

Using a yeast two-hybrid system to identify FTCD as a new regulator for HIF-1 α in HepG2 cells



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ABSTRACT

HIF-1 α is implicated in hepatocellular carcinoma (HCC) pathologies. Here, we screened a human liver cDNA library for HIF-1 α -interacting partners using a yeast two-hybrid system. We identified 53 genes, including formiminotransferase cyclodeaminase (FTCD), which was confirmed by co-immunoprecipitation. Moreover, our data indicated that HIF-1 α mediated the effects of hypoxia on FTCD induction via binding to the hypoxia-responsive elements of the FTCD promoter. Knockdown of FTCD reduced the effects of HIF-1 α in hypoxia and enhanced chemosensitivity in HepG2 cells. Our findings suggested crosstalk between FTCD and HIF signaling and promoted HCC progression, thus implicating FTCD as a therapeutic target for HCC.

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1. Introduction

Hypoxia-inducible factors (HIFs) play an important role in hepatocellular carcinoma (HCC) [1]. HIFs are frequently upregulated in HCC and seem to control tumor progression and sensitivity to radiation therapy [2,3]. The homeostatic response to hypoxia is predominantly mediated by the transcription factor hypoxia-inducible factor (HIF). HIF consists of two basic helix–loop–helix proteins of the PAS family – a hypoxia regulated subunit (HIF-1 α or HIF-2 α) and a constitutive β -subunit (HIF-1 β) [4]. HIF-1 α is the key regulatory component which is hydroxylated on proline residues by a class of prolyl-hydroxylases under normoxic conditions. Then, prolyl-hydroxylated HIF-1 α is recognized and targeted by the von Hippel–Lindau (VHL) tumor suppressor protein, which is an E3 ubiquitin ligase [5]. HIF-1 α remains unmodified by prolyl-hydroxylases under hypoxic conditions, and thereby escapes recognition by VHL and destruction [6]. HIF-1 α dimerizes with HIF-1 β , and translocates to the nucleus to activate hypoxia response genes by binding to the core sequence (RCGTG) of the hypoxic responsive element (HRE) with additional recruitment of the co-activators CBP/p300 [7].

Hydroxylation of HIF-1 α at asparagine, which is catalyzed by FIH-1 in normoxic cells, blocks the binding of the transcriptional coactivator p300 to HIF-1 α [8]. HIF-1 α enhancing has been reported to promote angiogenesis and invasiveness in HCC [9,10]. Furthermore, HIF-1 regulates hundreds of genes encoding proteins that play vital roles in many aspects of cancer biology [11].

Formiminotransferase cyclodeaminase (FTCD) is expressed in every mammalian cell type, but is most highly enriched in the liver [12,13]. The enzyme comprises two domains (FT and CD) and catalyzes histidine degradation during folate metabolism [14–16]. In addition to its metabolic function, FTCD associates with the Golgi complex [17,18]. FTCD binds to vimentin filaments, and causes bundling of the filaments, which originate in the Golgi [19]. FTCD is associated with several human diseases. FTCD is one of the key antigens in AIH type 2 [20–22]. FTCD is associated with deficiency of glutamate formiminotransferase, which is the second most common inborn error of folate metabolism [23]. FTCD is identified as a novel immunohistochemical tumor marker of primary hepatocellular carcinoma [24]. FTCD is also over-expressed in many malignant cancer cells [25–27].

To understand the role of the HIF-1 α protein in HCC, a yeast two-hybrid system was used to screen for the interacting partners of the HIF-1 α protein using a human liver cDNA library. We identified 53 different HIF-1 α -interacting partners, including the FTCD protein. Co-immunoprecipitation was performed to confirm the interaction

