUMOylation is involved in the ZFHX3-HIF1A interaction is also an interesting but unanswered question. Finally, whether genes other than VEGFA are transcriptionally regulated by ZFHX3 and HIF1A remains to be identified.

The ZFHX3-HIF1A interaction has clinical implications in human HCC
The association between HIF1A and ZFHX3 also appeared to occur in human HCC, implicating ZFHX3 in HCC progression. HIF1A and VEGFA are overexpressed in HCC, and the HIF1A/VEGFA axis clearly plays an important role in the development and progression of human HCC (30). ZFHX3 not only promoted angiogenesis and tumor growth of HCC cells in a xenograft model (Fig. 6, 7, S5), it was also upregulated in human HCC (Fig. 8) and its upregulation correlated with HIF1A upregulation as well as worse HCC patient survival (Fig. 8). It is thus likely that ZFHX3 also plays a role in human HCC via its function as part of the HIF1A/VEGFA axis. The role of ZFHX3 in HCC development and progression could have clinical implications. For example, as a therapeutic approach, inhibition of HIF1A activity can be achieved by selectively cutting off its functional dependence on its coactivator (33), and the HIF1A-ZFHX3 interaction could provide a similar opportunity for targeted therapy of HCC. In addition, reagents targeting ZFHX3 could be developed to constrain tumor angiogenesis and treat other hypoxia-related diseases.

Roles of ZFHX3 in tumorigenesis is tissue type dependent
Interestingly, the role of ZFHX3 in human tumorigenesis appears to be tissue dependent. As stated in the introduction section, ZFHX3 clearly plays a tumor suppressor role in prostate cancer because its inactivating deletions/mutations not only frequently occur in advanced human prostate cancers (13,14) but also cause neoplastic lesions in mouse prostates (15). The findings in this study, on the other hand, indicate an oncogenic role of ZFHX3 in HCC (Fig. 1-8, S1-S6), even though genetic alterations of ZFHX3 are infrequent in HCC. A gene can clearly have opposing functions in tumor cells. For example, while the wild-type KLF5
transcription factor slows cell proliferation and tumorigenesis, its deacetylated mutant has opposing functions (36,37). Our unpublished study suggests that ZFHX3 also plays an oncogenic role in breast cancer. Molecularly, whereas interaction with HIF1A to upregulate VEGFA was established in this study as a mechanism by which ZFHX3 promotes HCC tumor growth, interaction of ZFHX3 with ERβ to repress MYC and regulate other genes is an important mechanism underlying ZFHX3’s tumor suppressor function in prostate cancer (38). Both mechanisms depend on ZFHX3’s transcription factor function, yet the outcomes are opposing. Therefore, understanding the molecular mechanisms through which interactions of ZFHX3 with different transcription factors lead to different functions in different types of cancers is important not only to the field of gene regulation but also to the field of cancer biology. ZFHX3 provides a unique opportunity for addressing this question.

In this study, we examined whether and how ZFHX3 plays a role in the angiogenesis of human HCC cells. We found that ZFHX3 was dramatically upregulated by hypoxia in HCC, and the upregulation depended on the binding of HIF1A to the promoter of ZFHX3. Functionally, ZFHX3 was necessary for hypoxia to promote angiogenesis and tumor growth, and ZFHX3 exerted such functions by coordinating with HIF1A to induce the transcription of VEGFA in HCC cells. In human HCC, ZFHX3 was upregulated, and the upregulation correlated with HIF1A upregulation and worse patient survival. ZFHX3 is thus a newly identified angiogenic factor that could lead to novel therapeutic opportunities for the treatment of HCC.

EXPERIMENTAL PROCEDURES

Cell lines, plasmids and transfection

HCC cell lines HepG2 and Huh-7, along with the BEL-7402/HeLa cell line and human umbilical vein endothelial cells (HUVECs) were purchased from the BeNa Culture Collection (BNCC, Beijing, China). Human embryonic kidney 293T cells were purchased from ATCC (Manassas, VA). These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA) in a humidified incubator (37°C and 5% CO2). They were authenticated using short tandem repeats (STRs) DNA profiling.

