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Article

CXXC5 suppresses hepatocellular carcinoma by promoting TGF-β-induced cell cycle arrest and apoptosis

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Evading TGF-β-mediated growth inhibition is often associated with tumorigenesis in liver, including hepatocellular carcinoma (HCC). To better understand the functions and the underlying molecular mechanisms of TGF-β in HCC initiation and progression, we carried out transcriptome sequencing (RNA-Seq) to identify the target genes of TGF-β. CXXC5, a member of the CXXC-type zinc finger domain-containing protein family, was identified as a novel TGF-β target gene in Hep3B HCC cells. Knockdown of CXXC5 attenuated the expression of a substantial portion of TGF-β target genes and ameliorated TGF-β-induced growth inhibition or apoptosis of Hep3B cells, suggesting that CXXC5 is required for TGF-β-mediated inhibition of HCC progression. Analysis of the TCGA database indicated that CXXC5 expression is reduced in the majority of HCC tissue samples in comparison to that in normal tissues. Furthermore, CXXC5 associates with the histone deacetylase HDAC1 and competes its interaction with Smad2/3, thereby abolishing the inhibitory effect of HDAC1 on TGF-β signaling. These observations together suggest that CXXC5 may act as a tumor suppressor by promoting TGF-β signaling via a positive feedback loop, and reveal a strategy for HCC to bypass TGF-β-mediated cytostasis by disrupting the positive feedback regulation. Our findings shed new light on TGF-β signaling regulation and demonstrate the function of CXXC5 in HCC development.

Keywords: hepatocellular carcinoma (HCC), TGF-β, CXXC5, HDAC, signaling regulation

Introduction

Hepatocellular carcinoma (HCC) accounts for ~80% cases in liver cancer, which ranks as the fifth most common cancer type and the third cause of cancer-related death worldwide (Dooley and ten Dijke, 2012; El-Serag, 2012; Li and Wang, 2016; Marquardt et al., 2015). Distinct from other cancers that usually arise from normal tissues, HCC is often developed in a setting of chronic liver diseases like hepatitis, fibrosis and cirrhosis. As a consequence, most HCC patients have a bad outcome due to late diagnosis. Similar to other cancers, HCC encompasses all the hallmarks of cancer, and deregulation of transforming growth factor-β (TGF-β) signaling has been documented to contribute to several of the hallmarks, such as evading growth suppressors, inducing angiogenesis, escaping from immune surveillance, activating invasion and metastasis, and others (Massague, 2008; Hanahan and Weinberg, 2011; Katz et al., 2016).

TGF-β is capable of eliciting various cellular responses, including cell cycle arrest, apoptosis, differentiation, extracellular matrix deposition, cell migration, etc. (Massague, 2008; Hata and Chen, 2016; Heldin and Moustakas, 2016; Hill, 2016; Morikawa et al., 2016). TGF-β initiates signal transduction by binding to two types of transmembrane receptors with intrinsic Ser/The kinase activity, namely the type I receptor TβRI and the type II receptor TβRII. In the complex, TβRII phosphorylates and activates TβRI, which then propagates signaling by phosphorylating Smad2 and Smad3 and inducing their binding to Smad4. The heteromeric Smad complex are accumulated in the nucleus and function as transcription factors to regulate target gene transcription. In addition, TGF-β can also exert its pathophysiological

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functions by activating other intracellular signaling molecules, such as PI3K-Akt, MAPKs, PAK2, small GTPases, and LIMK1/2, depending on cell types and contexts (Zhang et al., 2013; Zhang, 2017).

TGF-β/Smad signaling suppresses development of a broad spectrum of cancer types, especially those derived from epithelial cells including hepatocytes, by inhibiting cell proliferation and inducing apoptosis (Massague, 2008; Giannelli et al., 2011; Katz et al., 2016; Zhang et al., 2017). Thus, TGF-β receptors and Smads are hot spots for cancerous mutations (Levy and Hill, 2006; Katz et al., 2016). Alternatively, tumor cells evade TGF-β-mediated growth inhibition via downregulation of TGF-β/Smad signaling or upregulation of mitogenic signaling (Giannelli et al., 2011; Hanahan and Weinberg, 2011; Dooley and ten Dijke, 2012; Katz et al., 2016).

CXXC5 belongs to the CXXC-type zinc finger domain-containing protein family, which also includes KDM2A/2B, MLL1/2, DNMT1, TET1/3, and others (Blackledge et al., 2013; Long et al., 2013). These factors can regulate gene transcription by binding to CpG islands in gene promoters and modulating DNA methylation or histone methylation via their intrinsic chromatin-modifying abilities or recruiting chromatin-modifying enzymes. Although CXXC5 has been shown to modulate the expression of several genes like COX4, myelin, and CD40L (Aras et al., 2013; Tsuchiya et al., 2016; Kim et al., 2016b), the molecular mechanism is less understood. CXXC5 has also been shown to regulate cellular signaling pathways. CXXC5 is a downstream target and mediator of BMP signaling in neural stem cells and endothelial cell differentiation (Andersson et al., 2009; Kim et al., 2014). CXXC5 has been reported to interact with Smad2 and Smad3 to regulate zebrafish heart development (Wang et al., 2013; Peng et al., 2016). In addition, CXXC5 is a binding partner of Dishevelled and ameliorates Wnt/β-catenin signaling in neural stem cells, zebrafish embryonic kidney development, osteoblast differentiation, and cutaneous wound healing (Andersson et al., 2009; Kim et al., 2010, 2015; Kuhnl et al., 2015; Lee et al., 2015). Furthermore, disrupting the Dvl-CXXC5 interaction with small molecule inhibitors has been proposed for bone anabolic osteoporosis therapy (Kim et al., 2016a). In agreement with the important functions in different tissues, altered expression of CXXC5 has been connected with cancer development. CXXC5 is downregulated in acute myeloid leukemia, while it is overexpressed in a subset of breast cancers (Knappskog et al., 2011; Bruserud et al., 2015; Kuhnl et al., 2015).

Evading TGF-β-mediated growth inhibition is critical for HCC initiation and progression, but the underlying molecular mechanism is yet to be clarified. In this study, we identified CXXC5, but not other members of the CXXC-type zinc-finger family, as a novel target gene of TGF-β in HCC cells. CXXC5 in turn promotes TGF-β/Smad signaling, forming a novel positive feedback regulatory loop. It plays an important role in TGF-β-induced cell cycle arrest and apoptosis. In mechanism, CXXC5 associates with HDAC1 in competition with Smad2/3, thereby enhancing the transcriptional activity of Smad2/3 proteins. Together, these results shed new light on TGF-β signaling regulation and provide evidence that CXXC5 acts as a tumor suppressor in HCC.

Results

Genome-wide identification of TGF-β target genes in Hep3B cells

The context-dependent target genes play a pivotal role in mediating the pathological functions of TGF-β in HCC initiation and development (Giannelli et al., 2011; Dooley and ten Dijke, 2012). Therefore, we attempted to identify the target genes of TGF-β in hepatoma cells. To this end, Hep3B cells were treated with 100 pM TGF-β1 for 4 h, and RNAs were extracted for sequencing (RNA-Seq). Using >1.4-fold change for upregulation and <0.65-fold change for downregulation as cutoff, 474 genes were identified as TGF-β targets as shown in the heat map (Figure 1A) and volcano plot (Figure 1B). Among them, 375 genes are transcriptionally activated whereas 99 genes were repressed (Supplementary Dataset S1). Some of known TGF-β target genes were found among them, such as JUNB, INHBE, FST, Furin, Smad7, Serpine1 (PAI1), and c-myc, verifying the reliability of the experiment (Figure 1C).