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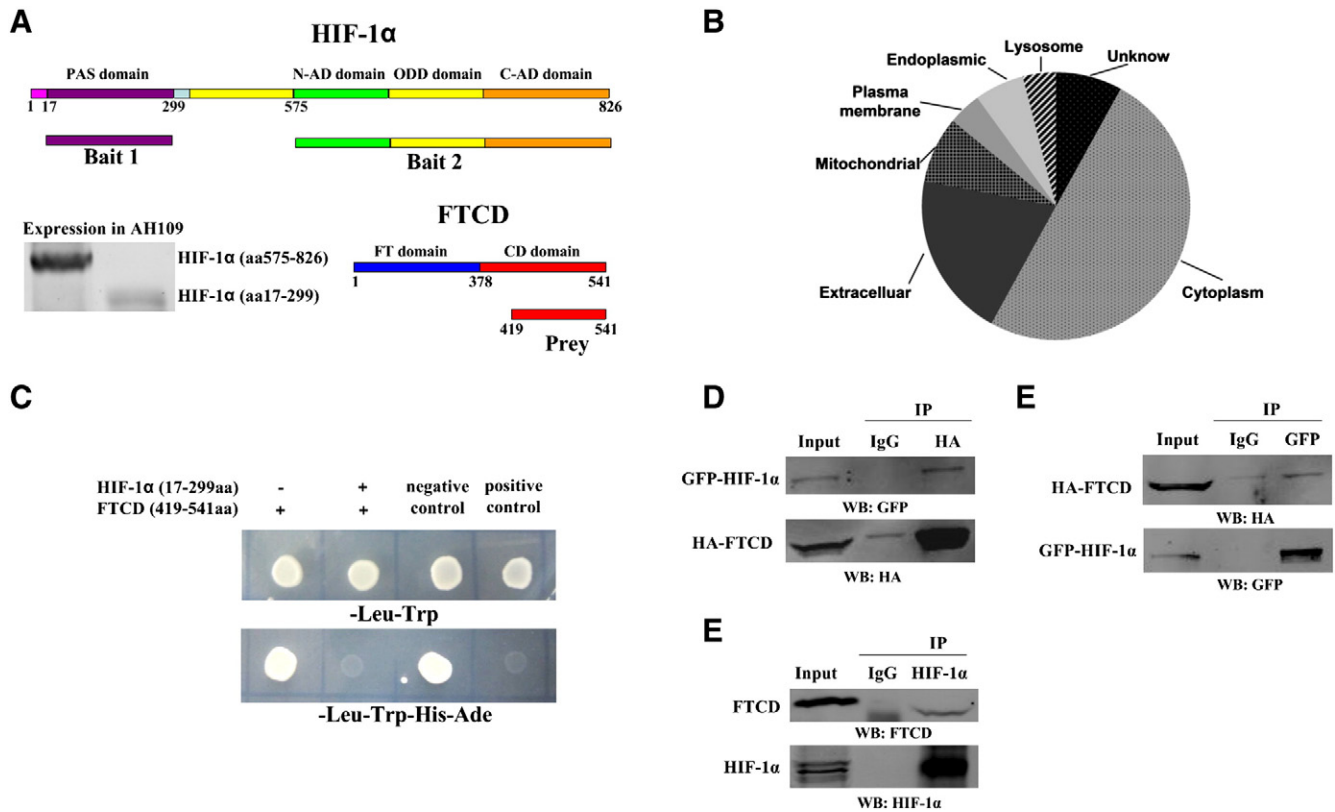


Fig. 1. Identification of HIF-1 α -interacting partners using the yeast two-hybrid system. (A) The location of the PAS homology domain, N-terminal transactivation domain (TAD-N), ODD (inhibitory domain), and N-terminal transactivation domain (TAD-C) of HIF-1 α are shown. The bait vector used for yeast two-hybrid screening encoded aa 17–299 (bait 1) and aa 575–826 (bait 2) of human HIF-1 α . The identified prey domain of FTCD is also shown. (B) Cellular localization of HIF-1 α interacting partners identified by yeast two-hybrid screening. (C) Interaction between HIF-1 α (aa 17–299) and FTCD (aa 419–541) was re-examined by yeast two-hybrid screening using SD-Leu-Trp or SD-Leu-Trp-His-Ade culture medium. Vector pGADT7-T with pGBKT7-p53 or pGBKT7-Lam was used as positive and negative controls, respectively. (D) Interaction between GFP-tagged HIF-1 α and HA-tagged FTCD full-length proteins was examined by co-IP followed by Western blotting using anti-GFP or anti-HA antibodies. Tagged proteins were over-expressed in HEK293T cells by transient transfection. (E) The association of endogenous HIF-1 α and FTCD in HepG2 cells was analyzed by co-IP followed by Western blotting using anti-HIF-1 α and anti-FTCD antibodies.

between HIF-1 α and FTCD. Our data indicated that FTCD levels were markedly increased in response to hypoxia. HIF-1 α , as an upstream transcription factor, initiated the transcription of FTCD under hypoxic condition. Furthermore, inhibition of FTCD significantly affected hypoxia-stimulated HIF-1 α transcriptional activity. Importantly, the induction of FTCD by hypoxia correlated with glycolysis and cell survival under hypoxic condition. Our findings suggest that a crosstalk between FTCD and HIF-1 α through a positive feedback loop regulates hypoxia-induced cellular adaptive responses.

2. Materials and methods

2.1. Materials

Rabbit anti-FTCD and anti-GFP antibodies were purchased from GeneTex (GTX109972 and GTX113617), rabbit anti-HIF-1 α antibody from Novus (NB100-479), mouse anti-HA and β -actin antibodies from Sigma (H3663 and A5441), and rabbit or mouse IgG from Santa Cruz Biotechnology. Goat anti-mouse or anti-rabbit IgG secondary antibodies were purchased from LI-COR Biosciences. The plasmids used in this study were generated by subcloning the indicated human cDNA fragments into expression vectors (pCDNA3.1-HA and pFLAG-cmv-4). All plasmids were verified by DNA sequencing. The sequences for siRNA and PCR primers are listed in Table S2.