Mammalian expression plasmid for HIF1A pFLAG-CMV-HIF1A and that for hypoxia response element (HRE) luciferase reporter pGL3-HRE-luciferase were kindly provided by Dr. Yushan Zhu of Nankai University (39). Expression plasmid for HA-tagged ZFHX3 pKUXa1-HA-ZFHX3 and those for the 6 fragments with HA tag, i.e., pcDNA3-HA-ZFHX3-A, -B, -C, -D, -E and -F, were constructed in our previous study (40). The pZFHX3-Luc promoter luciferase reporter plasmid for ZFHX3, which was constructed and name as pATBF1-Luc1 in another previous study (41), was used as the template to generate mutants (CGTG to TACA) for the 6 HREs in ZFHX3 promoter using PCR-based cloning. These mutants were named pGL3-ZFHX3-Luc-mHRE1 to pGL3-ZFHX3-Luc-mHRE6, and primer sequences for PCR are shown in Table 1.

Plasmids were transfected into cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Small interfering RNAs (siRNAs) were synthesized by RiboBio (Guangzhou, China) and transfected into cells using the Lipofectamine RNAiMax reagent (Invitrogen). Sequences of siRNAs used in this study are listed in Table 2. Hypoxic conditions were achieved by culturing cells in a hypoxia chamber (Billups Rothenberg, Del Mar, CA) with a mixed gas of 1% O2, 5% CO2, and 94% N2. Chemical deferoxamine mesylate (DFO) (Cat# ab120727, Abcam, Cambridge, MA) was also used to treat cells to mimic hypoxic conditions. All cell lines were authenticated by the short tandem repeats DNA profiling.
Antibodies and Western blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors (Roche, Indianapolis, IN). Equal amounts of cellular protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. After incubation with 5% nonfat milk in TBST buffer (25 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hour at room temperature, the membrane was probed with primary antibodies overnight, and washed three times with TBST (each for 10 min). The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (Cat# 7074S, Cell Signaling Technology, Danvers, MA) or anti-mouse (Cat# 7076S, Cell Signaling Technology) antibodies at 1:5000 dilution for 2 hours. The blots were washed three times with TBST and incubated in Western Bright ECL substrate (Advansta, Menlo Park, CA), and images were captured using the Image Quant LAS 4000 system (GE Healthcare Biosciences, Pittsburgh, PA). For the detection of ZFHX3 protein, 4% SDS-PAGE and the spectra multicolor high range protein marker (Cat# 26625; Thermos Fisher, Santa Clara, CA) were used. For other proteins, 8–12% SDS-PAGE and the PageRuler Plus Prestained protein marker (Cat# 26616; Thermos Fisher, Santa Clara, CA) were used. The following antibodies were used in this study: anti-HIF1A (Cat# NB100-479, 1:2000 dilution, Novus Biologicals, Littleton, CO), anti-HIF1B (Cat# 5537, 1:1000 dilution, Cell Signaling Technology, Danvers, MA), β-actin (Cat# A1978200UL, 1:10000 dilution, Sigma, St Louis, MO), and ZFHX3 (homemade, 1:800 dilution).

Immunoprecipitation

RNA isolation and real-time PCR

Total RNA was extracted from cultured cells with the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The first-strand cDNA was synthesized with oligo dT and random primers using the Moloney murine leukemia virus reverse transcriptase system (Promega, Madison, WI). The mRNA levels of indicated genes were quantified by qRT-PCR using the SYBR Green MasterMix reagent (Takara, Tokyo, Japan) on the Mastercycler ep Realplex thermal cycler system (Eppendorf, Shanghai, China). Expression levels were calculated using the 2−ΔΔCt method and normalized to that of β-actin. Primer sequences used for qRT-PCR are shown in Table 3.

