

# COMMD7 activates CXCL10 production by regulating NF- $\kappa$ B and the production of reactive oxygen species

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**Abstract.** While >80% of the incidence occurs in sub-Saharan Africa and East Asia, cases of hepatocellular carcinoma (HCC) have been rapidly increasing in Western countries. Despite its global importance, HCC is relatively under-researched compared with other lethal cancer types, which is possibly due to the high complexity and heterogeneity of HCC. It has been reported previously that COMM domain-containing protein 7 (COMMD7) is upregulated in HCC and promotes HCC cell proliferation by triggering C-X-C motif chemokine 10 (CXCL10) production. However, the value of targeting CXCL10 signal transduction in treating COMMD7-positive tumors, or the molecular mechanisms underlying COMMD7-mediated CXCL10 expression, has not been completely addressed. In the present study, it was demonstrated that disruption of the CXCL10/C-X-C chemokine receptor type 3 axis reduces COMMD7-mediated HCC cell proliferation. Furthermore, COMMD7 modulates CXCL10 production by activating nuclear factor (NF)- $\kappa$ B. Additionally, it was demonstrated that intracellular reactive oxygen species (ROS) are required for NF- $\kappa$ B activation and CXCL10 production. In conclusion, COMMD7 activates CXCL10 production by regulating NF- $\kappa$ B and the production of ROS. The present study highlighted the role of COMMD7 in the development of HCC, and provides novel options for anticancer drug design.

## Introduction

While >80% of the incidence occurs in sub-Saharan Africa and East Asia, cases of hepatocellular carcinoma (HCC) have been rapidly increasing in Western countries (1). Despite its global importance, HCC is relatively under-researched compared with other lethal cancer types, which is possibly

due to the high complexity and heterogeneity of HCC (2). It is widely accepted that chronic liver diseases, including liver cirrhosis and hepatitis, are important steps in HCC tumorigenesis. A clinical investigation demonstrated that >80% of patients with HCC had suffered from liver cirrhosis, which is caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcoholism, metabolic disorders or exposure to toxic chemicals (3). Among these factors, HBV infection is most prevalent in Asia, while HCV infection is more common in Western countries. The majority of aflatoxin-associated liver cirrhosis occurs in Southeast Asia (4). Therefore, an improved understanding of the pathogenesis of HCC is required in order to develop novel treatment strategies.

A previous study identified a gene termed COMMD7, which is mapped to 20q11.22, by sequence analysis and homology comparison. COMMD7 cDNA fragments were observed to be highly expressed in HCC samples via suppression subtractive hybridization. The pro-cancer properties of COMMD7 were additionally demonstrated, since knock-down of this gene by short hairpin RNA reduced HCC cell proliferation and tumor growth in a mouse model (5). It was additionally observed that COMMD7 is capable of stimulating C-X-C motif chemokine 10 (CXCL10) expression and, in turn, may promote HCC cell proliferation and metastasis in an autocrine manner (Zheng *et al.*, unpublished data). However, the value of targeting CXCL10 signal transduction in treating COMMD7-positive tumors, or the molecular mechanisms underlying COMMD7-mediated CXCL10 expression, has not been completely addressed.

In the present study, using multiple *in vitro* and *in vivo* models, it was demonstrated that inhibition of the CXCL10/C-X-C chemokine receptor type 3 (CXCR3) axis attenuated COMMD7-mediated HCC proliferation. The present mechanistic study demonstrated that COMMD7 activated CXCL10 by modulating nuclear factor (NF)- $\kappa$ B and oxidative stress.

## Materials and methods

**Cell culture.** The human HCC cancer cell line Huh7 was purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA),

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penicillin ( $10^7$  U/l) and streptomycin (10 mg/l) at 37°C in a humidified chamber containing 5% CO<sub>2</sub>.

**Reagents.** Rabbit anti-human Ki67 monoclonal antibody (cat. no. ab92742), rabbit anti-human total p65 monoclonal antibody (cat. no. ab32536), rabbit anti-human phosphorylated p65 monoclonal antibody (cat. no. ab76302) and rabbit anti-human  $\beta$ -actin monoclonal antibody (cat. no. ab8227) were purchased from Abcam (Cambridge, MA, USA). The CXCL10 ELISA kit (cat. no. ab83700) was purchased from Abcam. The NF- $\kappa$ B inhibitor, celastrol, was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The NF- $\kappa$ B inhibitor, Bay 11-7085, was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). A pan inhibitor of ROS production, N-acetyl cysteine (NAC), was purchased from Abcam (cat. no. ab143032). As CXCR3 is the principal receptor for CXCL10, a commercially available chemical antagonist, NBI-74330 (6) was used in the present study (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**pGenesil-COMMD7 plasmid establishment.** The COMMD7 cDNA was extracted from the human HCC cell lines of Huh7 cells. The primer sequences for the COMMD7 was listed as: Forward, 5'-AGTGGCTTTCTCCTCACTAAGACC-3' and reverse, 5'-GGAAAGATTCTGGCTCAGCTC-3'. The amplified COMMD7 was directly cloned into the plasmid pGenesil-1 vector (Genesil Biotechnology, Wuhan, China), and then transformed into the DH5 $\alpha$  *E. coli* (Tiangen, Beijing, China). The transformed colonies were selected by the kanamycin resistance. The established pGenesil-COMMD7 plasmid was transfected into the Huh7 by using the Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at the condition of 5% CO<sub>2</sub> and at 37°C. Then, the over-expression of the COMMD7 in Huh7 cells were observed.