2.2. Yeast two-hybrid screen

The N-terminal (aa 17–299) or C-terminal portion (aa 531–826) of the human HIF-1 α cDNA sequence was subcloned into the yeast

expression vector pGBKT7 (Clontech) in frame with the Gal4 DNA binding domain. This bait was used to screen the Human Liver Matchmaker[®] cDNA Library pACT2 (Clontech). The yeast two-hybrid screens were performed according to the manufacturer's instructions (Clontech).

2.3. Cell culture and transient transfection

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 mg/mL penicillin, and 100 mg/mL streptomycin sulfate (GIBCO) at 37 °C under 5% CO₂. Hypoxic treatment was performed in a specially designed hypoxia incubator (Thermo Electron, Forma, MA, USA) under 1% O₂, 5% CO₂ and 94% N₂. Lipofectamine 2000 (Invitrogen) was used in transient transfections according to the manufacturer's protocol.

2.4. Quantitative real-time PCR

Total RNA was isolated using a TRIzol kit (Omega), and cDNA was synthesized using a cDNA synthesis kit (TaKaRa). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (TaKaRa) on the Roche 480 system (Roche). The primers used in this study are listed in Table S2.

2.5. Co-immunoprecipitation assay

Cell extracts were incubated with antibodies and Protein A agarose (Pierce) overnight at 4 °C. Normal mouse or rabbit IgG (Santa Cruz