Chromatin immunoprecipitation (ChIP) assay

The SimpleChIP® Enzymatic Chromatin IP Kit (Cat# 9003S, Cell Signaling Technology) was used for ChIP according to the manufacturer’s protocol. Briefly, cultured cells were treated with 1% formaldehyde at room temperature for 10 min, 10 x glycine was added to quench crosslinking, and cells were then washed, harvested, and lysed in the kit’s lysis buffer. Cell lysates were digested with micrococcal nuclease, sonicated, and centrifuged to remove debris. ChIP was performed with the anti-
HIF1A antibody (Cat# NB100-479, Novus Biologicals, Littleton, CO), anti-ZFHX3 antibody, and IgG (negative control) overnight, and protein A-agarose beads were added. After 2 hours incubation, beads were washed sequentially with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer. The eluted immunocomplex was incubated with 5 M NaCl with proteinase K at 65°C for 2 hours, and DNA was purified and used as a template for PCR. Primer sequences for VEGF promoter (from −1216 to −883) are 5’-CACAGACCTTCACAGCCATC-3’ and 5’-CCCAGCGTAGACAGTTGAGT-3’; and those for ZFHX3 promoter are listed in Table 4.

**Enzyme-linked immunosorbent assay (ELISA)**
For the detection of secretory factors, conditioned culture media were collected from 100% confluent cells in 10-cm dishes, and a commercial ELISA kit against VEGF (Cat# DVE00, R & D, Minneapolis, MN) was used to determine the expression level of VEGF following the manufacturer’s instruction.

**Luciferase reporter assay**
ZFHX3 and HRE promoter activities were measured by the promoter luciferase assays using HepG2 cells, in which wildtype pZFHX3-Luc, mutants of pZFHX3-Luc with each of the HREs mutated, and the pGL3-HRE-luciferase reporter plasmid were transfected in combination with the pRT-TK Renilla luciferase plasmid (Promega) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 hours transfection, cells were incubated in normoxic or hypoxic conditions for 12 hours, and then collected. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System Kit (Promega) according to the manufacturer’s protocol. Each treatment was performed in triplicate and mean ± standard deviation (S.D.) was calculated for each group.

**Transwell assay**
For the migration assay, transwell inserts (pore size: 8 μm, BD Biosciences, San Jose, CA) in 24-well plates were used, with 1 × 10^5 HUVECs seeded into each upper chamber. HUVECs were treated with conditioned medium from normoxic or hypoxic conditions. After 24 hours, the migrated cells were fixed with 4% paraformaldehyde, cells on the upper surface of the membrane were scraped with a cotton swab, cells on the lower side were stained with 0.1% crystal violet (Sigma) for 0.5 hours and eluted in 250 μl of 10% acetic acid for 10 min, and the absorbance was measured at 570 nm. An equal number of seeded cells were also stained and measured for absorbance, and the reading was used to divide that of migrated cells. Each treatment was performed in triplicate, and each experiment was performed three times.

**Tube formation assay in HUVECs**
HCC cells were cultured in 10-cm dishes to 90% confluence after 48 hours, washed with PBS 3 times, and cultured in 4 ml of medium with 1% serum under normal or hypoxic conditions for another 24 hours to reach 100% density. Conditioned media were collected after centrifugation at 1500 rpm for 5 min. A total of 5 × 10^4 HUVECs were seeded into each well of a 24-well plate coated with growth-factor reduced Matrigel (BD Biosciences), and cultured for 6 hours in conditioned medium. Images were captured using microscopy, and analyzed for the extent of tube formation by measuring the tube length and counting the number of tube nodes using the ImageJ software. At least 10 fields were examined for each group.

