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Heat Shock Factor 1 Epigenetically Stimulates Glutaminase-1-Dependent mTOR Activation to Promote Colorectal Carcinogenesis

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Heat shock factor 1 (HSF1) generally exhibits its properties under stress conditions. In tumors, HSF1 has a pleiotropic feature in regulating growth, survival, and aggressiveness of cancer cells. In this study, we found HSF1 was increased in colorectal cancer (CRC) and had a positive correlation with shorter disease-free survival (DFS). Knockdown of HSF1 in CRC cells attenuated their growth while inhibiting mTOR activation and glutamine metabolism. HSF1 inhibited the expression of microRNA137 (MIR137), which targeted GLS1 (glutaminase 1), thus stimulating GLS1 protein expression to promote glutaminolysis and mTOR activation. HSF1 bound DNA methyltransferase DNMT3a and recruited it to the promoter of lncRNA MIR137 host gene (MIR137HG), suppressing the generation of primary MIR137. The chemical inhibitor of HSF1 also reduced cell growth, increased apoptosis, and impaired glutamine metabolism *in vitro*. Moreover, both chemical inhibition and genetic knockout of HSF1 succeeded in increasing MIR137 expression, reducing GLS1 expression, and alleviating colorectal tumorigenesis in azoxymethane (AOM)/dextran sulfate sodium (DSS) mice. In conclusion, HSF1 expression was increased and associated with poor prognosis in CRC. By recruiting DNMT3a to suppress the expression of MIR137 that targets GLS1 mRNA, HSF1 stimulated GLS1-dependent mTOR activation to promote colorectal carcinogenesis. Therefore, targeting HSF1 to attenuate glutaminolysis and mTOR activation could be a promising approach for CRC treatment.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer, and its mortality rate has been ranked at fourth worldwide.[1] Other than genetic and dietary elements, new factors involved in colorectal carcinogenesis, such as altered gut microbiota, are emerging.[2] Dysregulated metabolism, such as obesity, also predisposes the development of CRC.[3,4] However, the underlying mechanism and potential clinical relevance remain largely unclarified.

Heat shock factor 1 (HSF1) is an essential factor responsible for inducing the expression of heat shock proteins (HSPs) in response to heat shock.[5] As a "pioneer" transcription factor, HSF1 could first access its regulatory elements at the promoter and act as a scaffold to facilitate recruitment of other activators or repressors.[6,7] Recently, HSF1 has been revealed to be involved in response to other stimuli, such as tumorigenesis. HSF1 is indispensable for malignant transformation in multiple tumors, including breast cancer,[8] prostate cancer,[9] hepatocellular carcinoma (HCC),[10,11] and endometrial cancer.[12] Furthermore, high levels of HSF1 are associated with poor prognosis and may serve as a therapeutic target for cancer patients.[13] HSF1 is a versatile factor in tumorigenesis, such as acting as a signal modulator,[14] enhancement of polyploidy,[15] resistance of apoptosis,[16] and generation of epithelial-mesenchymal transition (EMT).[17] More importantly, several studies have linked HSF1 to the aberrant metabolism, a hallmark of cancer cells. It was also shown to have a profound effect on glycolysis[18] and lipid metabolism.[19] Yet no studies exist about its role in glutamine metabolism, which is crucial for energy generation as well as protein and nucleotide synthesis.[20,21]

DNA methylation is a fundamental epigenetic mechanism for regulating gene transcription.[22] Despite a wealth of knowledge on DNA methylation, the mechanism of the recruitment of DNA methyltransferases (DNMTs) to DNA regions to be specifically methylated is still poorly understood. Genome-wide DNA methylation profiles have revealed that methylated DNA regions are enriched with binding sites for transcription factors, suggesting potential roles of transcription factors in specifically modulating the changes of DNA methylation landscape.[23,24] For instance, EZH2 or E2F6 could bind to DNA in a sequence-dependent manner, thus allowing the formation of gene-specific DNA methylation.[25,26]

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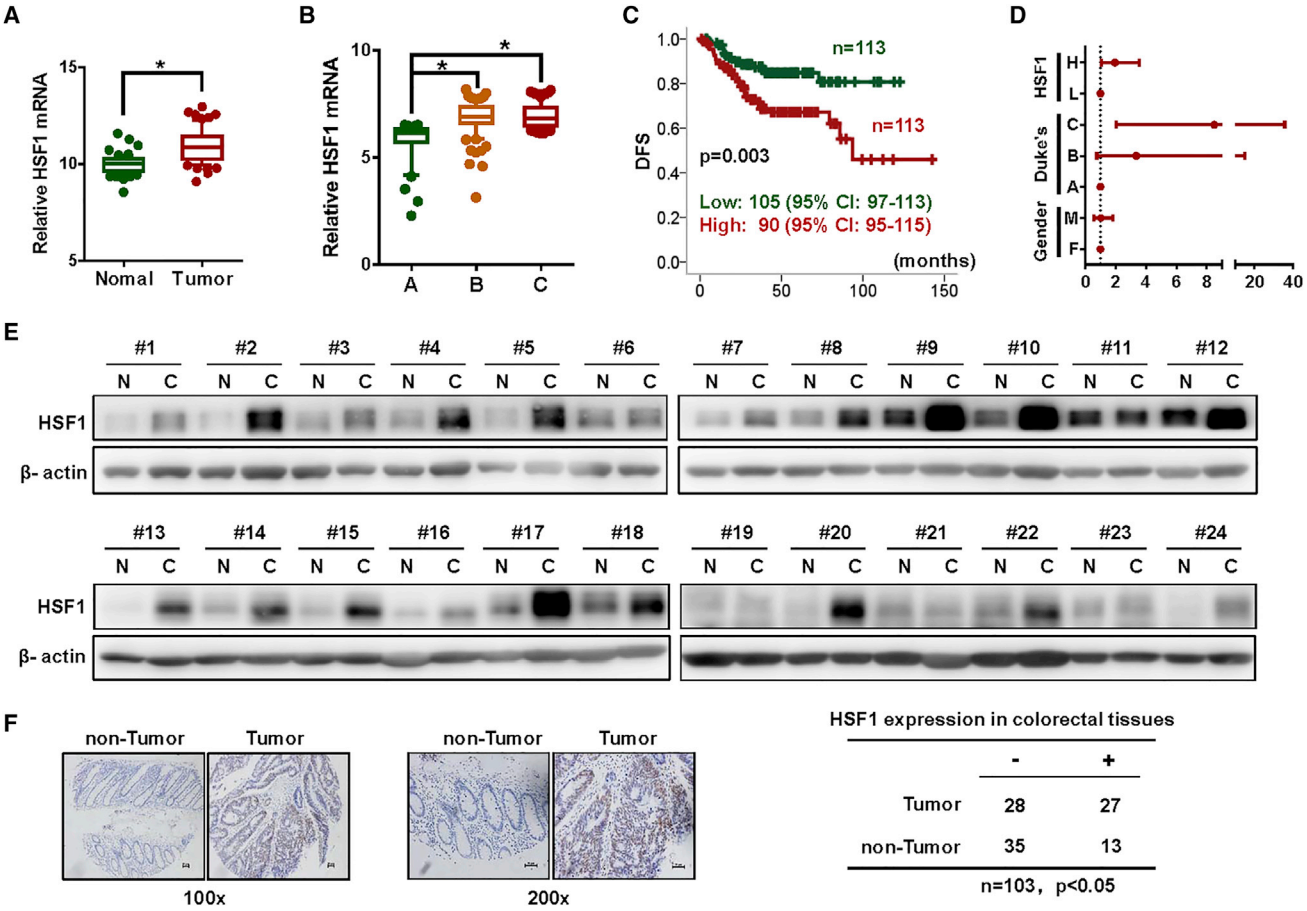