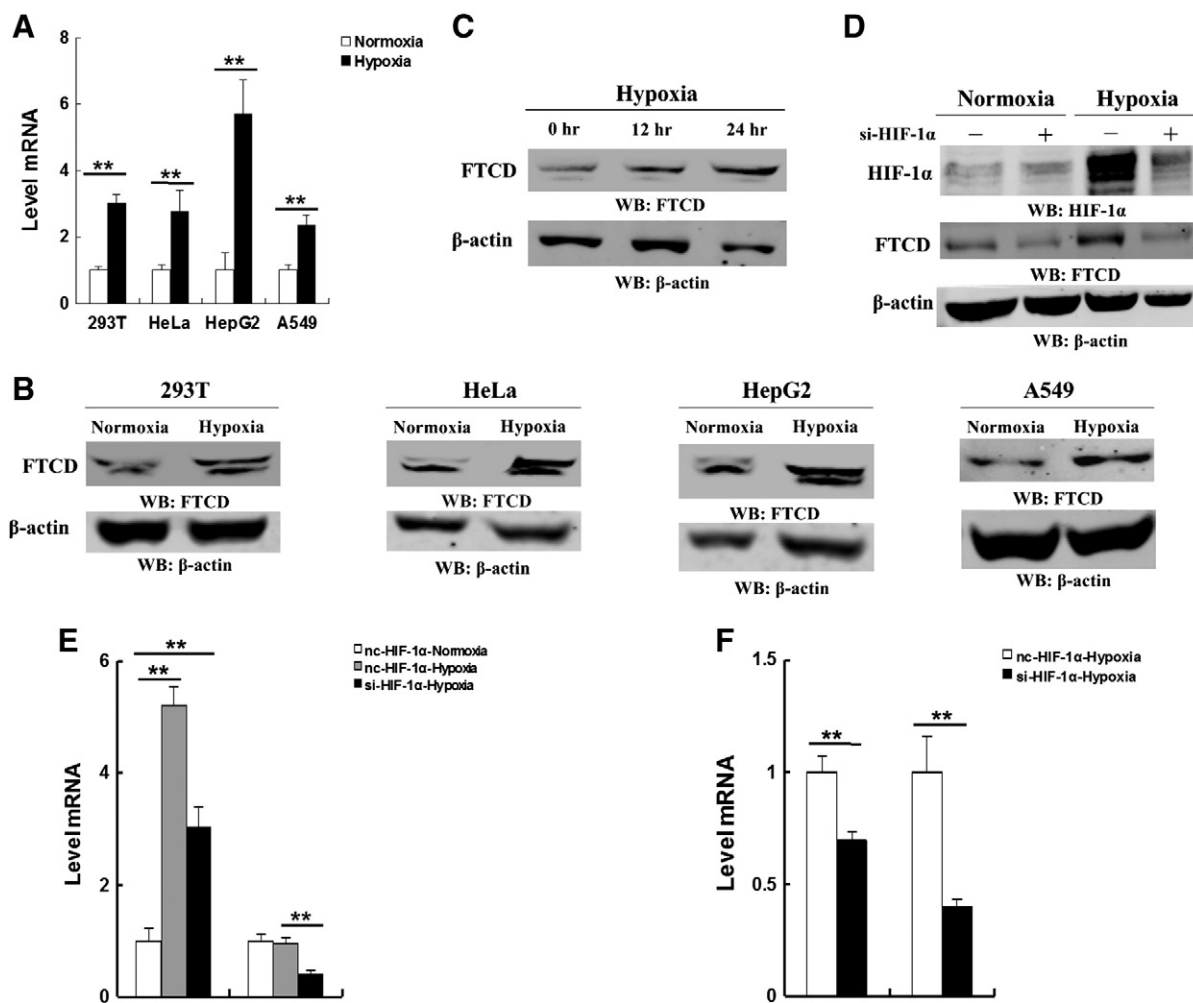


Fig. 2. FTCD expression was induced by hypoxia. (A) The mRNA levels of FTCD in HEK293T, HeLa, HepG2 and A549 cells under normoxia (20% O₂) or hypoxia (1% O₂) for 24 h were determined in real-time PCR assays. (B) The protein levels of FTCD in HepG2 cells under normoxia or hypoxia condition for 24 h were followed by immunoblot assays using the indicated antibodies. (C) HepG2 cells were cultured under hypoxia for the indicated length of time. Cell lysates were analyzed in immunoblot assays using the indicated antibodies. (D) HepG2 cells were transfected with HIF-1α siRNA for 24 h and cultured under normoxia or hypoxia for a further 24 h. Protein levels were determined in immunoblot assays using the indicated antibodies. (E) HepG2 cells were transfected with si-HIF-1α for 24 h and cultured under normoxia or hypoxia for a further 24 h. Levels of FTCD and HIF-1α mRNA were determined in real-time PCR assays. (F) HepG2 cells were transfected with si-HIF-1α for 24 h and cultured under hypoxic conditions for a further 24 h. Levels of FTCD and HIF-1α mRNA were determined in real-time PCR assays. All data represent the means ± SEM of three independent experiments, **p* < 0.05 and ***p* < 0.01.

Biotech) was used as a negative control. After washing five times with lysis buffer (RIPA), the beads were eluted with the 2 × SDS sample buffer and boiled for 8 min at 100 °C. The samples were then analyzed by Western blotting [28,29].

2.6. Luciferase reporter assays

Cells were seeded onto 6-well plates, transfected with nc-FTCD or si-FTCD together with the reporter plasmid p2.1 [30] and control pSV40-Renilla for 24 h, and then exposed to either 20% or 1% O₂ for a further 24 h. Cell extracts were analyzed using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions.

2.7. Glucose consumption and lactate production

Cells were seeded onto 6-well plates and transfected with siRNAs. Approximately 48 h after transfection, cells were seeded again and cultured in serum-free DMEM under 20% or 1% O₂ for a further 16 h [31]. Glucose and lactate levels in medium were measured using a glucose

assay kit (Sigma) and a Lactate assay kit (CMA, Microdialysis), respectively. These readouts were normalized to the corresponding amounts of protein (Beyotime).

2.8. Cell invasion assays

Cell invasion was assessed using 6.5 mm transwell chambers equipped with 8.0 μm pore-size polycarbonate membranes. Briefly, 5 × 10⁵ cells pre-starved in serum-free medium for 12 h were placed into the upper chamber with 0.2 mL of serum-free medium. The lower chambers were filled with 0.6 mL medium supplemented with 10% FBS. After culturing for 48 h, non-invasive cells were physically scraped from the membrane with cotton swabs and counted. The cells that were attached to the underside of the membranes were stained with crystal violet and counted [32].

2.9. Cell proliferation analysis

Cells were seeded onto 6-well plates, transfected with nc-FTCD or si-FTCD. After 24 h 4 × 10⁴ cells were harvested and seeded in triplicates