**Deletion of ZFHX3 in HepG2 cells**
The CRISPR-cas9 system was used to introduce a deletion in ZFHX3 following the protocol from the Feng Zhang laboratory (42).
Briefly, sgRNA-encoding oligos for the ZFHX3 genome, 5'-CACCGGGCAGATCTTCACCATCCGC-3' (forward) and 5'-AAACGCGGATGGTGAAGATCTGCCC-3' (reverse), were synthesized and annealed; and annealed DNA was digested with BsmBI and cloned into the CRISPR-cas9 lentiviral vector, which was kindly provided by Dr. Yushan Zhu of Nankai University. For the preparation of lentiviral particles, 293T cells in 6-cm dishes were transfected with 1 µg CRISPR-cas9 lentivirus-ZFHX3 plasmid, 750 ng psPAX2 packaging plasmid, and 250 ng pMD2.G envelope plasmid using the FuGENE 6 transfection reagent (Promega). HepG2 cells were seeded onto 6-well plates, grown to approximately 80% confluency, infected with lentiviral supernatant containing 8 µg/mL of polybrene, and selected in the medium containing puromycin (2 µg/ml) for 96 hours. Single clones were then isolated and deletion of ZFHX3 was confirmed by Western blotting and DNA sequencing after PCR amplification with primers 5'-TTTCCAGCCAGTAGCCCTTTGCA-3' (forward) and 5'-GTTGGTGTAGTAGTCACAGGCGTTG-3' (reverse).

Preparation of lentiviruses expressing ZFHX3 shRNAs

Two shRNAs for ZFHX3, which were validated in a previous study (43) with the following pairs of oligonucleotides: 5'-CCGCGGTAGCTCTTGAAGTCTCTGGAGATCAGTCTAAGAGCAAGACG-3' (forward) and 5'-GGTGGTGTAGTAGTCACAGGCGTTG-3' (reverse).

Tumorigenesis assay

BABL/c nude mice aged 4-5 weeks were used for the tumorigenesis assay. For each mouse, a total of 2 × 10⁶ HepG2 cells (control shRNA or ZFHX3 shRNAs) and a total of 5 × 10⁶ HepG2 cells (wild type or ZFHX3 knock out) were injected subcutaneously on both sides. Four mice were successfully injected for each group. Tumor volumes were measured twice a week for 4 weeks, and the size of a tumor was calculated using the following formula: tumor volume (cm³) = (length × width²)/2. At the end of the experiment, mice were euthanized, and tumors were surgically dissected and weighed. Use of mice was approved by the Institutional Animal Care and Use Committee at Nankai University, and all mice were maintained by facility technicians at the Center for Experimental Animal facility.

Immunohistochemistry

Tissue sections were rehydrated and boiled in a pressure cooker for 3 min in a citrate buffer (10 mM of sodium citrate, pH 6.0) for antigen retrieval, treated with 0.3% H₂O₂ for 20 min to block endogenous peroxidase activity, and incubated with 5% normal goat serum to block nonspecific antibody binding, incubated with primary antibodies at 4°C overnight and then with the EnVision Polymer-HRP secondary antibodies (Dako, Glostrup, Denmark) at room temperature for 1 hour, visualized with a DAB Substrate Kit (Maixin-Bio, Fuzhou, Fujian, China), stained with hematoxylin, and dehydrated and mounted. The following antibodies were used for immunohistochemistry: anti-CD31 (Cat# ab28364,
ZFHX3 is integral to HIF1A/VEGFA function

1:500 dilution, Abcam), anti-VEGFA (Cat# ab46154, 1:2000 dilution, Abcam), and anti-CA9 (Cat# ab15086, 1:1000 dilution, Abcam).

Statistical and bioinformatic analyses

All experiments except that for tumorigenesis were performed three times unless stated otherwise. Each treatment in an experiment was in triplicate. For all real-time qPCR, each biological sample was analyzed in triplicate. For quantification of Western blotting results, we used ImageJ software to measure the relative intensity of each band, and relative protein levels were normalized to that of the loading control. Data are presented as mean ± SD unless otherwise indicated. Details of statistics are provided in each figure legend.