Then, we continued to analyze genes that are differentially expressed in response to TGF-β stimulation using gene ontology (GO) and KEGG enrichment analyses (DAVID website) (Huang da et al., 2009; Jiao et al., 2012). Results of GO classification showed that the most enriched biology processes include gene transcription and regulation, cell adhesion, signal transduction, regulation of cell proliferation and cell cycle progression, and apoptosis (Figure 1D). The KEGG pathway analysis revealed that genes involved in TGF-β signaling were the most significantly enriched, a strong indication of feedback regulation of TGF-β signaling in HCC. Besides, p53, cell cycle, Wnt, and PPAR pathways were also highly enriched (Figure 1E), all of which have been shown to play crucial roles in HCC development. Quantitative RT-PCR (q-PCR) was performed to verify the RNA-seq data, and representative q-PCR validation is shown in Figure 1F, including some known target genes and some newly identified genes like CXXC5, Bcl2-modifying factor (BMF), and sterile α motif domain-containing 11 (SAMD11).

TGF-β induces CXXC5 expression in HCC

To validate the transcriptional regulation of CXXC5 gene by TGF-β, we examined the CXXC5 mRNA expression after TGF-β1 stimulation in Hep3B cells. As shown in Figure 2A, TGF-β1 induced the mRNA expression of Smad7 and PAI1, two typical TGF-β target genes, in distinct modes. The Smad7 mRNA level culminated at 1 h after TGF-β treatment and then decreased, while the PAI1 mRNA expression was elevated gradually. Similar to PAI1 expression, the CXXC5 mRNA expression was also increased gradually by TGF-β1. Accordantly, TGF-β1 enhanced the CXXC5 protein levels in both Hep3B and HepG2 cells (Figure 2B), and inhibition of TGF-β signaling by SB431542, a chemical inhibitor of TβRI, decreased CXXC5 protein expression level in Hep3B cells (Figure 2C). To explore the universality of TGF-β induction of CXXC5 expression, the protein levels of CXXC5 were examined upon TGF-β1 stimulation in different cell

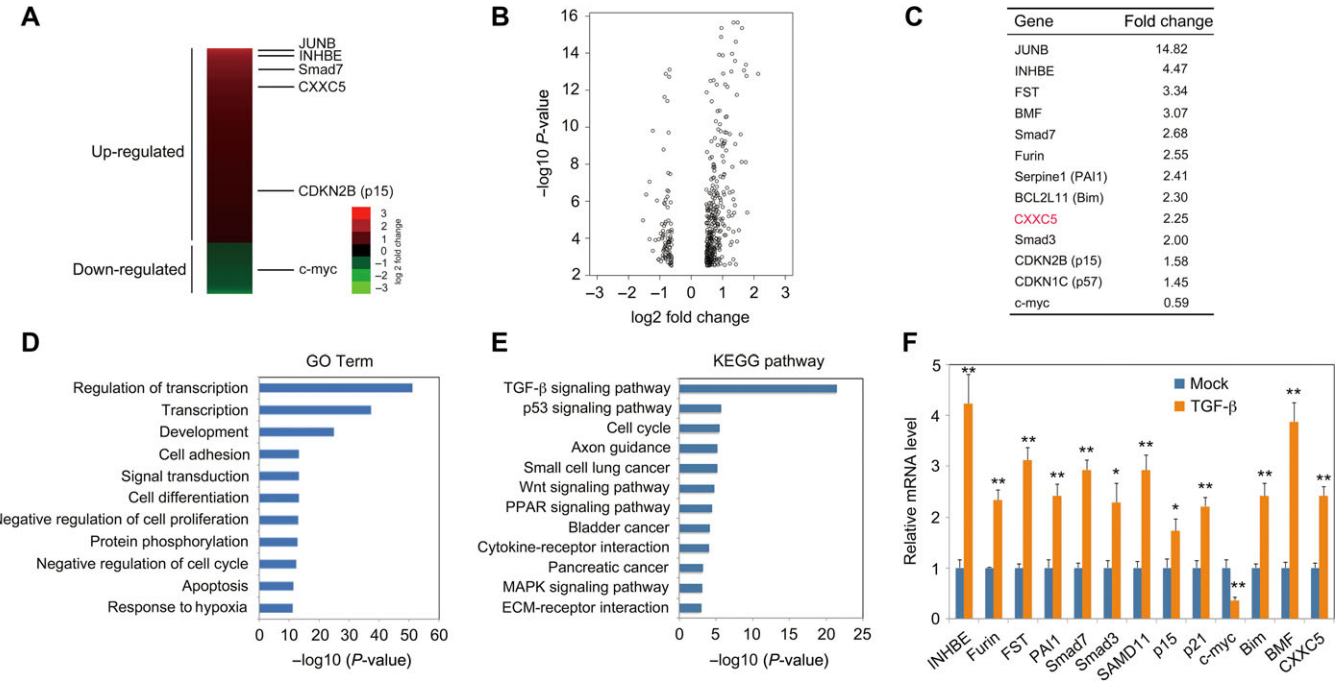

Figure 1 Genome-wide identification of TGF-β target genes in Hep3B cells by transcriptome sequencing. (**A**) Heat map classification of DEGs upon TGF-β1 treatment in HCC cells. Hep3B cells were stimulated with or without 100 pM TGF-β1 ligand for 4 h before being subjected to RNA isolation and transcriptome sequencing. Genes whose expression was altered by a fold change >1.4 for upregulated genes and <0.65 for downregulated genes, with a *P*-value <0.05, were regarded as biologically significant and TGF-β target genes. Color encoded relative gene expression levels are expressed in log2 scale. (**B**) Volcano plots for all DEGs ($P < 0.05$, fold change <0.65 or >1.4) in comparison. The differential expression (log2 fold change for TGF-β-stimulated samples compared to control) is plotted against the –log10 *P*-value for each gene. Each point represents a single gene. Of the DEGs, upregulated genes were shown by solid circles and downregulated genes by open circles. The circles represent significance for both false-discovery rate of $P < 0.05$ and fold change >1.4 or <0.65. (**C**) Representatives of TGF-β target genes, known or newly identified. (**D** and **E**) GO analysis (**D**) and KEGG pathway enrichment (**E**) of TGF-β target genes. (**F**) Experimental verification of representative TGF-β target genes. After being stimulated with 100 pM TGF-β1 for 4 h, Hep3B cells were lysed for RNA extraction and gene expression analysis by q-PCR. Gene expression levels were normalized to that of GAPDH, and *P*-value was calculated for the expression of all the tested genes. *$P < 0.05$, **$P < 0.01$.

lines. TGF-β enhanced CXXC5 expression in a normal hepatocyte cell line HL-7702 and some HCC cell lines including Hep3B, HepG2, and Huh7, but not in MHCC97L or MHCC97H cells (Figure 2D). Together, these results demonstrate that CXXC5 is a novel target gene of TGF-β in normal hepatocyte and a portion of HCC cell lines.