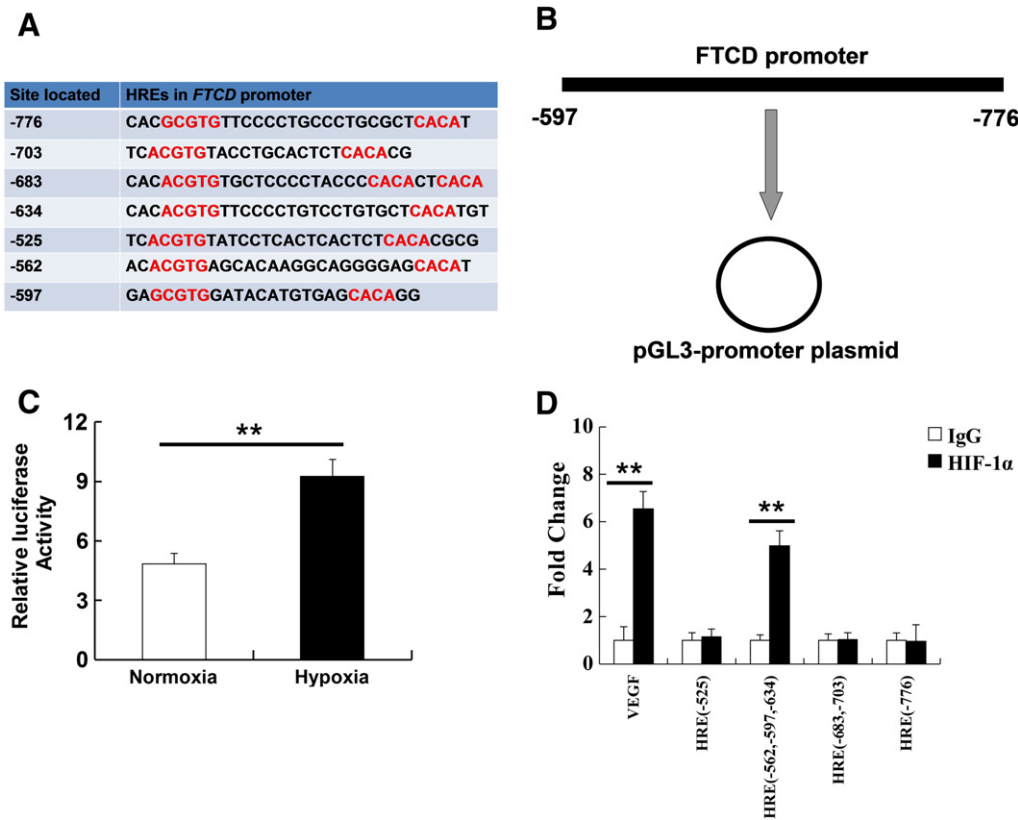


Fig. 3. FTCD was a HIF-1 α target gene. (A) Schematic representation of the hypoxic response element (HRE) binding sites within the FTCD promoter region. Sequences for putative binding sites and their positions are indicated. (B) The HREs in the FTCD promoter region were cloned into the pGL3-promoter plasmid. (C) HepG2 cells were transfected with the HRE (–776 to –597)-luciferase reporter plasmid under normoxia or hypoxia. After 48 h, luciferase activity was measured. Transfection efficiency was normalized against Renilla luciferase expression. (D) Anti-IgG and anti-HIF-1 α antibodies were used in the chromatin immunoprecipitation (ChIP) assays using HepG2 cells treated with hypoxia for 24 h. All data represent the means $\pm$ SEM of three independent experiments, * p < 0.05 and ** p < 0.01.

onto new 6-well plates, and cell numbers were counted every 24 h over a four-day period [33].

2.10. CCK-8 assay

The cell survival rate was examined using Cell Counting Kit-8 (Dojindo Molecular Technologies) [34]. Cells were plated in 96-well plates at 6000 cells/well. After 24 h of culture, the medium was replaced with DMEM containing 10% FBS and drugs at the indicated concentrations. After incubation for 24 h, 10 μ L CCK-8 was added to each well, and cells were further incubated for 2 h. Absorbance was read at 450 nm using an enzyme micro-plate reader. The IC₅₀ value was calculated using GraphPad Prism version 5.0 software.

2.11. Statistical analysis

We determined the significance of differences using Pearson's correlation test and Student's *t*-test (two-tailed). p < 0.05 was considered to be significant. Statistical significance is displayed as * p < 0.05, ** p < 0.01.

3. Results

3.1. Identification of HIF-1 α -interacting partners using the yeast two-hybrid system

Considering the importance of the HIF-1 α protein in HCC and its possible involvement with liver dysfunction [35], we aimed to understand its role in HCC. Therefore, we performed a yeast two-hybrid screen of a human liver cDNA library to identify novel HIF-1 α interacting partners. First, to determine whether the recombinant yeast AH109 cells