Groups of means among different treatments were compared using the Student’s t-tests (2-tailed, unpaired), except that one-way analysis of variance was used for tumor growth curves (Fig. 7D, 7G). The GraphPad Prism (Version 5.0, San Diego, CA) was used for analyses. P value smaller than 0.05 was considered statistically significant.

Three independent liver hepatocellular carcinoma (LIHC) gene expression profiles (GSE14323, GSE14520 and GSE36376) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) to investigate the mRNA expression distribution of ZFHX3 in hepatocellular carcinoma (LIHC). Furthermore, the Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn) website, which is an online analysis tool based on the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from TCGA and the GTEx projects was used to validate the mRNA expression of ZFHX3 between LIHC and adjacent normal tissues. Student’s t-tests were utilized for the comparison of two sample groups. Differences were considered as statistically significant when P < 0.05.

Spearman correlation test from the GraphPad Prism was performed to determine the correlation between ZFHX3 and HIF1A mRNA levels in the LIHC of TCGA. The 369 patients’ samples from TCGA, in which both gene expression data and patient survival data are available, were used to test whether ZFHX3 expression levels correlate with disease-free survival using the Kaplan–Meier method and the log-rank test from the GEPIA.

Data availability: All the data are contained within the article.
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CONFLICT OF INTEREST

All authors declare no conflict of interest to this work.
ZFHX3 is integral to HIF1A/VEGFA function

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ZFHX3 is integral to HIF1A/VEGFA function

FOOTNOTES
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ABBREVIATIONS
HIF1A: Hypoxia-inducible factor-1 alpha; HIF1B: Hypoxia-inducible factor-1 beta; HCC: hepatocellular carcinoma; DFO: Deferoxamine mesylate
ZFHX3 is integral to HIF1A/VEGFA function

Table 1. Primer sequences for cloning HRE mutants of pGL3-ZFHX3-Luc.

| Primer name | Forward primer (5'-3')/reverse primer (5'-3') |
|-------------|------------------------------------------------|
| mHRE1       | ACCCGGCCCCTACACTCTCACTCGTGACT/CACTGAGAGTGAGCTAGGGGCCGGGTGGGGG |
| mHRE2       | CGTGCTCTCATACTGACTGAATTGGGCT/AATTCACTGCTATGAGAGCAGCGGGGCG |
| mHRE3       | CTGGGCTGTGATACAGCCGCCCAGGGGA/GGCGGCCTCTGTATACTACAGCCAGCAGCCAC |
| mHRE4       | TGGGAAAGGGGTGTAATCTTTGTATCTCT/TTAAGAGATTACACCATATAGGAGGAG |
| mHRE5       | CCCGTCGCTCTATGACTGACTGAATTGGG/TTGCTACGGTACAGAGCAGCAAGGCTGGGAGG |
| mHRE6       | CTGCAGCGGCTGTAAGTGGGTATCAGAGG/CTATGACGGTACACCGGGCGAGGAGC |

Table 2. List of sequences for siRNAs against different genes.

| Gene name | siRNA sequences (5'-3') |
|-----------|------------------------|
| HIF1A-1   | GGACACAGAUUUAGACUUG    |
| HIF1A-2   | GAUGGAAGCACUAGACAAA   |
| HIF2A     | GCAAAUGUACCCCAUGUUA   |
| ZFHX3     | AGAAUAUCCUGCUAGUAC    |

Table 3. Primer sequences for real-time q-PCR analyses of different genes.

| Gene name | Forward primer (5'-3')/reverse primer (5'-3') |
|-----------|------------------------------------------------|
| ZFHX3     | TGTTCCAGAATCGAGATGGGAT/CTTCCCCAGATCCTGTAGTTT |
| VEGFA     | GGCTGGCAATACGAGATGGA/CCCCACATCTATACACCTCC |
| HIF1A     | CACCACAGGACAGATGGA/CTTCTGAGATATAGCAATCCACCTCA |
| ACTIN     | ATGGGCAATGAGCGGT/CGTGTCTGATGAGTCCACCCAC |