Figure 1. HSF1 Expression Was Elevated in Colorectal Cancer

(A) Expression of HSF1 mRNA in CRC tissues was analyzed in GEO database (Wilcoxon test; $p < 0.01$). (B) The expression of HSF1 mRNA in different CRC Duke's stages was analyzed in GEO database (one-way ANOVA test; $p < 0.01$). (C and D) The impact of HSF1 expression on disease-free survival (DFS) was analyzed by Kaplan-Meier survival curve analysis in (C) (patients were grouped based on median HSF1 expression; median survivals were shown; log rank test; $p < 0.01$) and Cox-regression analysis in (D) (relative risk; HSF1 low: 1, high: 1.95, 95% confidence interval [CI]: 1.07–3.56, $p < 0.05$; gender: female: 1, male: 1.03, 95% CI: 0.58–1.80; Duke's stage, A: 1, B: 3.36, 95% CI: 0.76–14.83, C: 8.50, 95% CI: 2.03–35.60, $p < 0.05$). The original data were extracted from NCBI GEO: GSE14333. (E) Expression of HSF1 protein in 24 pairs of fresh CRC tissues and adjacent non-tumor tissues was analyzed by western blotting. (F) Expression of HSF1 protein in colorectal cancer tissues and non-tumor tissues was analyzed by immunohistochemical staining (chi-square test; $p < 0.05$). The asterisks indicate statistical difference ($p < 0.05$).

In this study, we explored the biological effect, molecular mechanism, and clinical implication of HSF1 in CRC. We found that HSF1 stimulated glutaminase 1 (GLS1)-dependent mTOR activation to promote colorectal carcinogenesis by recruiting DNMT3a to epigenetically suppress the expression of microRNA137 (MIR137) that targets GLS1. Thus, targeting HSF1-driven metabolism appears to be a promising strategy for the clinical management of CRC patients.

RESULTS

HSF1 Expression Is Elevated in CRC

In order to understand the relevance of HSF1 in CRC, we obtained the gene expression profile of HSF1 mRNA from NCBI GEO: GSE20842)[27] and found that higher HSF1 expression was seen in the tumors than in the adjacent non-tumor colorectal tissues ($p < 0.05$; Figure 1A). Additionally, there was a positive correlation between HSF1 levels and Duke's stage ($p < 0.01$; Figure 1B). More importantly, high HSF1 mRNA levels were associated with shorter disease-free survivals (DFSs) in patients with CRC (NCBI GEO: GSE14333;[28] http://www.prognoscan.org/; Figures 1C, Kaplan-Meier survival curve, and 1D, Cox-regression analysis). On the other hand, western blotting analysis revealed higher protein levels of HSF1 in CRC tissues than in adjacent non-tumor tissues (19/24; Figure 1E). A similar trend was observed in a tissue array containing 103 cases of colonic tissues, including 55 CRC tissues and 48 non-tumor colon tissues, by immunohistochemical staining (IHC) ($p < 0.05$; Figure 1F). These data suggest that HSF1 expression was upregulated in CRC.

HSF1 Promotes mTOR Activity and Glutaminolysis in CRC

To evaluate the biological relevance of HSF1 in CRC, we detected the expression of HSF1 in CRC cell lines. Likewise, HSF1 was expressed

abundantly in CRC cell lines (Figure 2A). Knockdown of HSF1 inhibited cell growth in 3 CRC cell lines with high HSF1 expression (HCT116, SW480, and SW620; Figure 2B). However, there were minimal effects of HSF1 knockdown on extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway, two major signal pathways important to cell proliferation (Figure S1A). In order to identify the molecular basis of growth inhibition induced by loss of HSF1 function, we screened chemical inhibitors that affected genome-wide gene expression in a similar manner as HSF1 inhibition by the connective map (CMap; https://portals.broadinstitute.org/cmap/).[29,30] In addition to some HSF1 inhibitors, such as cephaeline and emetine,[31] sirolimus, a well-known mTOR inhibitor, also generated a similar gene expression pattern as HSF1 inactivation did (Figure 2C), indicating the potential connection of HSF1 with mTOR signaling. Indeed, mTOR activity was greatly reduced once HSF1 was knocked down (Figure 2D). However, glucose consumption was not affected by HSF1 depletion in CRC cells (Figure S1B). In contrast, a significant reduction of glutamine consumption and alpha-ketoglutarate (α-KG) (a glutaminolysis metabolite) generation were observed (Figures 2E and 2F), implicating that HSF1-depletion-induced inactivation of mTOR might be a result of glutaminolysis reduction rather than glycolysis inhibition. Furthermore, supplementation of cell-permeable α-KG, dimethyl 2-oxoglutarate (DMK), was able to restore HSF1-depletion-induced mTOR inactivation (Figure 2G). Overall, these findings indicated that HSF1 stimulated glutaminolysis and mTOR activation in CRC cells.

HSF1 Stimulates GLS1 Protein Expression

Because GLS1 is the rate-limiting enzyme for converting glutamine to glutamate,[32] we suspected that HSF1-promoted glutaminolysis was dependent on GLS1. Protein levels of GLS1 protein were elevated in CRC cell lines and CRC tissues (Figures 3A and 3B). Moreover, HSF1 expression was positively correlated with GLS1 expression ($p < 0.01$; Figure 3C). Importantly, knockdown of HSF1 resulted in a marked decrease of GLS1 protein levels in CRC cells (Figure 3D). Moreover, wild-type rather than enzyme-activity-deficient mutant (S286A) GLS1[33] rescued mTOR inactivation in CRC cells with HSF1 depletion (Figure 3E), implying the dependence of HSF1-depletion-induced mTOR inactivation on GLS1-mediated glutaminolysis. Taken together, HSF1 stimulated GLS1 expression to promote glutaminolysis and mTOR activation in CRC.

HSF1 Inhibits the Expression of MIR137 that Targets GLS1

We further dissected how HSF1 stimulates GLS1 protein expression in CRC. There were no apparent alterations in the GLS1 mRNA level after HSF1 knockdown (Figure 4A), excluding the regulation of GLS1 expression at the level of transcription or mRNA stability. We then used a protein synthesis inhibitor cycloheximide (CHX) to detect whether HSF1 is able to increase GLS1 protein stability. However, the half-life of GLS1 protein also showed no discrepancy before and after HSF1 knockdown (Figure S2A). Therefore, other mechanisms, such as translation regulation by small non-coding microRNAs (miRNAs), might be responsible for the regulation of GLS1 expression by HSF1. Subsequently, we analyzed the effect of HSF1 knockdown on the expression of *in silico* predicted miRNAs that might target GLS1 3′ UTR (Figure 4B). This low-throughput screen identified MIR137 as a candidate of GLS1-targeting miRNAs potentially regulated by HSF1, as its expression was greatly increased in three CRC cell lines after HSF1 knockdown (Figure 4C). In addition, MIR137 reduced GLS1 protein level (Figure 4D) and inhibited CRC cell viability (Figure S2B). Furthermore, MIR137 hampered the activity of a luciferase reporter driven by wild-type GLS1 3′ UTR, but not mutated GLS1 3′ UTR lacking MIR137 binding sites (Figures 4E and S2C), indicating that GLS1 is a direct target of MIR137. Importantly, HSF1-depletion-induced reduction of GLS1 protein expression was reversed by MIR137 inhibitors (Figure 4F). As a result, mTOR activity was inhibited by MIR137 mimics (Figure 4G), which was reversed by wild-type GLS1 rather than GLS1 mutant lacking enzyme activity (Figure 4H). Therefore, HSF1 stimulated GLS1 protein expression by relieving the targeted inhibition from MIR137.