CXXC5 promotes TGF-β/Smad signaling

Feedback regulation plays a critical role in TGF-β/Smad signaling, whereas its deregulation has been associated with HCC development (Giannelli et al., 2011; Yan and Chen, 2011; Dooley and ten Dijke, 2012; Miyazawa and Miyazono, 2017). To explore whether CXXC5 could modulate TGF-β/Smad signaling, reporter assays were performed in HEK293FT cells. Ectopic expression of CXXC5 promoted TGF-β1-mediated expression of three TGF-β/Smad-responsive reporters (CAGA-luciferase, ARE-luciferase, and 3TP-luciferase), in a dose-dependent manner (Figure 3A). Similar results were obtained with the CAGA-luciferase reporter in both the normal liver cell line HL-7702 (Figure 3B) and Hep3B HCC cells (Figure 3C). To consolidate the regulatory role of CXXC5 in TGF-β signaling, we established three Hep3B-derived cell lines that stably expressing two CXXC5-targeting shRNAs (#1 and #2) or the non-specific (NS) shRNA (Figure 3D). CAGA-luciferase reporter assay showed that TGF-β signaling was significantly ameliorated in the two CXXC5-depleted cell lines (Figure 3E).

To test whether CXXC5 functions at the Smad level, we carried out Gal4-luciferase reporter assay, in which Smad3 is fused to the DNA-binding domain (DBD) of Gal4 and recruited to the Gal4-binding element on the reporter plasmid, thus affecting luciferase expression (Yan et al., 2016). Overexpression of CXXC5 enhanced Gal4-Smad3-induced reporter expression in a dose-dependent manner in both Hep3B and HEK293FT cells (Figure 3F), suggesting that CXXC5 modulates TGF-β signaling at the Smad level.

CXXC5 facilitates TGF-β signaling to regulate gene expression

We then assessed the biological consequence of this regulation in terms of gene transcriptional regulation. The Hep3B cell

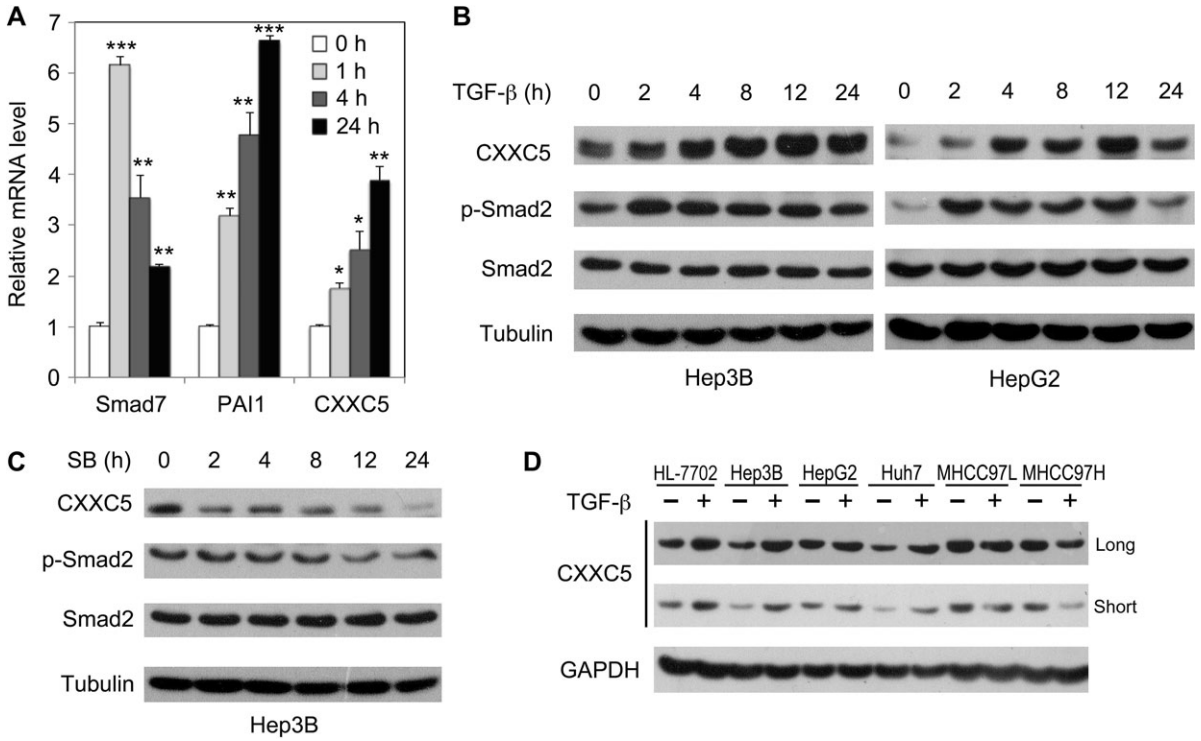

Figure 2 TGF-β induces CXXC5 expression in HCC cells. (**A**) Hep3B cells were treated with or without 100 pM TGF-β1 for the indicated time periods, and then harvested for total RNA extraction and gene expression analysis as in Figure 1**F**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**B** and **C**) Hep3B and HepG2 were treated with 100 pM TGF-β1 (**B**) or 10 μM SB431542 (**C**) for the indicated time periods before being harvested for immunoblotting analyses of protein expression levels. (**D**) Western blot analysis of CXXC5 protein expression in hepatocyte and HCC cells. Cells were treated with 100 pM TGF-β1 for 8 h.

lines stably expressing the non-specific (NS) or the CXXC5-targeting (#1) shRNAs (Figure 3D and E) were treated with or without TGF-β1 for 4 h, followed by total RNA isolation and RNA-Seq. As shown in the heat map (Figure 4A), TGF-β-regulated genes in the control cell line (lane S3 in Figure 4A; fold change >1.5 and <0.6 as the cutoff) were further classified into four clusters based on the effects of TGF-β (activation or repression) and CXXC5 deficiency (Supplementary Dataset S2). As a result, a large portion of the genes, classified in Cluster 1 and Cluster 3, exhibited an attenuated sensitivity to TGF-β upon CXXC5 depletion, in accordance with the above observation that CXXC5 positively regulates TGF-β signaling. GO term analysis of the genes in Cluster 1 and Cluster 3 showed that the most highly enriched GO terms includes apoptosis regulation, transcription regulation, signal transduction, drug response, cell proliferation and differentiation, etc. (Figure 4B). KEGG pathway analysis indicated that TGF-β, Hippo, and FoxO pathways were highly enriched (Figure 4C). Validation of gene expression with q-PCR confirmed the essential role of CXXC5 in TGF-β-mediated transcriptional regulation (Figure 4D). Intriguingly, a portion of TGF-β target genes (Figure 4A, Cluster 2 and Cluster 4) were more sensitive to TGF-β upon CXXC5 knockdown, and the most highly enriched GO terms and KEGG pathways were different to those of Cluster 1 and Cluster 3 (Supplementary Figure S1).

CXXC5 depletion renders HCC cells insensitive to TGF-β-mediated cell cycle arrest and apoptosis

TGF-β inhibits HCC initiation by inducing cell cycle arrest and/or apoptosis (Dooley and ten Dijke, 2012; Katz et al., 2016). To assess the cellular functions of CXXC5, we monitored proliferation of Hep3B cells. As shown in Figure 5A, TGF-β apparently inhibited cell proliferation in control sh-NS Hep3B cells. However, this inhibition was significantly attenuated upon knockdown of CXXC5, indicating an important role of CXXC5 in TGF-β-induced anti-proliferation. Cell cycle analysis by flow cytometry showed that TGF-β was able to induce G2/M arrest in Hep3B cells, in accordance with previous studies (Hashimoto et al., 2003), but this effect was ameliorated by CXXC5 knockdown (Figure 5B). In addition, in cell apoptosis analyses by PI staining or PI/Annexin V double staining followed by cell cytometry analysis, TGF-β induced apoptosis in the control sh-NS Hep3B cells, but this effect was blunted upon CXXC5 knockdown (Figure 5C, D and Supplementary Figure S2). The results together indicate that CXXC5 plays a critical role in TGF-β-mediated cytostatic effects in Hep3B HCC cells.