19
ZFHX3 is integral to HIF1A/VEGFA function

| Primer name | Forward primer (5’-3’)/reverse primer (5’-3’)                              |
|-------------|----------------------------------------------------------------------------|
| P1          | AAACCGCTGTACTGTGA/ATTCTACCGAGCCAACC                                      |
| P2          | CAGCCAGCTCGTGTTAG/CTCGCAGTTCTCCATACCC                                    |
| P3          | TAGTCCCTCATTTCCTATAATCTGCAACTTCCCAAAG                                   |
| P4          | CACCTGTTCCTGGCCTAGTCAG/GGGAATCATGTCCGATTA                                |
| P5          | ATACTGCTTTCCCTGCT/ATCGAATCCCACCTCA                                      |
| P6          | GGGAGATAGAAGGCGGCC/ATAGCAAATCGTGCCC                                     |
| P7          | CCGCTTTAAATCTTTACC/TTGAGGCCAGAGAAAG                                     |
| P8          | AAAGCAGTTAAATAGGATGGG/ATCGGGCGAGAAGAAGG                                  |
| P9          | AAAGCAGTTAAATAGGATGGG/ATCGGGCGAGAAGAAG                                  |
| P10         | CACATTGGCTCTCTTGCC/CTCGCTCATCAAAGGTCA                                   |
| P11         | ACCTTTGATGAGCGAGGGGTCA/CTCGCCTCGCTCGCGTTG                               |
| P12         | CTTCCGCTTTTGCTGTG/TCACCACGGGGCGGC                                       |
ZFHX3 is integral to HIF1A/VEGFA function

Figure 1. Hypoxia upregulates ZFHX3 expression in HCC cells. (A, B) HCC cell lines HepG2 and Huh-7 were cultured under hypoxia for the indicated times, and the expression of ZFHX3 and two regulators of
ZFHX3 is integral to HIF1A/VEGFA function

hypoxia-induced angiogenesis, HIF1A and VEGFA, detected by western blotting and real-time PCR for protein (A) and mRNA (B) respectively. ZFHX3 band intensities were quantified and normalized to β-actin, and the results are shown below the ZFHX3 bands in panel A. (C-F) HepG2 and Huh-7 cells were treated with hypoxia-mimetic agent deferoxamine mesylate (DFO) for the indicated times at 50 μM (C, D) or the indicated concentrations for 24 hours (E, F), and expression of the same set of molecules was analyzed as in panels A and B. Data are shown in mean ± SD. Band intensity ratios under each lane of western blots in panels A, C and E were the average from 3 independent experiments, and their scatter plots and statistical details are shown in Figure S1 (S1K-S1M). *, P<0.05. The statistical analysis of real-time PCR was based on three independent experiments (i.e., n=3), and the value for each group in an experiment was the average of triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
Figure 2. HIF1A mediates hypoxia-induced ZFHX3 transcription in HCC cells. (A-D) Knockdown of HIF1A by RNAi in HepG2 (A, C) and Huh-7 (B, D) cells indicates that HIF1A is responsible for hypoxia- (A, B) or DFO- (C, D) induced ZFHX3 upregulation, as measured for the expression of both protein (A-D, upper) and mRNA (A-D, lower) by western blotting and real-time PCR respectively. siHIF1A #1 and siHIF1A #2, siRNAs against HIF1A. (E, F) Ectopic expression of HIF1A by plasmid transfection in HepG2 cells with HIF1A silencing partially restored hypoxia-induced ZFHX3 expression, as measured by western blotting (E) and real-
ZFHX3 is integral to HIF1A/VEGFA function

time PCR (F) in HepG2 cells. Data are shown in mean ± SD. The statistical analysis for real-time PCR was based on three independent experiments (i.e., n=3), and the value for each group in an experiment was the average of triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