HSF1 Recruits DNMT3a to Facilitate the Promoter Methylation of lncRNA MIR137HG

We next investigated the underlying mechanism of HSF1-driven inhibition of MIR137 expression. Because MIR137 is derived from the long non-coding RNA (lncRNA) MIR137 host gene (MIR137HG), which encodes the primary MIR137,[34] we wondered whether lncRNA MIR137HG shares a concordant expression pattern with miR137. Indeed, we found that MIR137HG was upregulated after HSF1 depletion (Figure 5A). HSF1 is inclined to bind heat shock response elements (HSEs), nGAAnnTTCn.[35] Interestingly, a classical HSE is located in the promoter of lncRNA MIR137HG (Figure 5B), indicating that HSF1 might directly bind to MIR137HG promoter. Chromatin immunoprecipitation (ChIP) assay did reveal a direct association of HSF1 with MIR137HG promoter (Figures 5C and S3D). We were also interested to discover a CpG island in MIR137HG promoter (Figure 5B). Bisulfite genomic sequence (BGS) subsequently confirmed promoter methylation of MIR137HG in CRC cells (Figure S3A). After demethylation treatment with DNA methyltransferase inhibitor (5-aza-2'-deoxycitydine [AZA]) and histone deacetylase inhibitor (trichostatin A [TSA]), the methylation level at MIR137HG promoter was decreased, accompanied with marked increase of MIR137HG and MIR137 expression (Figures S3A and S3B), therein confirming the relevance of promoter methylation to MIR137HG-MIR137 expression. Notably, methylation intensity at the MIR137HG promoter was decreased after HSF1 knockdown (Figures 5D and S3C), suggesting that the methylation of MIR137HG promoter was regulated by HSF1.

Whereas DNMTs, such as DNMT1, DNMT3a, and DNMT3b, are important for the formation or maintenance of DNA methylation,[36] none of them contain DNA-binding capability. This prompted us to consider whether HSF1 facilitates promoter methylation by recruiting DNMTs to MIR137HG promoter. What we found was that HSF1 interacted with DNMT3a, one DNMT critical for *de novo* DNA methylation, but not DNMT3b and DNMT1 (Figure 5E). DNMT3a did interact with MIR137HG promoter (Figures 5F and S3D), which

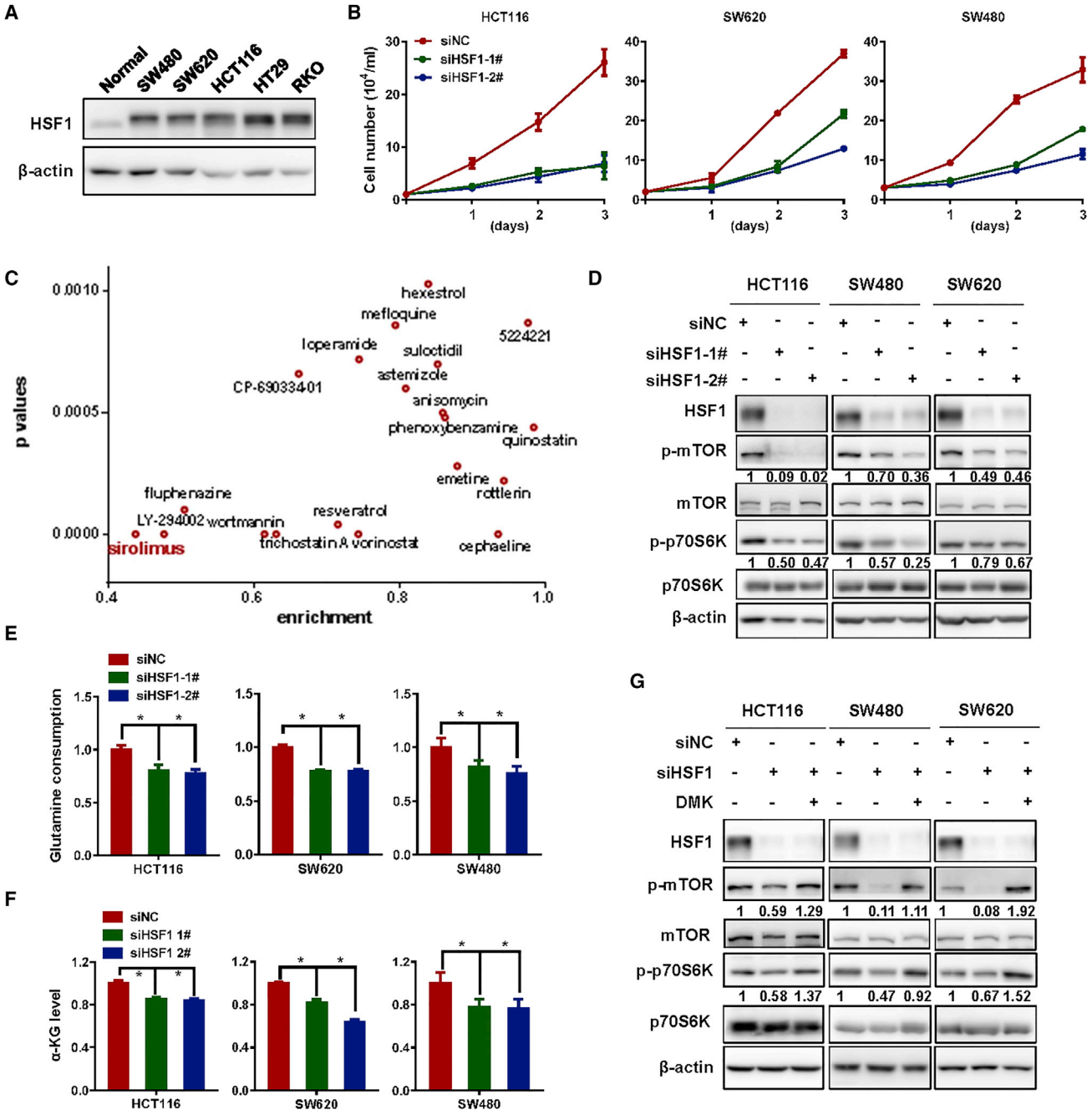

Figure 2. HSF1 Promotes mTOR Activation and Glutaminolysis in CRC

(A) The expression of HSF1 in colorectal cancer cell lines was determined by western blotting. Normal indicates normal colon mucosa tissues. (B) Cells growing in the presence or absence of HSF1 were counted for three days. (C) Chemicals influencing gene expression in a similar manner to HSF1 depletion were screened by connective map analysis. (D) The effect of HSF1 knockdown on mTOR activation in CRC cells was analyzed by western blotting. The band intensities were quantified and normalized their counterparts to calculate the relative intensity as shown below the band. (E and F) The effect of HSF1 knockdown on glutamine metabolism was detected by metabolites analyses (E, glutamine consumption; F, α-KG level). (G) The effect of HSF1 knockdown on mTOR activity in CRC cells treated with exogenous a-KG (DMK) was analyzed by western blotting. All experiments were repeated 3 times and the representative results shown. The asterisks indicate statistical difference ($p < 0.05$). (B, E, and F) Representative results from three experiments were shown as mean ± SD.