In line with these observations, gene expression analysis using the TCGA database showed that CXXC5 mRNA levels are significantly decreased in a majority of human HCC tissue samples in comparison to that of normal tissues (Figure 5E and F). Together with the results that CXXC5 depletion attenuated TGF-

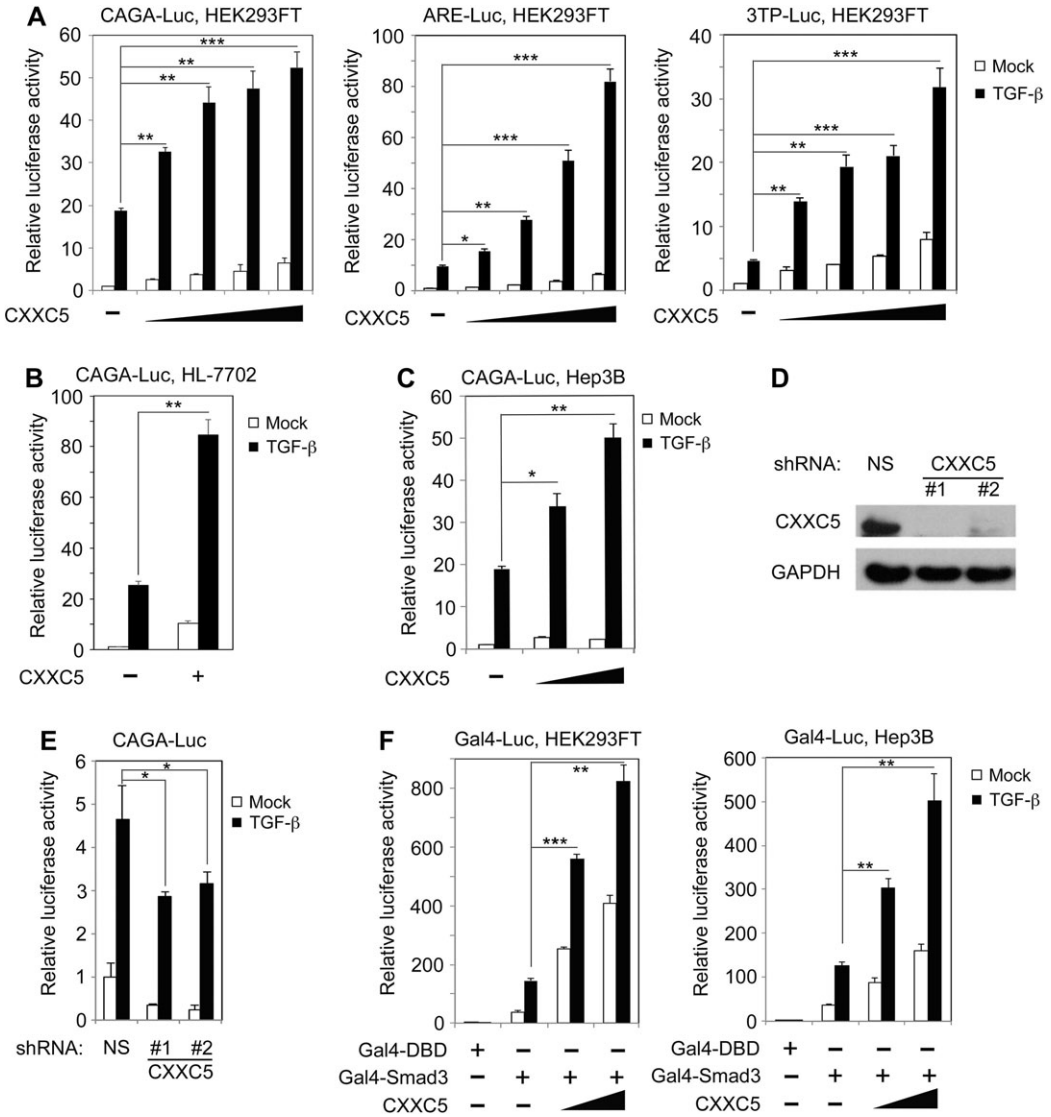

Figure 3 CXXC5 promotes TGF-β signaling. (**A**) HEK293FT cells were transfected with constructs encoding CAGA-luciferase reporter (200 ng, left), ARE-luciferase reporter (200 ng, middle), or 3TP-luciferase reporter (200 ng, right), together with Renilla-luciferase (50 ng), CXXC5-expressing plasmid (10, 25, 50, and 100 ng), or empty vector as indicated. At 20 h post-transfection, cells were treated with or without 100 pM TGF-β1 for another 20 h before harvested for luciferase activity determination. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**B** and **C**) CAGA-luciferase reporter assays were performed in HL-7702 cells (**B**) and Hep3B HCC cells (**C**), respectively. (**D**) Hep3B cells stably carrying the non-specific (NS) or CXXC5-targeting shRNAs were lysed for immunoblotting detection of endogenous CXXC5 protein. GAPDH was detected as loading control. (**E**) The above stable cells were transfected with CAGA-luciferase (200 ng) and Renilla plasmids. After being treated with or without 100 pM TGF-β1 ligand for 20 h, the cells were subjected to luciferase activity measurement. (**F**) HEK293FT cells (left) or Hep3B cells (right) transfected with plasmids encoding Gal4-luciferase (pFR-luciferase) reporter (200 ng) together with Renilla-luciferase (20 ng), Gal4-DBD (50 ng), Gal4-Smad3 (50 ng), and CXXC5 (50 and 100 ng) as indicated were treated with 100 pM TGF-β1 for reporter analyses.

β-induced anti-proliferation and apoptosis in Hep3B cells, these data suggest that CXXC5 may act as a tumor suppressor in HCC.

CXXC5 promotes Smad2/3 activity by disrupting their interaction with HDAC1

To elucidate how CXXC5 facilitates TGF-β/Smad signaling, we assessed the interaction between CXXC5 and Smad2/3 proteins, but found no interaction (Supplementary Figure S3). As histone acetylation plays a crucial role in regulating transcription (Xu et al., 2016), we examined whether CXXC5 could associate with the enzymes with histone acetyltransferase activity or deacetylase activity, including p300, CBP and HDAC1. Intriguingly, CXXC5 was capable of interacting with the histone deacetylase HDAC1 as shown by co-immunoprecipitation (co-IP) (Figure 6A and B), but not with p300 or CBP (data not shown). Domain mapping indicated that the carboxyl terminal CXXC domain