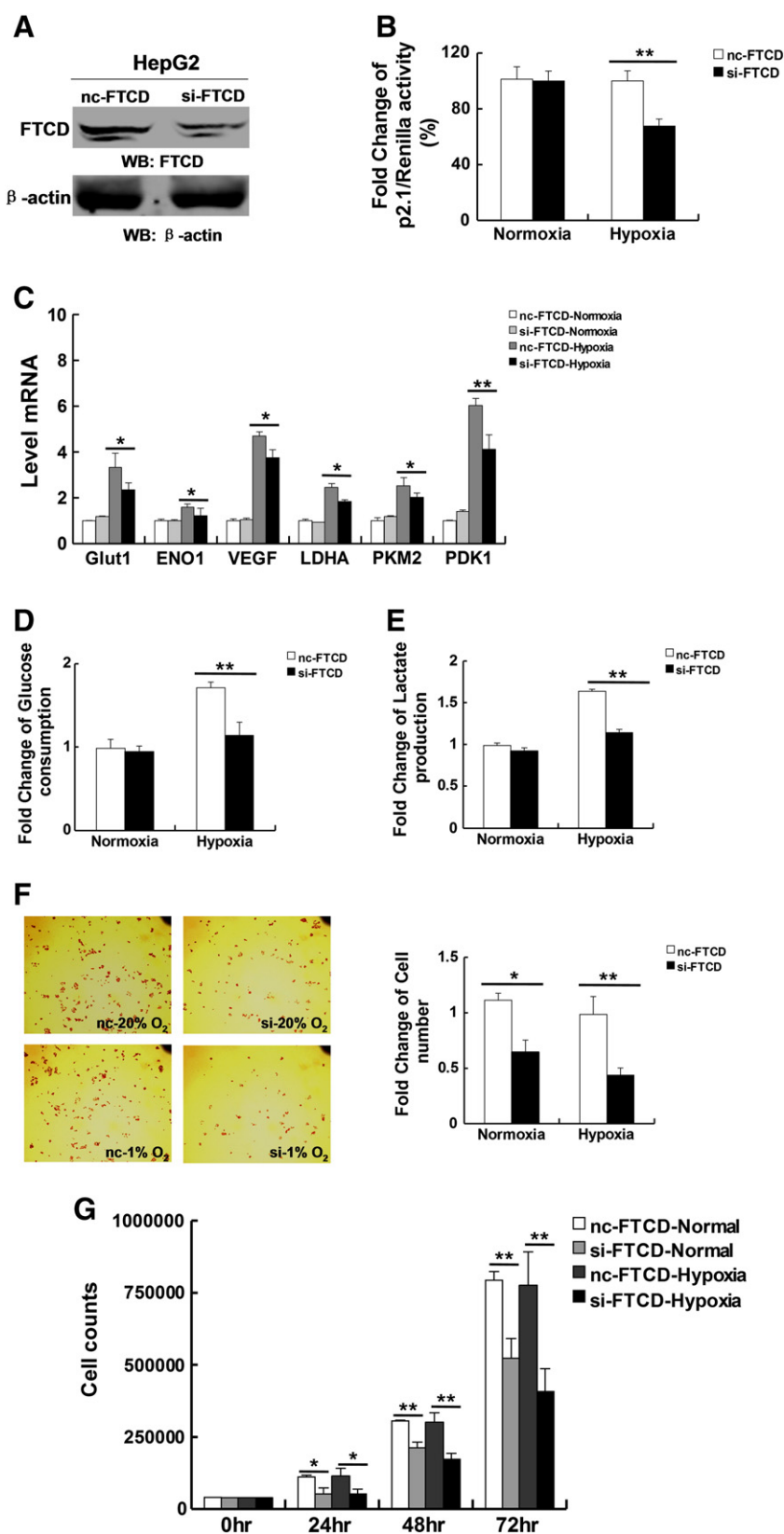
expressed the HIF-1 α (aa 17–299) [36] and HIF-1 α (aa 531–826) [8] peptides, we performed a Western blot assay using the anti-c-myc polyclonal antibody (Fig. 1A). Next, using drop-out media (–Trp–His–Ade–Leu), we identified 53 interacting partners of HIF-1 α (Table S1). Interestingly, the interaction of HIF-1 α with the OS-9 [37] or MCM7 [38] proteins has been described previously. As shown in Fig. 1B, these were arranged according to their primary cellular localization. These results indicate the ability of the HIF-1 α protein to interact with different classes of host proteins, primarily those localized in the extracellular and cytoplasm regions. Because FTCD was one of the strongest binding partners of HIF-1 α in AH109 (data not shown) and this interaction might play an important role in liver, we focused our studies on confirmation of the HIF-1 α and FTCD interaction. First, the interaction between HIF-1 α and FTCD was validated in an independent yeast two-hybrid assay (Fig. 1C). To further analyze their interaction, we transiently over-expressed GFP-tagged HIF-1 α and HA-tagged FTCD in HEK293T cells under hypoxia for 24 h. By co-immunoprecipitation (co-IP) analysis, we showed that FTCD pulled down HIF-1 α (Fig. 1D) and vice versa (Fig. 1E). Moreover, to determine whether endogenous FTCD interacted with HIF-1 α , we performed co-IP experiments with cell lysates from HepG2 cells which were cultured under hypoxia for 24 h. We showed that endogenous FTCD pulled down endogenous HIF-1 α in HepG2 cells using an anti-HIF-1 α antibody (Fig. 1F). These results confirm the interaction between FTCD and HIF-1 α .