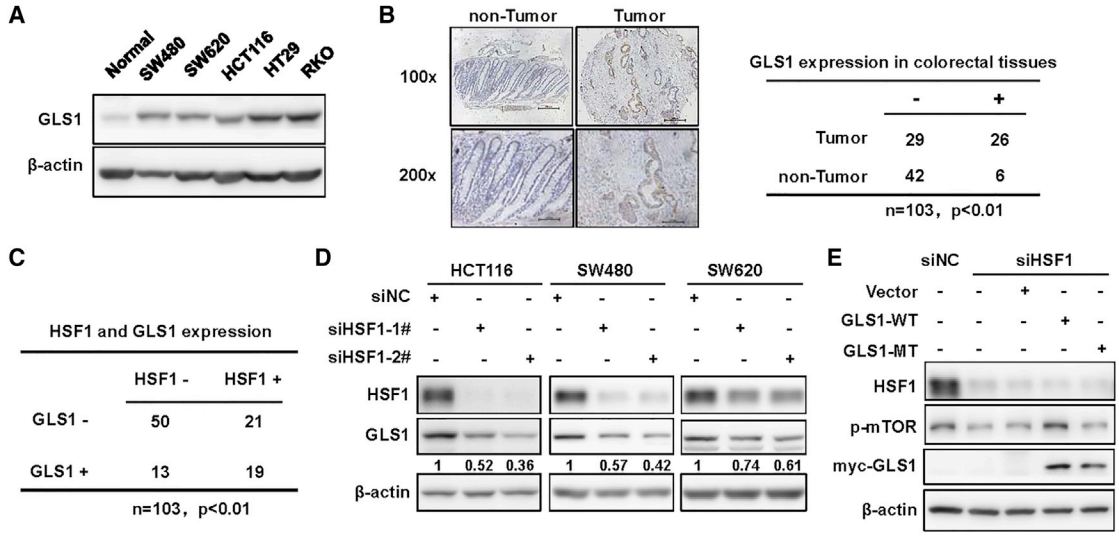

Figure 3. HSF1 Stimulates GLS1 Protein Expression

(A) The expression of GLS1 in colorectal cancer cell lines was determined by western blotting, as in Figure 2A. (B) The expression of GLS1 protein in colorectal tissues was analyzed by immunohistochemical staining (chi-square test; $p < 0.01$). (C) The correlation between HSF1 expression and GLS1 expression in colorectal tissues was analyzed by chi-square test ($p < 0.01$). (D) The protein level of GLS1 before and after HSF1 knockdown was analyzed by western blotting. (E) The effects of HSF1 knockdown on mTOR activation in CRC cells treated with wild-type (WT) or enzyme-activity-deficient mutant (MT) GLS1 were analyzed by western blotting.

was greatly attenuated once HSF1 was depleted (Figure 5G). To further prove the potential regulation of DNA methylation by HSF1, we analyzed GEO data of upregulated genes by pharmacological demethylation (NCBI GEO: GSE79041) as well as HSF1 ChIP sequencing (ChIP-seq) results (NCBI GEO: GSE57398) in CRC cells and identified several HSF1 target genes, such as NXT2, DUSP1, and KCTD11, were subjected to promoter methylation-dependent silencing (Figure 5H), thus indicating that HSF1 was capable of facilitating promoter methylation to inhibit target gene transcription. In summary, HSF1 recruited DNMT3a to facilitate the promoter methylation of lncRNA MIR137HG and in turn decreased MIR137HG-MIR137 expression.

Pharmacological Inhibition of HSF1 Retarded Cell Growth *In Vitro*

All of these results suggest that HSF1 could be a potential target for CRC treatment. As a fact, HSF1 inhibitor KNK437 inhibited viability (Figure 6A) and induced apoptosis (Figures 6B and 6C) in CRC cells. In line with genetic HSF1 depletion, KNK437 significantly upregulated the expressions of MIR137 and MIR137HG (Figure 6D) while inhibiting GLS1 expression and glutamine metabolism and inactivating mTOR signaling (Figures 6E and 6F). Overall, chemical inhibition of HSF1 phenocopied HSF1 depletion to inhibit glutaminolysis in CRC.

Pharmacological Inhibition or Genetic Deficiency of HSF1 Suppresses Colorectal Carcinogenesis *In Vivo*

In light of these *in vitro* findings, we further explored the relevance of HSF1 in colorectal carcinogenesis with azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced mice, a well-established animal model for colorectal carcinogenesis (Figure S4A).[37] Both HSF1 and GLS1 proteins were increased, whereas the level of Mir137 was decreased, in colon tissues from AOM/DSS-treated mice (Figures 7A and 7B), confirming the validity of this model for exploring the relevance of HSF1 to colorectal carcinogenesis. Strikingly, after 10 weeks of treatment with KNK437, AOM/DSS-induced mice showed a significant decrease in both the number and the size of colorectal tumors (Figure 7C). Meanwhile, KNK437 treatment decreased GLS1 protein levels whereas Mir137 expression was increased (Figures 7D and 7E). Concerning the potential off-target effects of the chemical inhibitor, we generated HSF1 knockout mice with CRISPR/Cas9 approach (Figure S4B). Like their KNK437-treated counterparts, mice with HSF1 deficiency, either heterozygous knockout or homozygous knockout, expressed less GLS1 protein but more Mir137 and developed less tumors after AOM/DSS induction (Figures 7F–7H). Therefore, by repressing the expression of MIR137 that targets GLS1, HSF1 is critical for the enhanced glutaminolysis in the development of colorectal tumors and could be targeted for the intervention of colorectal carcinogenesis.

DISCUSSION

HSF1 promoted cell survival and restored global protein quality under stress conditions, such as heat shock.[38,39] Recent evidence indicated that HSF1 has the capacity to facilitate the development of various cancers.[40] In this study, we found that HSF1 stimulated GLS1-dependent glutaminolysis and mTOR activation to promote colorectal carcinogenesis by recruiting DNMT3a to epigenetically suppress the expression of GLS1-targeting MIR137 (Figure 7I). Inhibition of HSF1, either chemically or genetically, succeeded in inhibiting colorectal carcinogenesis both *in vitro* and *in vivo*.