Figure 3. HIF1A binds to ZFHX3 promoter to mediate its induction by hypoxia. (A) Schematic of the ZFHX3 promoter from nucleotide −2954 to +597 relative to the transcription initiation site. Locations of 6 consensus HREs of pGL3-ZFHX3-Luc promoter are shown, which have the following sequences: H1, CCCCCGTGC; H2, TCACGTTG; H3, TGACGTTG; H4, CCCCCGTGCT; H5, AGAGTGCA; and H6, CCCCCGTGCT. Location of PCR primers for CHIP-PCR are indicated by arrows (P1F to P9R). (B) Mutation of the 4th, 5th or 6th hypoxia response element (HRE) (mH4, mH5 and mH6, respectively) in the ZFHX3 promoter abolished its transactivation activity induced by hypoxia, as indicated by the promoter-luciferase reporter assay in HepG2 cells. (C-D) HIF1A bound to the ZFHX3 promoter in HCC cells under hypoxia, as detected by ChIP-PCR in different cell lines (HepG2, C; Huh-7, D). EPO and SLC2A1, two known transcriptional targets of HIF1A, were used as positive controls. Data are shown in mean ± SD. The statistical analysis for luciferase assay was based on three independent experiments (n=3), and that for real-time PCR was based on four independent experiments (n=4). The value for each group in an experiment was the average of triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
Figure 4. ZFHX3 is required for transactivation of the hypoxia-responsive VEGFA in HepG2 cells. (A-B) Knockdown of ZFHX3 reduced hypoxia-induced VEGFA expression, as analyzed by both real-time PCR (A) and ELISA assay (B), and experiments were performed in duplicate for each group. (C-D) Similarly, in Huh-7 cells, knockdown of ZFHX3 reduced hypoxia- (C) or DFO- (D) induced VEGFA expression, as analyzed by real-time PCR. (E) Hypoxia-induced HRE promoter luciferase activity was reduced by the knockdown of ZFHX3 while increased by ectopic expression of ZFHX3 in HepG2 cells. (F) ZFHX3 was required for hypoxia to induce VEGFA expression in HepG2 cells under hypoxia, as measured by real-time PCR. (G) Knockdown of HIF1A dramatically reduced the binding of ZFHX3 to VEGFA promoter in HepG2 cells under hypoxia. Western blotting (lower panel) confirmed the knockdown effect. (H, I) Knockdown of ZFHX3 dramatically reduced, while ectopic expression of ZFHX3 increased, the binding of HIF1A to the promoter of VEGFA in HepG2 cells under hypoxia. IgG was used as the isotype control. Western blotting (lower panel) confirmed the knockdown effect. Data are shown in mean ± SD. The statistical analysis for both luciferase assay and real-time PCR was based on three independent experiments (n=3), and the value for each group in an experiment was the average of triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
**Figure 5. ZFHX3 physically interacts with the HIF1A complex.** (A) Detection of protein association between HIF1A, ZFHX3 and HIF1B in HepG2 cells by co-IP with HIF1A (left), HIF1B (middle), or ZFHX3 antibody.
ZFHX3 is integral to HIF1A/VEGFA function (right) and western blotting with indicated antibodies. Input (1/20 of whole cell lysate) indicates cell lysate not subjected to IP. (B) Schematic of full-length ZFHX3 (3703 residues, horizontal bar) with 4 homeodomains (black rectangle) and 23 zinc fingers (blue rectangle). The six overlapping fragments of ZFHX3 were named A to F. (C) Six HA-tagged overlapping ZFHX3 fragments and FLAG-tagged HIF1A were ectopically expressed in HepG2 cells under hypoxia, and IP and western blotting were applied to test ZFHX3-HIF1A interactions. (D) Association of HIF1A with different fragments of ZFHX3 in HeLa cells ectopically expressed HA-tagged ZFHX3 fragments under hypoxia. Nuclear and cytoplasmic fractions were separated, and IP and western blotting were applied to each fraction. Input (1/50 of cytoplasmic or nuclear lysate) indicates the lysate not subjected to IP. C, cytoplasm; N, nucleus.