**Bromodeoxyuridine (BrdU) labeling assay.** BrdU was purchased from Roche Diagnostics (Indianapolis, IN, USA). BrdU was added to the cell culture at a working concentration of 10 mM. Following incubation for 18 h, BrdU signaling was measured using the BrdU Labeling and Detection kit III (Roche Diagnostics).

**In vivo study.** The present study was approved by the Laboratory Animal Welfare and Ethics Committee of Third Military Medical University (Chongqing, China). A total of 7 Balb/C mice, weighting 25-30 g were humanely housed and treated at room temperature (about 20°C), 40% humidity and a 12-h light/dark cycle. All the mice were given free access to food and water. A total of  $5 \times 10^7$  Huh7 cells were subcutaneously injected into male athymic nude mice (n=7). Animals were sacrificed 20 days post-injection. The tumor volumes were evaluated as follows: Tumor volume (mm<sup>3</sup>)=(length x width<sup>2</sup>)/2.

**ELISA.** The CXCL10 levels were examined by using the ELISA kit (Abcam) according to the manufacturer's protocols.

**Western blotting.** Cells were washed and treated with lysis buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The

concentrations of protein samples were determined by using BCA assay (Thermo Fisher Scientific, Inc.) and 2  $\mu$ g protein samples were separated by 15% SDS-PAGE, and translocated to polyvinylidene fluoride membranes. Following incubation with 5% skimmed milk for 1 h at room temperature, the blots were incubated with primary antibodies (1:3,000) and rabbit anti-human  $\beta$ -actin monoclonal (1:2,000) for 2 h and secondary antibody (1:2,000) for 1 h at room temperature. Finally, the blots were visualized by enhanced chemiluminescence (Amersham; GE Healthcare, Chicago, IL, USA).

**Reactive oxygen species (ROS) measurement.** CM-H2DCFDA (general oxidative stress indicator; DCF) was purchased from Thermo Fisher Scientific, Inc. An inverted fluorescence microscope (IX73; Olympus Corporation, Japan; magnification, x200) was used to visualize the images. The glutathione (GSH)/oxidized glutathione (GSSG) assay kit was purchased from Abcam. Intracellular ROS were examined according to the manufacturers' protocols (with the cell density of  $10^5$  cells/ml).

**Statistical analysis.** The data in this study were analyzed by using SPSS statistical analysis software version 18.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis of data was performed using one-way analysis of variance followed by the Tukey test. Data are presented as the mean  $\pm$  standard error of the mean. All of the experiments were repeated at least 3 times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Disruption of the CXCL10/CXCR3 axis reduces COMMD7-mediated HCC cell proliferation.** To determine whether COMMD7-mediated HCC cell proliferation depended on CXCL10, the present study aimed to inhibit CXCL10 signal transduction. As presented in Fig. 1A, for the BrdU assay, the conditioned medium (CM) derived from the COMMD7-over-expressing Huh7 cells markedly induced the proliferation of naïve Huh7 cells. However, the CM-mediated proliferation was significantly reduced in NBI-74330-treated cells. This observation was further supported by the clonogenic formation assay. As in Fig. 1B, COMMD7-induced cell clone formation was eliminated when cells were treated with NBI-74330.

To further support the *in vitro* observations, a mouse xenograft model was employed to determine the role of CXCL10 in the COMMD7-mediated proliferation of HCC tumor growth. To this end, Huh7 sub-clones that stably expressed COMMD7 or mock vector were established. These two sub-clones were subcutaneously transplanted into nude mice, respectively, and the mice were treated with or without NBI-74330. As presented in Fig. 1C, as expected, the tumor growth was enhanced in the COMMD7-expressing group compared with the mock vector-expressing group. Notably, inhibition of CXCL10 signal transduction by NBI-74330 substantially reduced tumor growth (Fig. 1C). The tumor volume in the NBI-74330-treated group decreased by 41.17% ( $P < 0.001$ ), compared with the vehicle-treated group (Fig. 1D). To further examine the cell proliferation status in