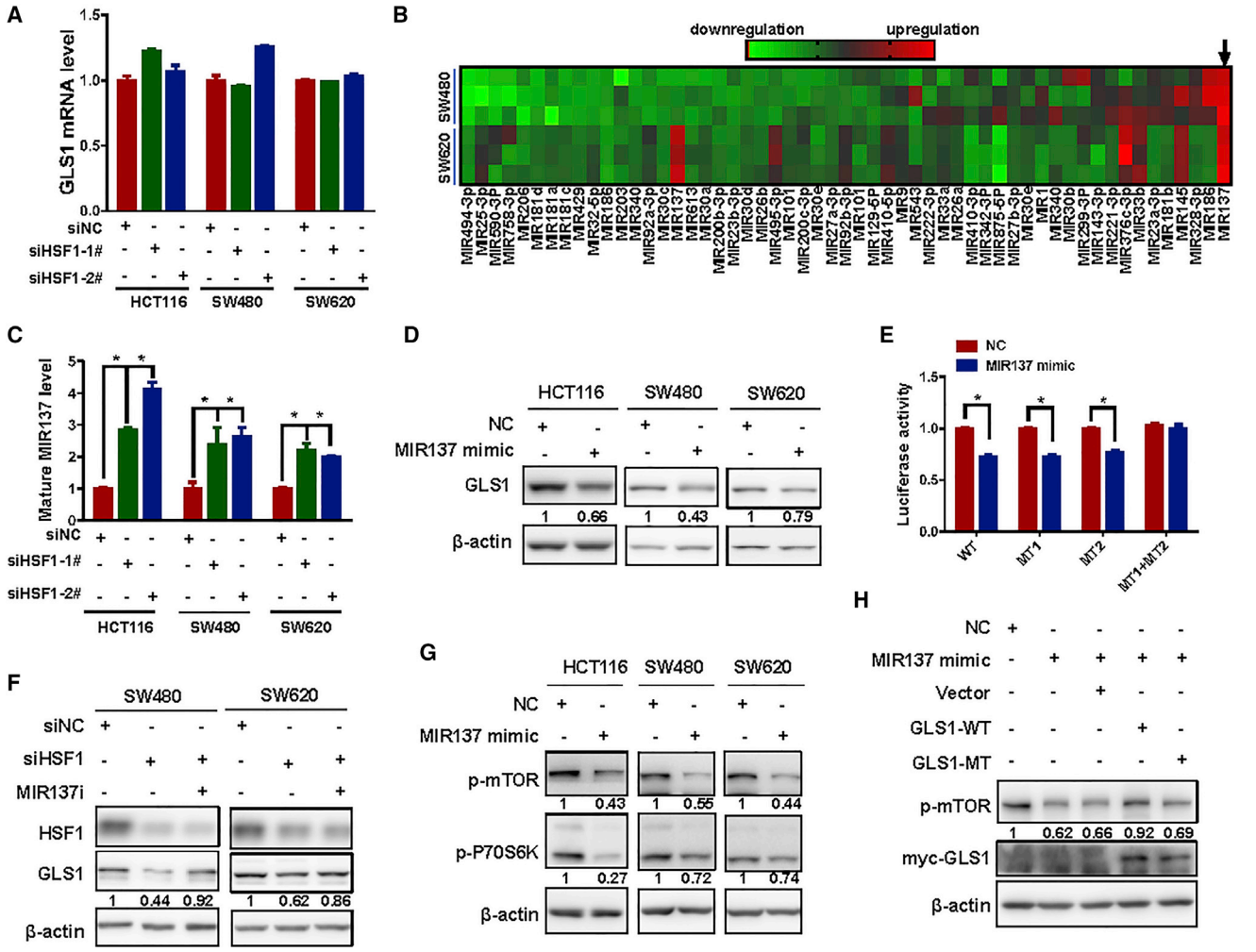

Figure 4. HSF1 Inhibits the Expression of MIR137 that Targets GLS1

(A) The mRNA level of GLS1 before and after HSF1 knockdown was analyzed by RT-PCR. (B) The effect of HSF1 knockdown on the expression of a group of microRNAs that may target GLS1 3′ UTR was analyzed by RT-PCR. (C) The expression of MIR137 in CRC cells with or without HSF1 knockdown was determined by RT-PCR (*$p < 0.05$). (D) Immunoblot analysis of GLS1 protein levels in CRC cells upon transfection of MIR137 mimics is shown. (E) Luciferase assays of GLS1 3′ UTR constructions with intact and mutated seed sequences for miR137 are shown (*$p < 0.05$). (F) The effect of MIR137 inhibitor (MIR137i) on HSF1 knockdown-induced GLS1 downregulation was analyzed by western blotting. (G) The mTOR activity in CRC cells treated with miR137 mimics was analyzed by western blotting. (H) The effect of MIR137 mimics on mTOR activity in CRC cells treated with WT GLS1 or MT GLS1 was analyzed by western blotting. (A, C, and E) Representative results from three experiments were shown as mean ± SD.

HSF1 has multifaceted roles in malignant transformation. It exerts its effect mainly by activating oncogenic proteins, regulating energy metabolism and promoting the formation of cancer cell polyploidy.[41] For instance, HSF1 could function downstream of HER2 to modulate glycolysis, whereas it decreased the activity of AMP kinase (AMPK) to foster lipid consumption in HCC.[19,42] Our study for the first time found that HSF1 has a profound influence on glutamine metabolism.

Though classified as one of the nonessential amino acids, glutamine is indispensable to cancer cells, not only for energy supply but also for macromolecular synthesis. In our previous reports, we found glutamine deprivation reduced the growth of CRC cells and GLS1 was overexpressed to promote glutaminolysis in CRC. Targeting GLS1 in combination with autophagy inhibition could effectively inhibit *in vitro* growth of CRC cells.[43] However, how GLS1 expression is upregulated in CRC remained unknown. Herein, we found that HSF1 stimulated GLS1 protein expression in CRC by suppressing the expression of MIR137. It has been reported that GLS1 was also regulated by other miRNAs, such as MIR23.[44] However, we failed to find the upregulation of these miRNAs upon HSF1 depletion in CRC cells. Therefore, distinct miRNAs might be involved in the epigenetic regulation of GLS1 expression in different cancers. Certainly, alternative mechanisms in addition to miRNA-mediated targeting may exist in other types of cells.

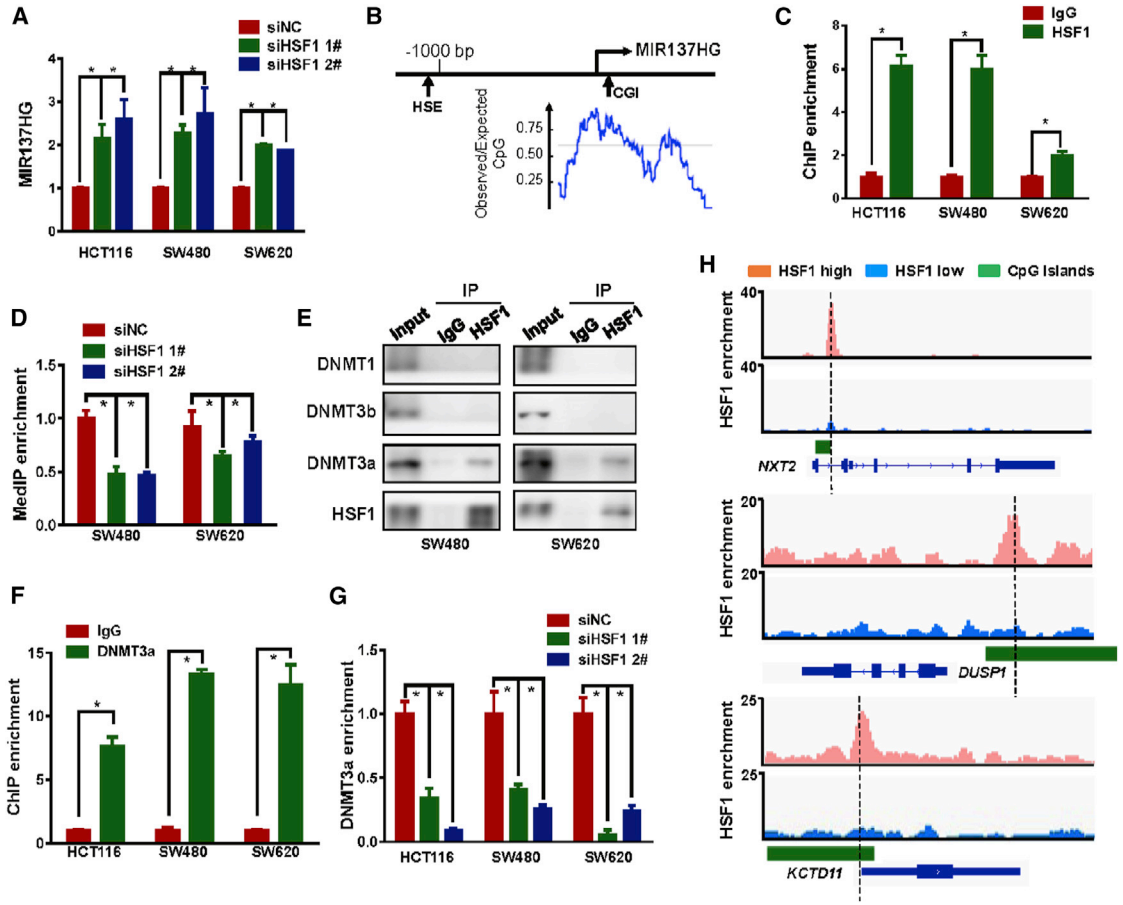

Figure 5. HSF1 Bound DNMT3a and Recruited It to Facilitate the Promoter Methylation of lncRNA MIR137HG