Figure 6. ZFHX3 plays a necessary role in the migration and tube formation of HUVECs involving VEGFA. (A–C) Tube formation of HUVECs did not increase after incubation with conditioned medium (CM)
ZFHX3 is integral to HIF1A/VEGFA function

from HepG2 cells with RNAi-mediated ZFHX3 silencing under hypoxia, as indicated by cell images (A), the total length of tubes (B), and the number of nodes (C). (D-F) Migration of HUVECs was not significantly affected by CM from HepG2 or Huh-7 cells treated with siZFHX3 as in panels A-C, as indicated by migrated cells (D) and their quantification in the transwell assay (E, F). (G-I) Addition of VEGFA to CM partially rescued the inhibitory effect of ZFHX3 knockdown on tube formation in HUVECs, as indicated by cell images (G), the total length of tubes (H), and the number of nodes (I). Statistical analysis was based on three independent experiments (n=3), and the value for each group in an experiment was the average of triplicate (transwell) or 5 fields (tube formation). Scale bar for panel D is 200 µm and those for A and G are 100 µm. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
ZFHX3 is integral to HIF1A/VEGFA function
Figure 7. Loss of ZFHX3 attenuates xenograft tumor growth of HCC cells likely via compromised angiogenesis. (A-D) ZFHX3 was knocked down by shRNAs against ZFHX3 (shZFHX3-1 and -2) in HepG2 cells, as confirmed by real-time qPCR (A), and subcutaneous tumorigenesis assay was performed. Tumor growth is indicated by tumor images (B) and tumor weights (C) at excision and tumor growth curves (D). (E-G) Clone KO-1 was subjected to tumorigenesis assay as in panels B-D. (H, I) Two clones of HepG2 cells with CRISPR-Cas9-mediated ZFHX3 truncation, KO-1 and KO-2, were confirmed for lack of ZFHX3 protein by western blotting (H) and mutations in ZFHX3 by DNA sequencing (I). In panel I, the CRISPR-Cas9 target sequence is underlined, nucleotide deletion in clone KO-1 is indicated by a dotted line, and the mutations in clone KO-2 are marked by arrows. (J, K) Detection of microvessels by IHC staining of CD31 (Upper) and VEGFA (Lower) expression in xenograft tumors by IHC staining. (L, M) The microvessel densities (MVD) were quantified based on CD31 staining of endothelial cells. (N-O) Quantitative analyses of VEGFA expression based on IHC staining in panels J and K. The statistical analysis for MVD (L and M) and VEGFA score of IHC staining was based on the average of 3 fields from all 8 tumors (n=8). Scale bar in J and K, 200 µm. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

Figure 8. Upregulation of ZFHX3 and its correlation with HIF1A upregulation and worse patient survival in HCC. (A) ZFHX3 mRNA levels were higher in HCC tissues than in normal liver tissues, as revealed by the analyses of microarray expression data from 3 GEO datasets (left) and RNA-Seq data (right) of HCC in the TCGA database. RNA sequence data for normal liver tissues was from the TCGA and GTEx database. (B) ZFHX3 mRNA levels correlated with HIF1A levels in HCC, as revealed by Spearman correlation analysis using the TCGA database. (C) HCC with higher ZFHX3 expression had poorer disease-free survival, as revealed by the survival analysis of HCC samples with both patient survival data and ZFHX3 expression information in the TCGA database. The Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn), an online tool, was used to test the relationship between ZFHX3 expression levels and disease-free survival. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.
The transcription factor ZFHX3 is crucial for the angiogenic function of hypoxia-inducible factor 1 α in liver cancer cells

Changying Fu, Na An, Jinming Liu, Jun A, Baotong Zhang, Mingcheng Liu, Zhiqian Zhang, Liya Fu, Xinxin Tian, Dan Wang and Jin-Tang Dong

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