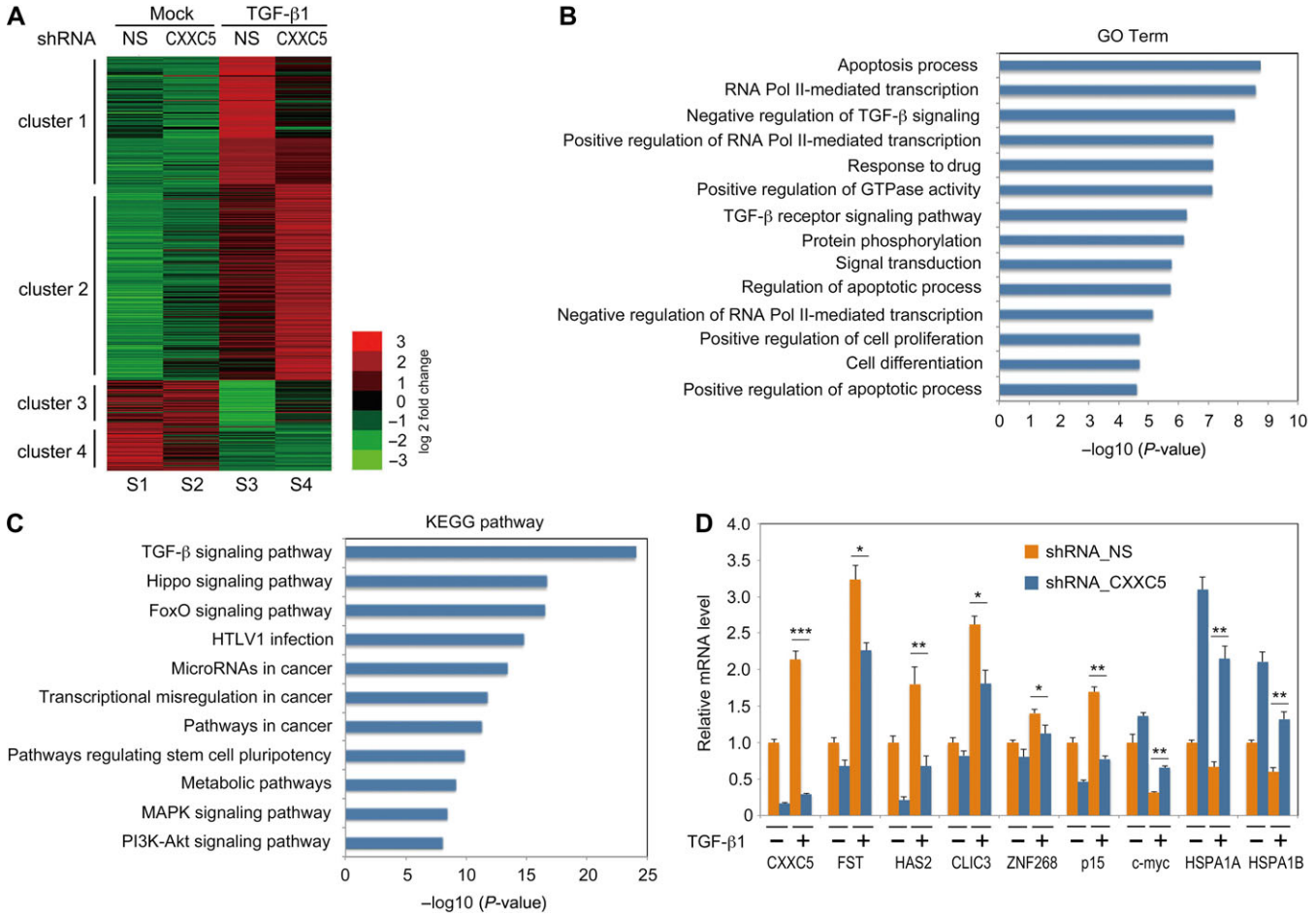

Figure 4 CXXC5 knockdown ameliorates TGF-β-mediated gene transcription. (**A**) Heat map representation of DEGs upon TGF-β1 treatment and CXXC5 knockdown. Hep3B cells stably carrying the non-specific (NS) shRNA or CXXC5-tergeting shRNA (#1) were treated with or without 100 pM TGF-β1 for 4 h. Then total RNAs were isolated for RNA-Seq and gene expression analysis. Similar as that in Figure 1A, DEGs upon TGF-β stimulation (line S3 vs. S1) are those with a fold change >1.5 for upregulated genes or <0.6 for downregulated genes, with a *P*-value <0.05. Relative gene expression levels are expressed in log2 scale. (**B** and **C**) GO analysis (**B**) and KEGG pathway enrichment (**C**) of CXXC5-dependent TGF-β target genes, including TGF-β-activated genes (Cluster 1) and TGF-β-repressed genes (Cluster 3). (**D**) Control or CXXC5 knockdown cell samples were treated with or without 100 pM TGF-β1 ligand for 4 h before being harvested for total RNA isolation and gene expression analysis by q-PCR. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

(255–322 amino acids) mediated the interaction of CXXC5 with HDAC1 (Figure 6C and D). Consistently, this region was necessary and sufficient to enhance TGF-β signaling (Figure 6E).

As HDAC1 can associate with Smad2/3 to inhibit their transcriptional activity (Wotton et al., 1999; Liberati et al., 2001; Xu et al., 2016), we then assessed whether CXXC5 could affect the binding of HDAC1 to Smad2/3 proteins. Indeed, overexpression of CXXC5 in Hep3B cells attenuated the Smad2/3–HDAC1 association as assessed by GST pull-down assay (Figure 6F), suggesting that CXXC5 may deprive HDAC1 away from R-Smad proteins. In concordance with this observation, CXXC5 deficiency enhanced the binding of ectopic Flag-HDAC1 to the promoters of two TGF-β target genes p15 and SnoN (Figure 6G). Functionally, CXXC5, like the histone acetyltransferases p300 and CBP, enhanced TGF-β signaling in CAGA-luciferase reporter assay (Figure 6H) and Smad3 activity in Gal4-luciferase reporter assay (Figure 6I). As shown previously (Wotton et al., 1999; Liberati et al., 2001), HDAC1 reduced the promoting effect of p300 or CBP, but the inhibitory effect of HDAC1 was counteracted by overexpression of CXXC5 (Figure 6H and I). To further validate the essential role of HDAC1 in CXXC5-mediated enhancement of TGF-β signaling, two HDAC1-targeting shRNAs were tested and one of them (#2) was able to reduce HDAC1 expression dramatically when assessed in HEK293FT cells (Supplementary Figure S4). As expected, knockdown of endogenous HDAC1 in Hep3B cells enhanced TGF-β-induced CAGA-luciferase expression, while the promoting effect of CXXC5 was less significant (Figure 6J), suggesting that CXXC5 acts through HDAC1 to promote TGF-β signaling.

Discussion

Although TGF-β/Smad signaling has been shown to act as a key tumor suppressive pathway in HCC (Senturk et al., 2010;