(A) The expression of MIR137HG in CRC cells with or without HSF1 knockdown was determined by RT-PCR (*$p < 0.05$). (B) Schematic diagraph depicting MIR137HG promoter is shown. (C) Binding of HSF1 to the MIR137HG promoter was analyzed by ChIP in CRC cells (*$p < 0.05$). (D) The methylation of the MIR137HG promoter before and after HSF1 knockdown was analyzed by MedIP (*$p < 0.05$). (E) The interaction between HSF1 and methyltransferases (DNMTs) was detected by co-immunoprecipitation (co-IP). (F) Binding of DNMT3a to the MIR137HG promoter was analyzed by ChIP in CRC cells (*$p < 0.05$). (G) Binding of DNMT3a to the MIR137HG promoter, before and after HSF1 knockdown, was analyzed by ChIP in CRC cells (*$p < 0.05$). (H) Representative HSF1 ChIP-seq tracks (NCBI GEO: GSE57398) for genes upregulated by demethylation in colorectal cancer cells (NCBI GEO: GSE79041) are shown. (A, C, D, F, and G) Representative results from three experiments were shown as mean ± SD.

HSF1 has been well known to execute its function by activating rapid transcription of HSP genes.[38,39] To our surprise, HSF1 actually inhibited the transcription of MIR137 by directly interacting with MIR137HG promoter. Importantly, promoter methylation has been known to be responsible for the epigenetic silencing of MIR137 in many cancers.[19,45,46] Whereas DNA methylation has been well recognized to play important roles in suppressing gene transcription, the regulation of *de novo* methylation was largely unknown. DNMTs generally lack classical DNA-binding motifs and are unable to directly interact with DNA. Therefore, the catalytic formation of DNA methylation should rely on the recruitment of DNMTs to the promoter by DNA-binding molecules, such as transcription factors or lncRNAs.[47] Intriguingly, we found that HSF1 interacted with DNMT3a and recruited it to the promoter of lncRNA MIR137HG, which can generate MIR137 through a series of maturation processes. Hence, our study indicated the scaffolding role of HSF1 and illuminated how DNMT3a mediates DNA methylation of HSF1 target genes. Based on *in silico* analysis of the high-throughput gene expression profiling and ChIP-seq data, some HSF1 targets were actually subjected to the regulation of DNA methylation. Therefore, by means of binding to HSE, HSF1 could epigenetically suppress gene expression in addition to activating gene transcription. Certainly, the detailed regulation of HSF1-mediated promoter methylation in response to various stimuli merits further investigation. Nonetheless, our study provides a model in which miRNA-generating lncRNA was regulated by DNA methylation through interplays between the transcription factor and DNA methylation machinery.

Given the crucial role of glutamine metabolism and mTOR signaling to human carcinogenesis, targeting HSF1 to inhibit GLS1-dependent mTOR activation represents a novel strategy for the treatment of human cancers. Both HSF1 knockdown and

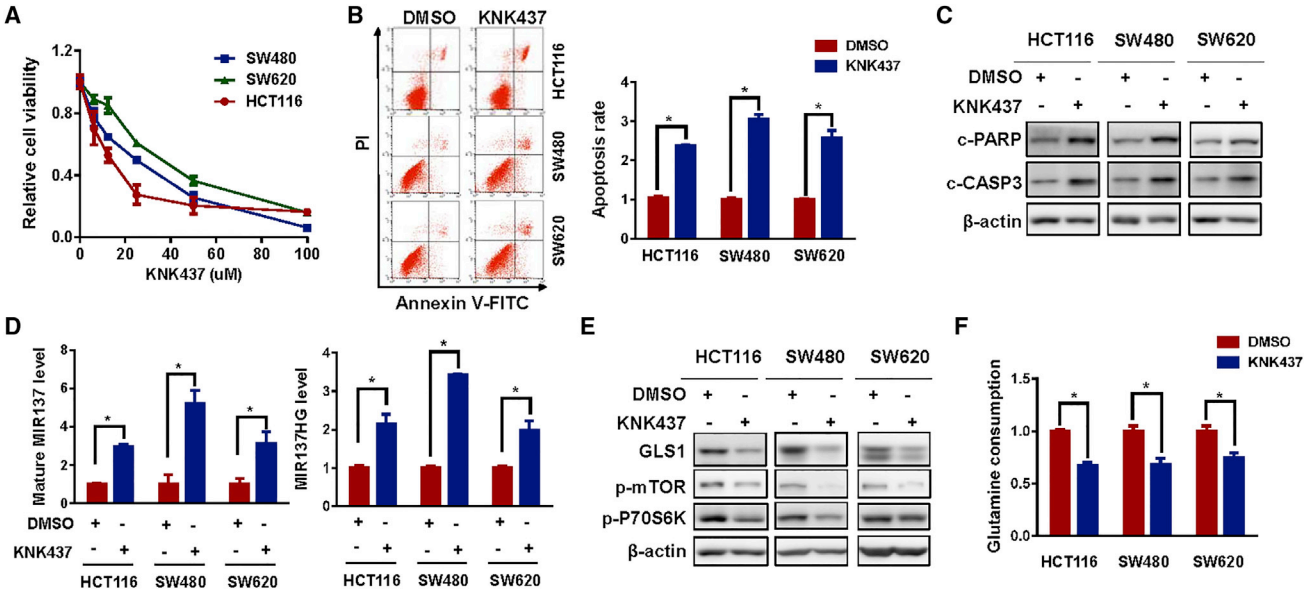

Figure 6. Pharmaceutical Inhibition of HSF1 Retarded Cell Growth *In Vitro*

(A) The effect of HSF1 inhibitor-KNK437 on viability of CRC cells was explored by MTS assay. (B) The effect of HSF1 inhibitor-KNK437 (40 μM) on apoptosis of CRC cells was assessed using flow cytometry after PI (propidium iodide) and annexin V-fluorescein isothiocyanate (FITC) staining. (C) Apoptosis of CRC cells treated with or without KNK437 was detected by western blotting. (D) The expressions of MIR137 and MIR137HG in CRC cells treated with or without HSF1 inhibitor-KNK437 was determined by RT-PCR (*$p < 0.05$). (E) The effects of HSF1 inhibitor-KNK437 on GLS1 expression and mTOR activation were detected by western blotting. (F) The effect of HSF1 inhibitor-KNK437 on glutamine metabolism was detected by metabolites analyses (*$p < 0.05$). (A, B, D, and F) Representative results from three experiments were shown as mean ± SD.

chemical inhibition suppressed the growth of CRC cells. Our data also demonstrated that HSF1 inhibitor KNK437 or genetic HSF1 deficiency led to a reduced number and size of tumors in AOM/DSS-induced mice. These results provided preclinical proof that targeting HSF1 could be a deliverable therapeutic approach in the clinical management of CRC. Nevertheless, there are still some key questions remaining open, including how HSF1 is activated in CRC. Under stress conditions, HSF1 is activated within a few seconds by posttranslational actions.[48,49] However, this paradigm could not be in accordance with the case of HSF1 activation in tumors where both mRNA and protein were increased.[8] It is likely that a series of processes, including the regulation of transcription, translation, and protein activation, work cooperatively to increase HSF1 activity in the chronic process of tumorigenesis.