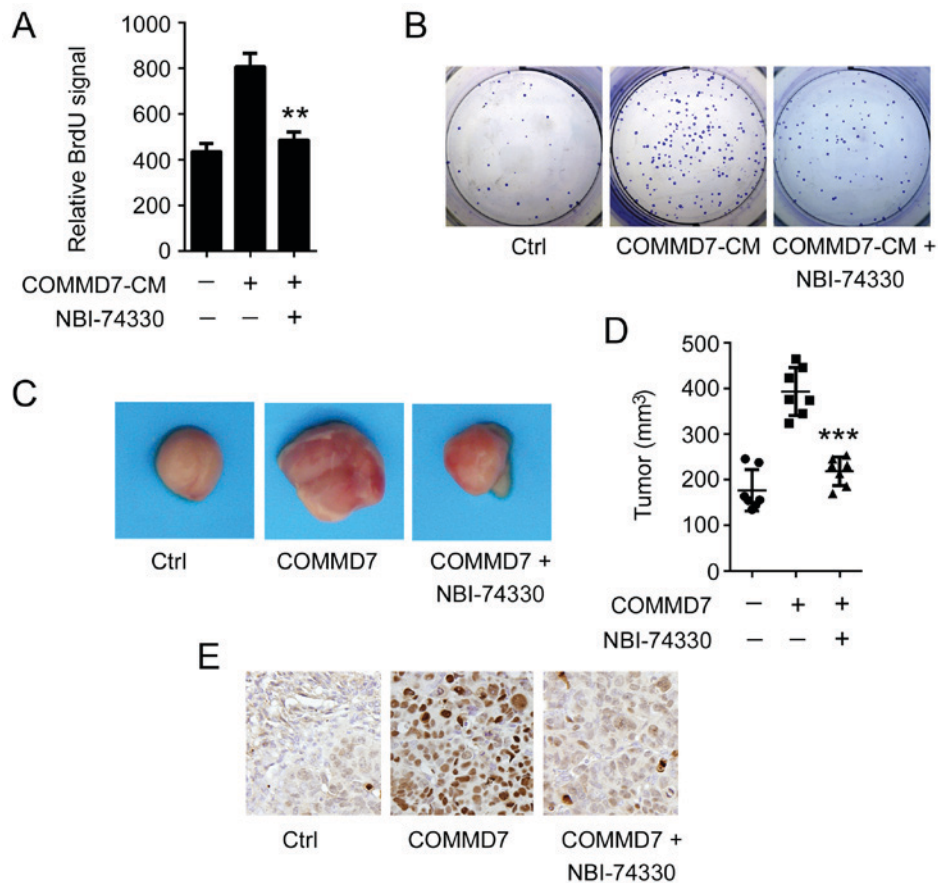


Figure 1. Observation of COMMD7-mediated hepatocellular carcinoma cell proliferation following disruption of the C-X-C motif chemokine 10/C-X-C chemokine receptor type 3 axis. (A) Huh7 cells were pretreated with 500 nM NBI-74330 and were transfected with COMMD7 vector or mock vector. The CM derived from the aforementioned cells was used to treat naïve Huh7 cells for a further 72 h. Cell proliferation was examined by BrdU assay. (B) Huh7 cells were transfected with COMMD7 vector or mock vector in the presence or absence of 500 nM NBI-74330. The CM derived from the aforementioned cells was used to treat naïve Huh7 cells for a further 72 h. Cell proliferation was examined by clonogenic assay. (C) Huh7 cells stably expressing COMMD7 or mock vector were injected into nude mice. The mice were treated with 100 mg/kg NBI-74330 every 2 days. Representative images of tumors in each group (n=7) are presented. (D) The mice were sacrificed and the tumor volume was measured. (E) Cell proliferation status in tumors was examined via the percentage of Ki67<sup>+</sup> cells (according to the immunostaining images). Magnification, x200. \*\*P<0.01, \*\*\*P<0.001 vs. COMMD7-CM (-)/NBI-74330 (-) group. COMMD7, COMM domain-containing protein 7; BrdU, bromodeoxyuridine; Ctrl, control; CM, conditioned medium.