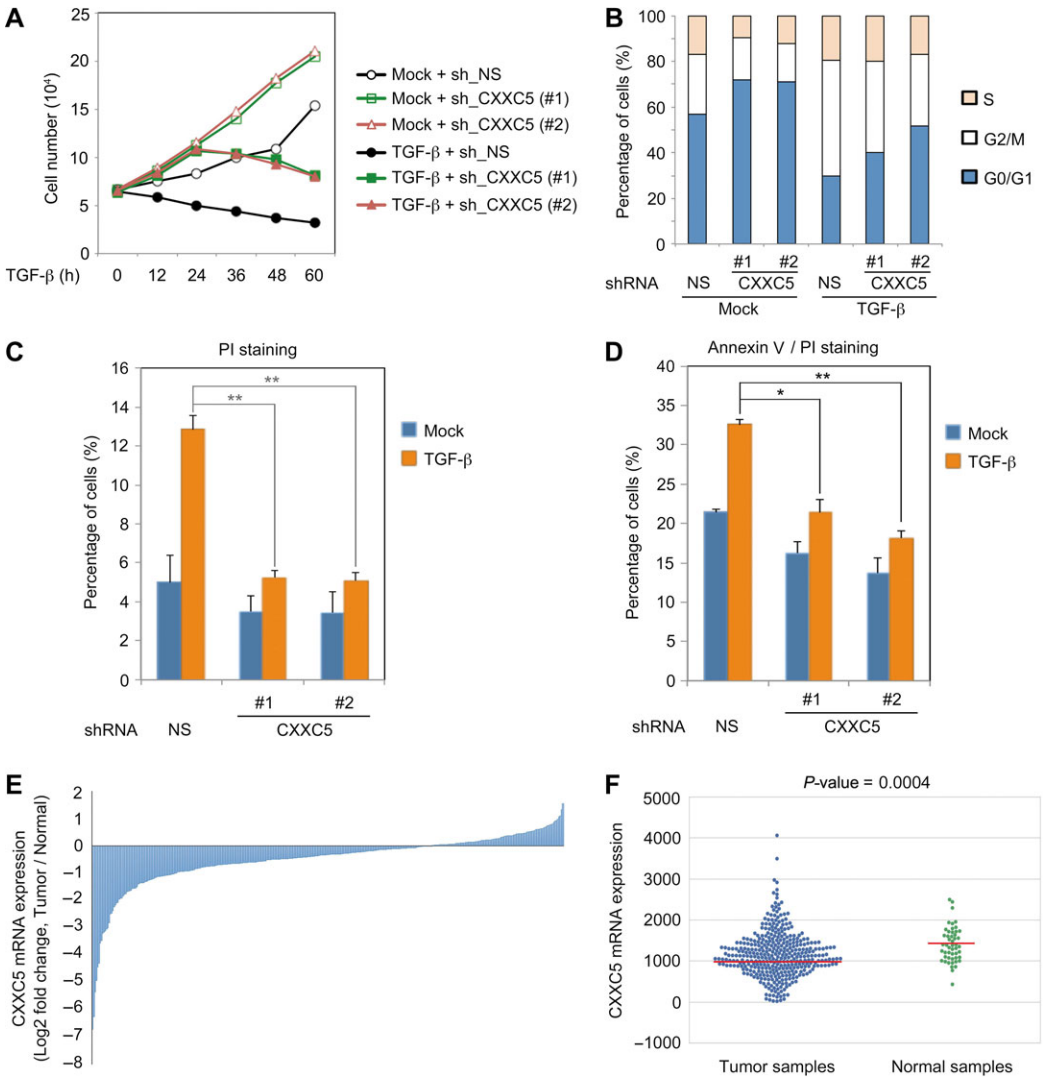

Figure 5 CXXC5 knockdown promotes proliferation and inhibits apoptosis of Hep3B cells. (**A**) Control and CXXC5 knockdown (#1 and #2) Hep3B cells were treated with or without 100 pM TGF-β1 ligand. The cell numbers were followed at the indicated time points by cell counting. (**B**) Cell cycle distribution of control or CXXC5 knockdown Hep3B cells were analyzed by treatment with 100 pM TGF-β1 for 24 h followed by flow cytometry analysis. (**C** and **D**) Hep3B cells treated with 100 pM TGF-β1 for 24 h were fixed and subjected to PI staining (**C**) or Annexin V/PI double staining (**D**), followed by flow cytometry analyses. *$P < 0.05$, **$P < 0.01$. (**E**) Gene expression analysis using human HCC samples collected in TCGA LIHC database shows decreased expression of CXXC5 mRNA in a majority of patients. (**F**) Expression of CXXC5 is significantly lower in human HCC samples in TCGA, in comparison to surrounding normal liver tissues ($P < 0.001$).

Dooley and ten Dijke, 2012; Sun and Irvine, 2014; Katz et al., 2016), TGF-β expression is quickly induced upon injury and maintained high in hepatitis, fibrosis, cirrhosis and HCC (Yoshida et al., 2014; Giannelli et al., 2016). However, how HCC cells escape from TGF-β-mediated cytostasis is not fully understood. In the present study, we identified CXXC5 as a TGF-β target gene in HCC, which in turn promotes TGF-β signaling. CXXC5 can limit growth of Hep3B cells by inducing cell cycle arrest and apoptosis, either upon TGF-β stimulation or at the resting state, as assessed by gene silence assays. Together with the observation that the CXXC5 gene expression level is downregulated in the majority of HCC tissue samples collected in the TCGA database, our findings suggest that CXXC5 might act as a tumor suppressor in HCC initiation and progression by potentiating TGF-β-mediated cytostasis.

Unlike in other cancer types like colon cancer, gastric cancer and pancreatic cancer, which usually disable the tumor suppressive function of TGF-β signaling by mutational inactivation or transcriptional silencing of TβRII, Smad4 and to a lesser extent Smad2/3, these genetic alterations are rare and the TGF-β signaling is retained in most HCC (Levy and Hill, 2006; Dooley and ten Dijke, 2012; Katz et al., 2016). Although cancer cells could evade the growth-inhibitory effect of TGF-β by obtaining enhanced mitogenic signaling (Massague, 2008; Katz et al., 2016; Zhang et al., 2017), reduction of positive regulators and amplification of inhibitors can also desensitize HCC cells to TGF-