3.2. FTCD expression was induced by hypoxia

Previous studies show that FTCD is a hypoxia-induced gene in malignant C6 glioma cells using microarray analysis [27], but its mechanism has not been fully elucidated. To investigate whether FTCD expression was oxygen-regulated, HepG2, HeLa, A549, and 293T cells were

incubated at 20% or 1% O₂ for 24 h. FTCD mRNA levels were induced under hypoxic conditions in these cell lines (Fig. 2A). Immunoblot assays indicated that FTCD protein also accumulated as cells were challenged by hypoxia (Fig. 2B). Moreover, hypoxia induced FTCD

expression in a time-dependent manner (Fig. 2C). HIF-1 α and HIF-2 α are the main transcription factors induced under hypoxic stress [39]. Therefore, we hypothesized that FTCD was upregulated by HIF-1 α and HIF-2 α . Hypoxia-induced FTCD expression was reduced in the absence



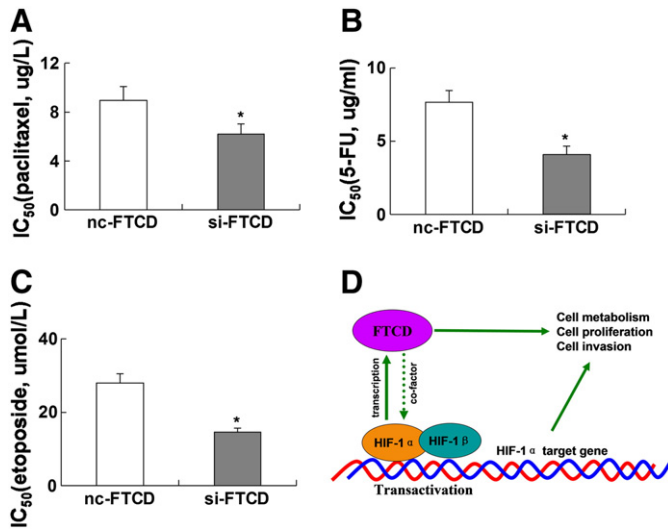


Fig. 5. Knockdown of FTCD enhanced chemosensitivity of HepG2 cells. A–C. HepG2 cells were transfected with nc-FTCD or si-FTCD. One day after transfection, cells were replated in 96-well plates and treated with the indicated drugs. After 24 h, the cell survival rate was examined in CCK-8 assays. The IC₅₀ values were calculated using GraphPad Prism version 5.0 software. All data represent the means $\pm$ SEM of three independent experiments, * p < 0.05 and ** p < 0.01. D. Schematic diagram of the proposed hypoxia-induced FTCD signaling pathway.

of HIF-1 α and HIF-2 α in HepG2 cells (Fig. 2E and Fig. S1A). Consistently, the mRNA and protein levels of FTCD were decreased while HIF-1 α (Fig. 2D and F) or HIF-2 α (Fig. S1B and C) was knocked down in HepG2 cells under hypoxia. Taken together, these results suggest that FTCD is upregulated during hypoxia.

3.3. FTCD was a HIF-1 α target gene

We next investigated whether FTCD was directly transcribed by the HIF-1 α transcription factor. We searched the human FTCD gene for matches to a consensus sequence (5'-RCGTG-3' followed by a 5'-CAC-3' sequence) [40]. Candidate HREs were identified in the FTCD promoter (Fig. 3A). To determine whether these HREs were functional in response to hypoxia, the fragment (–1520/+470) spanning of these HREs was subcloned into the luciferase reporter plasmid (Fig. 3B). The reporter plasmids were transfected into human HEK293T cells, which were exposed to 20% or 1% O₂ for 24 h. The luciferase assay showed that the promoter activity was induced by hypoxia (Fig. 3C). To determine whether the HIF-1 α protein binds at these sites, chromatin immunoprecipitation (ChIP) assays were performed in HEK293T cells subjected to hypoxia for 24 h to stabilize HIF-1 α protein. As shown in Fig. 3D, HIF-1 α bound at the HRE sites located at positions –634, –597 and –562. In summary, these data indicate that the FTCD gene is directly transcribed by HIF-1 α under hypoxia.

3.4. Knockdown of FTCD decreased HIF-1 α -mediated transcription, glycolysis, cell invasion and proliferation in HepG2 cells under hypoxia

To investigate whether FTCD regulates HIF-1 α transcriptional activity, HepG2 cells were co-transfected with the following: HIF-1 α -

dependent reporter plasmid p2.1, which contained an HRE from the human ENO1 gene upstream of the SV40 promoter and firefly luciferase coding sequences [30]; control reporter pSV40-Renilla; and nc-FTCD or si-FTCD. The ratio of p2.1/pSV40-Renilla activity represents a specific measure of HIF-1 α transcriptional activity [40]. HIF-1 α transcriptional activity was reduced significantly under hypoxia (Fig. 4B), following knockdown of FTCD (Fig. 4A). To determine whether FTCD regulates expression of HIF-1 α target genes involved in cancer progression, we knocked down FTCD and significantly down-regulated levels of the HIF-1 α target genes including *Glut1*, *ENO1*, *VEGF* and *LDHA* in hypoxic HepG2 cells (Fig. 4C). Under hypoxia, HIF-1 α shunts glucose toward glycolysis to maintain cell survival [41]. We then investigated whether FTCD augments adaptive responses to hypoxic conditions in cancer cells. Indeed, knockdown of FTCD decreased both glucose consumption (Fig. 4D) and lactate production (Fig. 4E) in response to hypoxia. Compared with cellular responses under normoxic conditions, cell invasion and proliferation were much more significantly decreased in the absence of FTCD when HepG2 cells were challenged with hypoxia (Fig. 4F and G). Taken together, these data suggest that FTCD is required for glucose metabolism, cell invasion and survival in response to hypoxia.