Collectively, by recruiting DNMT3a to epigenetically suppress the expression of MIR137 that targets GLS1 mRNA, HSF1 stimulated GLS1-dependent mTOR activation to promote colorectal carcinogenesis. Inhibition of HSF1, chemically or genetically, alleviated the development of CRC both *in vitro* and *in vivo*. Therefore, targeting HSF1 to inhibit mTOR activation might be a valuable approach for the clinical management of patients with CRC.

MATERIALS AND METHODS

Cell, Antibodies, and Chemicals

Human CRC cell lines were obtained from the American Type Culture Collection (ATCC). All cells were routinely cultured in RPMI-1640 (Invitrogen; 11875-093) or DMEM (Invitrogen; 11965-092) supplemented with 10% fetal bovine serum. All cells were incubated at 37°C with 5% CO2 and 95% humidity. The following antibodies were used for western blotting (WB; 1:500 or 1:1,000) and IHC studies: HSF1 (ab52757; Abcam; 1:250 for IHC); GLS1 (ap8809b; Abgent; 1:100 for IHC); cleaved poly ADP-ribose polymerase (PARP) (9541s; Cell Signaling Technology); cleaved caspase 3 (9661s; CST); p-mTOR (5536S; CST); p-p70s6K (9205s; CST); β-actin (4970L; CST); DNMT1 (ab13537; Abcam); DNMT3a (ab2850; Abcam); and DNMT3b (ab2851; Abcam). KNK437, AZA, and cycloheximide were purchased from Sigma-Aldrich (SML0964, A3656, and R750107, respectively). TSA was obtained from Selleck (S1045).

Plasmid Construction

The coding segment of GLS1 was cloned by PCR and inserted into the vector pPGKPuro02. The mutation in Ser286 (S286A) in GLS1 coding sequence (CDS) was generated by Quick Change Site-Directed Mutagenesis (600674-51; Stratagene, USA). The mutation primer was listed in Table S1.

siRNA and miRNA Mimics/Inhibitors Transfection

2×10^5 cells were plated overnight in 6-well plates and subsequently transfected with small interfering RNA (siRNA) or miRNA mimics/inhibitors using Lipofectamine RNAiMax (Invitrogen), according to the manufacturer's instructions. The medium was changed after 24 hr transfection, and the total time for transfection was 72 hr, unless

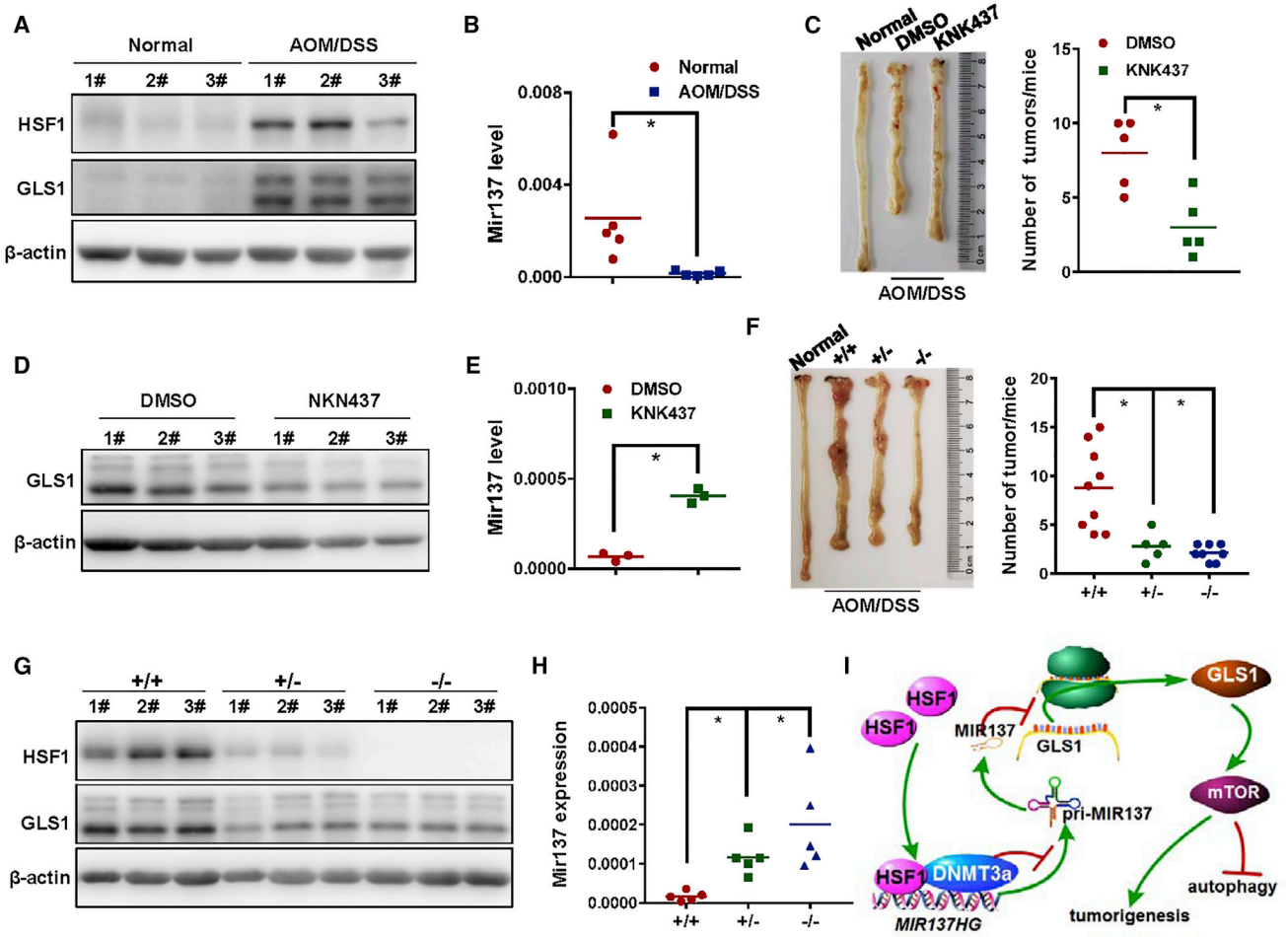

Figure 7. Pharmaceutical Inhibition or Genetic Knockout of HSF1 Suppressed Colorectal Carcinogenesis *In Vivo*

(A) Expression of HSF1 protein in colon mucosa of AOM/DSS-treated mice was analyzed by western blotting. (B) Mir137 expression in colon mucosa of AOM/DSS-induced mice was analyzed by RT-PCR (*$p < 0.05$). (C) The number of tumors per mouse in AOM/DSS-induced mice after DMSO or KNK437 treatment was counted (*$p < 0.05$). (D) Expression of GLS1 protein in colon of AOM/DSS-induced mice, treated with or without KNK437, was analyzed by western blotting. (E) Mir137 expression in colon of AOM/DSS-induced mice, treated with or without KNK437, was analyzed by RT-PCR (*$p < 0.05$). (F) The number of tumors per mouse in AOM/DSS-induced wild-type, $Hsf1^{+/-}$, and $Hsf1^{-/-}$ mice was counted (*$p < 0.05$). (G) Expression of HSF1 and GLS1 protein in colon mucosa of AOM/DSS-induced mice was analyzed by western blotting. (H) Mir137 expression in colon mucosa of AOM/DSS-induced wild-type, $Hsf1^{+/-}$, and $Hsf1^{-/-}$ mice was analyzed by RT-PCR (*$p < 0.05$). (I) Working model: HSF1 binds DNMT3a and recruits it to the promoter of MIR137HG, facilitating the methylation of MIR137HG promoter. In doing so, HSF1 increases GLS1 expression to promote glutamine metabolism and mTOR activation in CRC.

otherwise stated. The siRNA duplexes were purchased from Genepharma (Shanghai, China) for transient knockdown and listed in Table S1.