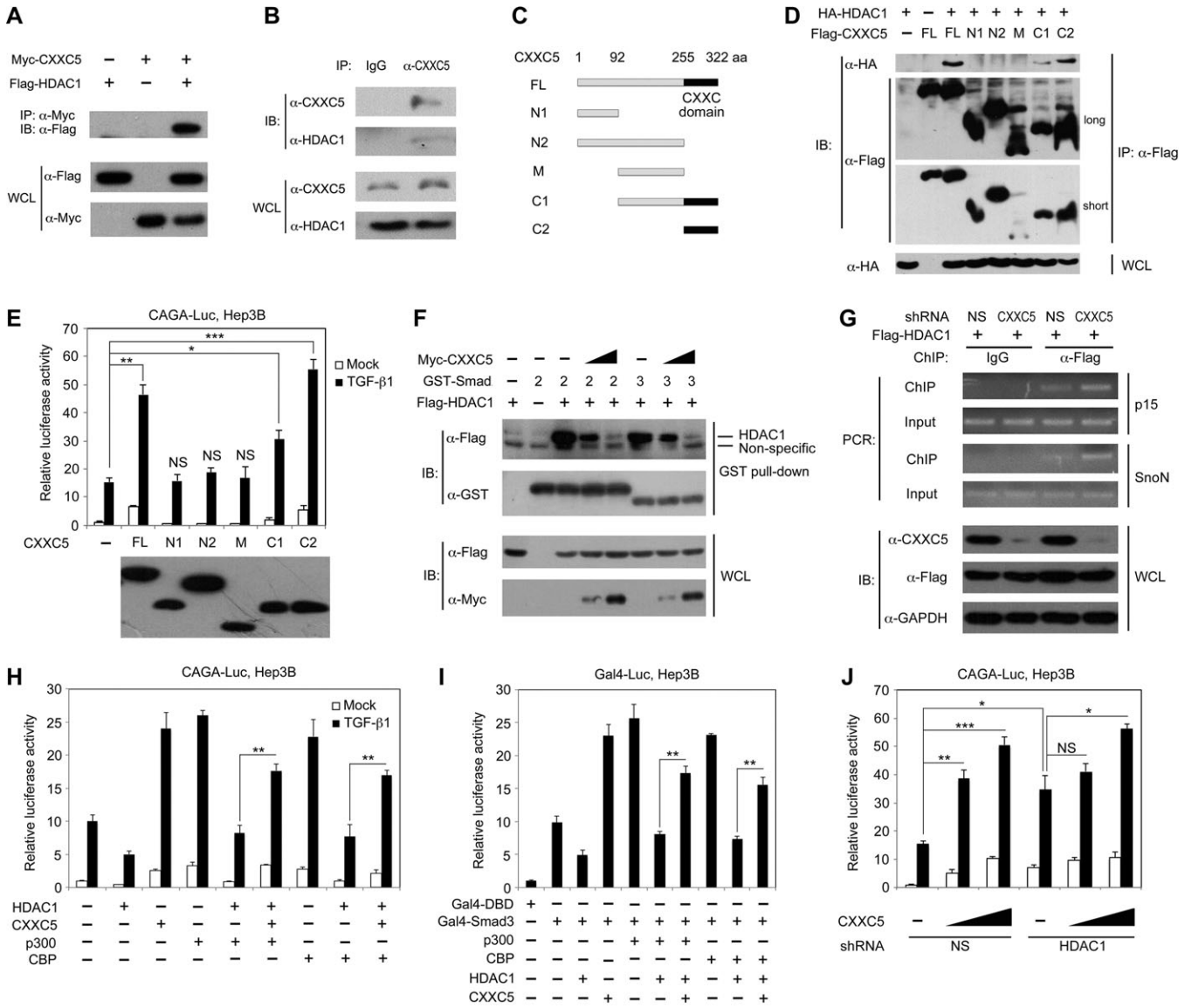

Figure 6 CXXC5 promotes Smad2/3 activity via its interaction with HDAC1. (**A**) At 40 h post-transfection, HEK293FT cells expressing Myc-CXXC5, Flag-HDAC1, or the empty vector were harvested for anti-Myc immunoprecipitation (IP) and anti-Flag immunoblotting (IB). (**B**) HEK293FT cells were lysed for immunoprecipitation with control rabbit IgG or anti-CXXC5 antibody, followed by anti-HDAC1 and anti-CXXC5 immunoblotting. Immunoblotting of whole-cell lysates (WCL) was performed to detect protein expression levels. (**C**) Diagrammatical representation of CXXC5 truncations. (**D**) Co-IP analysis in HEK293FT cells was similarly performed as in **A**. (**E**) CAGA-luciferase reporter assay was carried out as in Figure 3**A**. Plasmids expressing the full-length (FL) CXXC5 or its derivatives were transfected into Hep3B cells at an amount of 100 ng each. Protein expression was determined by immunoblotting. NS, no significance. (**F**) Plasmids were transfected into Hep3B cells by Lipofectamine 2000, and GST pull-down assay was carried out by the addition of Glutathione-Sepharose beads into cell lysates followed by gentle rotation at 4˚C overnight, beads washing with lysis buffer, and finally SDS-PAGE gel analyses. (**G**) Hep3B cells expressing non-specific (NS) or CXXC5 shRNA were transfected with Flag-HDAC1 and treated with 100 pM TGF-β1 for 4 h, and then lysed for chromatin immunoprecipitation (ChIP) assay using anti-Flag antibody or mouse IgG as indicated, followed by PCR amplification of the p15 or SnoN gene promoters. An aliquot of each cell lysate was taken for protein expression analysis by immunoblotting. WCL, whole cell lysates. (**H** and **I**) CAGA-luciferase (**H**) or Gal4-luciferase (**I**) reporter assays were carried out as in Figure 3. Cells were treated with 100 pM TGF-β1 for 20 h before being harvested for luciferase activity measurement. (**J**) CAGA-luciferase, Renilla-luciferase, CXXC5 (50 ng, 100 ng), HDAC1-specific shRNA (100 ng), or non-specific (NS) shRNA (100 ng) constructs were transfected into Hep3B cells. After being treated with 100 pM TGF-β1 for 20 h, the cells were harvested for luciferase activity measurement. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

β-mediated growth inhibition. Indeed, β2-spectrin (ELF), DACH1, TIEG1 (KLF10), and KLF17, all of which can facilitate TGF-β signaling, are downregulated in HCCs (Kitisin et al., 2007; Baek et al., 2011; Jiang et al., 2012; Zhu et al., 2013; Ali et al., 2015). On the other hand, EVI1, a TGF-β inhibitor, is amplified and highly expressed in HCC (Yasui et al., 2015). Our results reveal another strategy for HCC to bypass TGF-β-mediated inhibition by disrupting the positive feedback loop of CXXC5. It would be interesting to study whether CXXC5 is involved in the progression of other cancer types by regulating TGF-β signaling.

Feedback regulation is a common mechanism to finely tune signal transduction processes, including the TGF-β/Smad pathway (Xu et al., 2016; Yan and Chen, 2016; Miyazawa and Miyazono, 2017). Several key feedback regulators in TGF-β signaling have been identified and extensively studied, such as Smad7, SnoN, and TSC-22 (Yan and Chen, 2011; Xu et al., 2016; Miyazawa and Miyazono, 2017). We show here that CXXC5 is not only a downstream target gene of TGF-β signaling, but also acts as a positive regulator. Knockdown of CXXC5 attenuated TGF-β-stimulated transcriptional response in reporter assays and the expression of some of the target genes in Hep3B cells, indicating that CXXC5 plays an important role in full activation of TGF-β-mediated gene transcription and represents a novel positive feedback regulator. It is worthy noting that although the mechanism via HDAC1 can explain the promoting effect of CXXC5 on the TGF-β-induced gene expression (Figure 4A, Cluster 1), how CXXC5 enhances the suppressive function of TGF-β on the genes in Cluster 3 is unclear. Furthermore, a portion of the TGF-β target genes (Cluster 2 and Cluster 4) are regulated by CXXC5 in another manner, and the underlying mechanism remains to be explored. In addition to TGF-β, CXXC5 is also transcriptionally activated by BMPs and Wnt and participates in the pathophysiological functions of these factors (Andersson et al., 2009; Kim et al., 2014, 2015; Bruserud et al., 2015; Lee et al., 2015), suggesting that CXXC5 may serve as a crosstalk point of multiple cellular signaling pathways.

It has been reported that CXXC5 can associate with Smad2/3 when they are overexpressed (Wang et al., 2013; Peng et al., 2016). However, the interaction of CXXC5 with Smad2/3 was not detected in HCC cells (Supplementary Figure S3). Instead, we found that CXXC5 could associate through its C-terminal CXXC motif with the histone deacetylase HDAC1. This is in agreement with the notion that other CXXC-type zinc finger protein family members like TET1/3 and MLL1/2 act as epigenetic regulators (Blackledge et al., 2013; Long et al., 2013). Moreover, the CXXC motif of CXXC5 is essential for its promoting effect on TGF-β signaling as the truncated mutants lacking this domain lost the regulatory ability. Binding of CXXC5 leads to the dissociation of HDAC1 from Smad2/3, and concordantly, depletion of CXXC5 expression enhances binding of HDAC1 to the promoters of TGF-β target genes p15 and SnoN. In conclusion, this study defines a novel positive feedback loop of TGF-β signaling, and downregulation of CXXC5 disrupts the loop and thereafter enables HCC cells to escape from TGF-β-mediated cytostasis.