3.5. Knockdown of FTCD enhanced chemosensitivity in HepG2 cells

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide and represents half of the total number of cancers in China [42]. To explore the role of FTCD in chemoresistance, we knocked down FTCD in HepG2 cells with siRNA. This resulted in decreased resistance to paclitaxel, 5-FU and etoposide (Fig. 5A–C). Taken together, these data suggest that FTCD depletion is involved in paclitaxel, 5-FU and etoposide resistance.

4. Discussion

In the present study, we identified 53 putative interacting partners of HIF-1 α by screening a human liver cDNA library using a yeast two-hybrid system. In this screening, FTCD, a key enzyme of the folate metabolism, was found to interact with HIF-1 α . According to recent reports, FTCD is implicated as an important biomarker in HCC tissues [24]. Based on this information, we focused our efforts on confirmation of the HIF-1 α and FTCD interaction by co-immunoprecipitation. Using luciferase reporter and qRT-PCR assays, we found that this interaction enhanced HIF-1 α transcription. Surprisingly we also identified FTCD as a target gene of HIF-1 α . By ChIP and luciferase reporter assays, we showed that HIF-1 α bound to the HRE region of the FTCD promoter and upregulated transcription. Furthermore, FTCD increased proliferation and migration in HepG2 cells. Chemotherapy is one of the most successful treatments for HCC patients, although chemoresistance has become a significant problem for anti-cancer therapy [43]. In this study, we showed that si-RNA-mediated knockdown of FTCD decreased resistance of HepG2 cells to paclitaxel, 5-FU and etoposide, which are common chemotherapeutic agents for HCC. Taken together, these observations suggest that therapeutic strategies targeting FTCD might improve HCC sensitivity to chemotherapy.

In this study, we found that FTCD mRNA and protein levels increased significantly in response to hypoxia. Furthermore, following knockdown of FTCD, the proliferation rate of tumor cells decreased significantly under hypoxia. It can be speculated that FTCD levels are upregulated via

Fig. 4. Knockdown of FTCD decreased HIF-1 α -mediated transcription, metabolism, invasion and proliferation under hypoxia. (A) HepG2 cells were transfected with FTCD siRNA for 48 h. FTCD protein levels were determined in immunoblot assays using the indicated antibodies. (B) HepG2 cells were transfected nc-FTCD or si-FTCD with p2.1 luciferase reporter plasmids for 24 h and cultured under normoxia or hypoxia for a further 24 h before luciferase activity was measured. Transfection efficiency was normalized against Renilla luciferase expression. (C) HepG2 cells were transfected with nc-FTCD or si-FTCD for 24 h and cultured under normoxia or hypoxia for a further 24 h. Levels of *Glut1*, *ENO1*, *VEGF* and *LDHA* mRNA were determined in real-time PCR assays. (D) HepG2 cells were transfected with nc-FTCD or si-FTCD for 24 h and cultured under normoxia or hypoxia for a further 12–16 h. Glucose levels in medium were determined. (E) HepG2 cells were transfected with nc-FTCD or si-FTCD for 24 h and cultured under normoxia or hypoxia condition for a further 12–16 h. Lactate levels in medium were determined. (F) Representative images and statistical plots of transwell assays of HepG2 cells transfected with nc-FTCD or si-FTCD for 24 h and cultured under normoxia or hypoxia for a further 48 h. (G) HepG2 cells were transfected with nc-FTCD or si-FTCD. After 24 h, the cells were re-plated and cultured under normoxia or hypoxia for a further 72 h. Cell numbers were counted every 24 h for the analysis of cell proliferation. All data represent the means $\pm$ SEM of three independent experiments, * p < 0.05 and ** p < 0.01.

HIF-1 α to adapt to hypoxic conditions. There is a close relationship between FTCD, which is the main component of the Golgi body, and Golgi body morphology [17]. Thus, HIF-1 α and its regulation of FTCD expression may also have an important role in control of the Golgi body.

In summary, we have demonstrated that FTCD is a positive regulator of the hypoxia-HIF signaling pathway and has an important role in cell proliferation, metabolism and migration in HepG2 cells (Fig. 5D). These findings suggest that FTCD represents a novel molecular target for HCC therapy, which should be evaluated in clinical trials for HCC.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2014.03.016>.

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