RNA Isolation, Reverse Transcription, and qPCR

Total RNA was isolated using Trizol reagent (Invitrogen; 15596026), and miRNA was extracted by MIRNeasy Mini Kit (QIAGEN; 217004). After the quantification of RNA, 1 μg RNA was used for reverse transcription reaction by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; 4375222). The real-time qPCR was conducted by using SYBR Green Master Mix (Applied Biosystems, USA) to determine mRNA expression and miScript PCR system (QIAGEN; 218073) for miRNA expression analysis. GAPDH or U6 small nuclear RNA (snRNA) were used for the normalization. The primers used in this study were listed in Table S1.

Luciferase Activity Assay

3′ UTR segment of the GLS1 was cloned by PCR and inserted into the vector pMIR-REPORTER (Promega). The primers used were shown in Table S1. The mutation of two MIR137 binding sites in GLS1 3′ UTR were generated using the same methods as described above. The resultant plasmids were co-transfected with MIR137 mimics by using X-GENE (Roche; 06366236001). After 48 hr transfection, the luciferase activity was measured by the Dual-GLO Luciferase Assay System (Promega; E2940).

Metabolites Analyses

2×10^5 cells were seeded in 6-well plates overnight. After the indicated treatments, the concentrations of glutamine, ATP, and α-KG were measured by Glutamine Assay Kit (Abnova; KA1627), ATP Assay Kit (Beyotime; S0026), and α-Ketoglutarate Colorimetric/Fluorometric Assay Kit (BioVision Technologies; K677-100), based on the protocols provided in the kit.

Immunoblotting

CRC cells treated as indicated were washed once by PBS and lysed by radioimmunoprecipitation assay (RIPA) buffer. After protein quantification, cell lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes for primary antibody incubation overnight at 4°C, after blocking with 5% skim milk in Tris-buffered saline with Tween (TBST) (0.01M TRIS-HCl buffer; 8.8 g/L NaCl; 0.1% Tween-20). The membranes were washed three times by TBST and then incubated with the secondary antibody conjugated with horseradish peroxidase (HRP) (1:5,000; 111-035-003; Jackson ImmunoResearch Laboratories, USA) at 37°C for 2 hr. Finally, the membranes were visualized with enhanced chemiluminescence (EMD Millipore; 17-373SP).

Immunohistochemistry Analysis

Immunohistochemistry analysis was performed in a tissue array containing 103 cases of colonic tissues, including 55 CRC tissues and 48 non-tumor colon tissues. Briefly, anti-HSF1 antibody (1:250) and anti-GLS1 antibody (1:100) were incubated with tissue sections overnight at 4°C, following antigen retrieval and BSA blocking. After the binding of HRP-conjugated secondary antibody, the slide was incubated with diaminobenzidine (DAB). This was followed by hematoxylin counterstaining, the results of which were assessed by 2 independent pathologists. The results were evaluated by assigning a score of 0–3. Scores were defined as follows: 0, no staining in >10% of the tumor cells; 1, faint staining in >10% of the tumor cells; 2, weak staining in >10% of the tumor cells; and 3, strong staining in >10% of the tumor cells. Slides with scores of 0 and 1 were regarded as negative expression, whereas scores of 2 and 3 indicated positive expression.

MedIP

Methylated DNA immunoprecipitation (MedIP) was performed by a kit according to the manufacturer's instructions (D5101; Zymo Research). Briefly, 5 μg genomic DNA in 500 μL H_2O was fragmented into approximately 200–500 bp. Then, 160 ng sonicated DNA and 1.6 μg anti-5-methylcytosine monoclonal antibody were mixed with protein A beads on a rotator at 37°C for 1 hr. The enriched methylated DNA was collected and analyzed by PCR. The primers used to detect methylated DNA were listed in Table S1.

ChIP

ChIP assay was performed by a kit according to the manufacturer's instructions (no. 9003; CST), using antibodies (1 μg) for HSF1 and DNMT3a. Real-time PCR of the ChIP DNA was performed using the primers listed in Table S1.

Co-immunoprecipitation

Cells were lysed with 1% NP40 buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP40, and 10% glycerol) containing protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail Tablets; Roche). After adequate centrifugation, 500 μg supernatant was incubated overnight at 4°C with 5 μg rabbit monoclonal antibody for HSF1 (1 μg) . The mixture was then added to 50-μL agarose beads by rotating at 4°C for 3 hr. The resultant mixture was washed 3 times by NP40 buffer and subjected to western blotting.

Cell Growth Assay

5,000 cells seeded overnight in a 96-well plate were treated as indicated. The cell viability was measured by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega). The absorbance value of each well was detected by a microplate reader at 490 nm. For cell growth assay, cells were plated overnight in 6-well plates and subsequently transfected with HSF1 siRNA. The cells were then stained with 0.4% trypan blue and the number of cells counted daily.

Apoptosis Detection

Cell apoptosis was measured by flow cytometry analysis and western blotting. For flow cytometer analysis of apoptosis, cells treated as indicated for 72 hr were harvested by trypsin and re-suspended in 100 μL 1× binding buffer. 5 μL fluorescein isothiocyanate (FITC) annexin V and propidium iodide (PI) (556547; BD Biosciences, USA) were added to the cell suspension and then incubated for 15 min at room temperature. After dilution with 400-μL binding buffer, the samples were analyzed by ACS Calibur flow cytometer. For apoptosis detection by western blotting, cleaved caspase 3 and PARP were analyzed by the specific antibodies.

Animal Experiments

Animal care and experiments were conducted in compliance with Institutional Animal Care and Use Committee and NIH guidelines. Fifteen 5-week-old male C57BL/6J mice, purchased from Model Animal Research Center of Nanjing University (MARC, Nanjing, China), were divided randomly into 3 groups. The mice in no. 1 group were given the sterile saline as the control. The other 2 groups were given a signal dose of AOM (10 mg/kg, intraperitoneally [i.p.]) and 2% DSS (molecular weight of 36,000–50,000) diluted in water for 7 days. One week later, the water was replaced with the DSS-free water for two weeks. This DSS feeding cycle was carried out three times before starting the therapeutic protocol. The mice in no. 2 group were intraperitoneally injected with DMSO or KNK437 (10 mg/kg) twice weekly for 10 weeks. Mice were sacrificed at 6 weeks, after the last DSS cycle, and the body weights were measured. The colorectal tissues were dissected, flushed with PBS, and cut open longitudinally along the main axis. The number and size of tumors were recorded. The tumors were fixed in 4% formalin and prepared for IHC analysis. Histopathological changes were identified by H&E staining. HSF1 knockout mice were generated by the CRISPR/Cas9 strategy in the Model Animal Research Center of Nanjing University (MARC, Nanjing, China). $Hsf1^{+/-}$ or $Hsf1^{-/-}$ C57BL/6J mice, along with their

respective controls, were given the same AOM/DSS induction and subsequent operation.

Statistics

All data were expressed as mean ± SD. Unless otherwise stated, Student's t test was performed for statistical significance analysis. p value < 0.05 was considered as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.04.014.

AUTHOR CONTRIBUTIONS

J.L., X.W., and H.J. designed the project. J.L., P.S., T.J., D.D., H.W., J.Z.N., Z.Q.Y., W.X., and L.F. performed experiments. J.L., L.Z., V.Y.S., H.M., X.W., and H.J. analyzed data and drafted the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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