Materials and methods

Plasmids and reagents

Mammalian expression constructs of CXXC5, HDAC1, Smad2, and Smad3, fused with HA-, Flag-, or Myc-tags, were generated based on the vector pcDNA3.1(+). Plasmids expressing p300 or CBP were kindly afforded by Dr Xin-Hua Feng. Constructs encoding GST-Smad2, CAGA-luciferase, ARE-luciferase, 3TP-luciferase, Renilla-luciferase, Gal4-DBD, Gal4-Smad3, and Gal4-luciferase (pFR-luciferase) were described previously (Yan et al., 2012, 2016). CXXC5-specific shRNA constructs (seed sequences: #1 5′-GAAGCGGAAACGCTGCGGCAT-3′ and #2 5′-GAAAGACTGGCCATC AGATTT-3′), HDAC1 shRNAs (seed sequences: #1 5′-CGTTCTTAAC TTTGAACCATA-3′ and #2 5′-GCCGGTCATGTCCAAAGTAAT-3′), and the non-specific control shRNA plasmid were all based on vector pLKO.1-puro and purchased from the MISSION shRNA library (Sigma-Aldrich).

Recombinant human TGF-β1 peptide was purchased from R&D Systems Inc. Anti-Flag antibody (M2) was from Sigma. Antibodies recognizing CXXC5, phospho-Smad2, and Smad2 were from Cell Signaling Technology (CST). The antibody against HDAC1 was from Abcam. Other antibodies, including anti-HA, anti-Myc, anti-Tubulin, and anti-GAPDH antibodies, were all purchased from Santa Cruz Biotechnology.

Cell culture and transfection

HEK293FT and human HCC cell lines including Huh7, MHCC97L, and MHCC97H were maintained in Dulbecco's minimum essential medium (DMEM) (Corning) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a humidified, 5% CO_2 incubator. Human HCC cell lines Hep3B and HepG2 were maintained in minimum essential medium (MEM) (Corning), and normal human HL-7702 hepatocytes were maintained in RPMI 1640 (Corning), both supplemented with 10% fetal bovine serum. Cell transfection was conducted with VigoFect (Vigorous Biotechnology) or Lipofectamine 2000 (Invitrogen).

Stable cell line establishment

CXXC5 gene-targeting shRNA constructs or the control non-specific shRNA plasmid were transfected into Hep3B cells using Lipofectamine 2000. At 24 h post-transfection, the cells were subjected to puromycin selection (1 μg/ml) for a time period of 2–4 days, and then the drug-resistant cells were pooled as stable cells, which were further maintained by puromycin-containing (0.5 μg/ml) MEM growth medium.

Total RNA extraction, RNA-Seq and data processing

Hep3B cells (Figure 1) or the derivative cell lines stably carrying shRNAs (Figure 4) were cultured in 60-mm dishes before treated with or without 100 pM TGF-β1 ligand and harvested for total RNA extraction with Trizol (Life Technologies) (Yan et al., 2011). The RNA samples were subjected to transcriptome sequencing (RNA-Seq) with the Illumina HiSeq 2000 sequencer in BerryGenomics, Beijing.

Transcriptome construction from RNA-Seq raw data and subsequent analyses were conducted similarly as those described in

Qi et al. (2017), with updated databases or softwares as below. Briefly, raw single end reads were firstly trimmed of the first 13 bp from each end and mapped to the human genome (hg38) with Tophat2 (v2.1.1). Gene expression level was estimated and normalized with Cufflinks (v2.2.1) into an FPKM matrix using default parameters for the annotation GTF file downloaded from GENCODE (v24). Differentially expressed genes (DEGs) were generated with Cuffdiff (v1.3.0) with *P*-value <0.05 and filtered by corresponding threshold. DEGs were subjected to GO term clustering and KEGG pathway enrichment by the NIH Database for Annotation, Visualization and Integrated Discovery (DAVID), with default parameters as reported, and a *P*-value <0.01 (adjusted by Benjamini) was considered statistically significant (Huang da et al., 2009; Jiao et al., 2012).

The Cancer Genome Atlas (TCGA) data analyses

We used mRNA expression and somatic copy number variation (CNV) data generated by The Cancer Genome Atlas (TCGA) from LIHC (Liver hepatocellular carcinoma) specimens (377 tumors and 89 surrounding normal liver tissues). We downloaded the file containing level 3 normalized RNA-Seq by Expectation Maximization (RSEM) data and the SNP6 Copy number analysis (GISTIC2). The data version is 2016_01_28.

Reverse transcription (RT), and quantitative PCR (q-PCR)

Total RNA extraction and reverse transcription were carried out with TRIzol reagent (Invitrogen) and Revertra Ace (Toyobo), respectively (Yan et al., 2011), and quantitative PCR was performed in triplicate using the SYBR green detection method on a LightCycler 480 (Roche). The primers (for human) used were collected in Supplementary Dataset S3.

Luciferase reporter assay, immunoprecipitation, and immunoblotting

Luciferase reporter assay was conducted as described (Yan et al., 2016). The experiments were repeated in triplicate, and the data are presented as mean ± SD after normalization to the activity of Renilla luciferase.

For immunoprecipitation (IP), mammalian cells were plated in 6-well plates one night before transfection. At 40 h after plasmid transfection, cells were lysed with lysis solution (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate dodecahydrate, 0.5% Nonidet P-40, 1 mM EDTA, 10 mM NaF in addition to protease inhibitors), rotated on a table concentrator at 4°C for 20 min, and centrifuged for 10 min at 4°C in a microcentrifuge. After being taken by an aliquot for protein expression detection, the supernatant cell lysates were precleaned by addition of 30 μl protein A sepharose (GE Healthcare) and incubated for 2 h at 4°C. Then immunoprecipitation was performed by adding protein A sepharose and appropriate antibodies (2–5 μg) followed by incubation at 4°C overnight. The immune complex was isolated by gentle centrifugation and washed for four times with lysis buffer, and finally analyzed by SDS-PAGE gel followed by immunoblotting detection as described (Yan et al., 2016).

ChIP

ChIP was carried out according to the introduction of the EZ ChIP™ Kit (Upstate Biotechnology). Upon purification, the protein-bound DNA was analyzed by PCR reaction using the following primers: for human p15 promoter, 5′-CATGATTCTC GGGATTTTTCTC-3′ (forward) and 5′-GACAGCTCTGCACCTGTCAT-3′ (reverse); for human SnoN promoter, 5′-CTCGAGGAAGGAAGG AGGG-3′ (forward) and 5′-TACACACAGCCTCTGACGTC-3′ (reverse).

Cell proliferation, cell cycle, and apoptosis analyses

Hep3B cells stably carrying the CXXC5-targeting shRNAs or the control non-specific shRNA were cultured in 24-well plates before treated with or without 100 pM TGF-β1. Then, cell number was followed at indicated time point by cell counting. To analyze cell cycle progression, Hep3B stable cells were synchronized by treatment with 2.5 mM Thymidine (TdR) for 15 h, followed by a wash step with PBS. After being treated with 100 pM TGF-β1 for 24 h, cells were harvested and fixed in 90% ethyl alcohol, followed by propidium iodide (PI) (Polysciences) staining for 10 min and cell cycle analysis on a BD FACSCalibur Flow Cytometer. For apoptosis analysis, Hep3B stable cells were fixed by 90% ethyl alcohol and stained with PI and/or AnnexinV (Beyotime) for 10 min, and then analyzed by flow cytometry.

Statistic analyses

All the experiments were repeated at least three times. For reporter assay, q-PCR, and apoptosis results, the values were presented as mean ± SD, and the significance between the means was calculated using two-tailed Student's *t* test. In analyzing CXXC5 gene expression in TCGA database, Mann–Whitney test was exploited. For all of the statistic analyses, a *P*-value < 0.05 was considered as statistically significant.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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