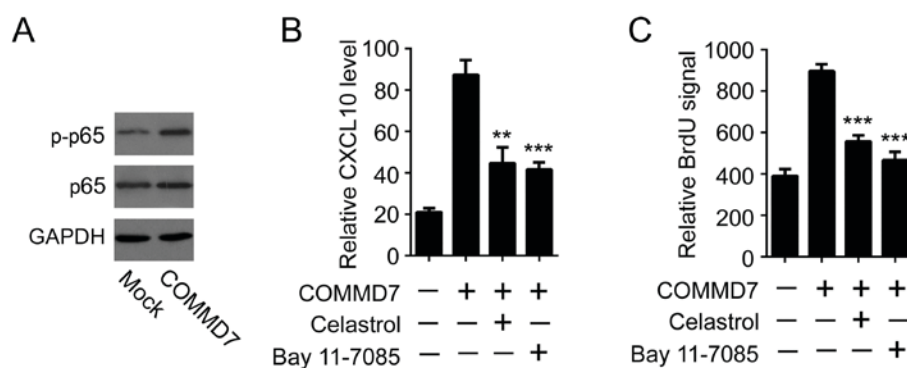


Figure 2. Examination of CXCL10 expression and cell proliferation. (A) Huh7 cells were over-expressed with COMMD7. The phosphorylation of p65 was examined. (B) Huh7 cells were induced to overexpress COMMD7, in the presence or absence of 0.5  $\mu$ g/ml celastrol or 10  $\mu$ M Bay 11-7085. CXCL10 production was examined by ELISA. (C) Huh7 cells were pretreated with 0.5  $\mu$ g/ml celastrol or 10  $\mu$ M Bay 11-7085, and were transfected with COMMD7 vector or mock vector. The conditioned medium derived from the aforementioned cells was used to treat naïve Huh7 cells for a further 72 h. Cell proliferation was examined by BrdU assay. \*\*P<0.01, \*\*\*P<0.001 vs. COMMD7-CM (-)/Celastrol (-)/Bay 11-7085 (-) group. COMMD7, COMM domain-containing protein 7; CXCL10, C-X-C motif chemokine 10; BrdU, bromodeoxyuridine.

tumor xenografts, immunostaining of Ki67 was performed. As shown in Fig. 1E, overexpression of COMMD7 induced more Ki67-positive cells, while treatment with NBI-74330

markedly reduced the number of Ki67-positive cells. These results suggested that the CXCL10/CXCR3 axis may be required for COMMD7-mediated HCC cell proliferation.

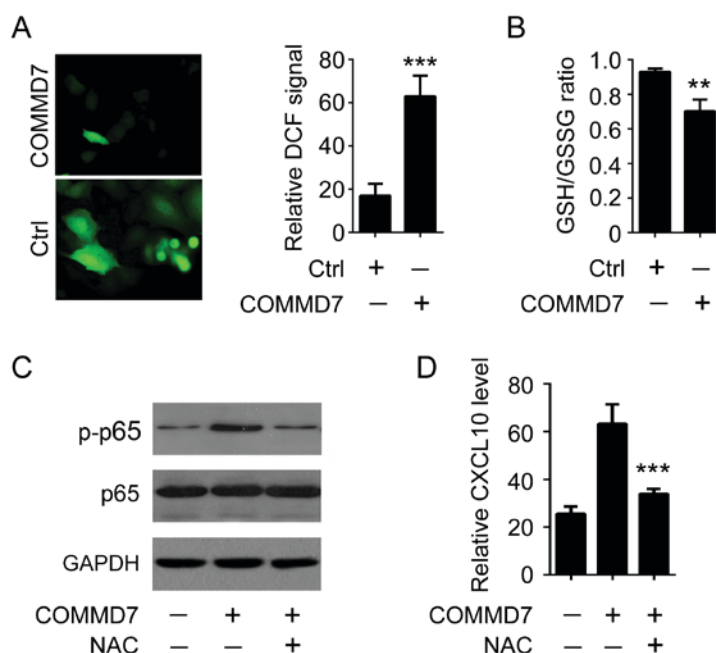


Figure 3. COMMD7 triggers nuclear factor- $\kappa$ B expression by modulating intracellular ROS. (A) Huh7 cells were induced to overexpress COMMD7. Intracellular ROS was measured by DCF probing. \*\*\* $P < 0.001$  vs. Ctrl (+)/COMMD7 (-) group. (B) Huh7 cells were induced to overexpress COMMD7. Intracellular ROS was examined by measuring the GSH/GSSG ratio. \*\* $P < 0.01$  vs. Ctrl (+)/COMMD7 (-) group. (C) Huh7 cells were induced to overexpress COMMD7 in presence or absence of 10 mM NAC. The phosphorylation of p65 was examined by western blotting. (D) Huh7 cells were induced to overexpress COMMD7 in the presence or absence of 10 mM NAC. CXCL10 production was examined by ELISA. \*\*\* $P < 0.001$  vs. COMMD7 (-)/NAC (-) group. ROS, reactive oxygen species; COMMD7, COMM domain-containing protein 7; DCF, CM-H2DCFDA; GSH, glutathione; GSSG, oxidized glutathione; Ctrl, control; NAC, N-acetyl cysteine; CXCL10, C-X-C motif chemokine 10.

*COMMD7 activates CXCL10 by modulating NF- $\kappa$ B.* The present study aimed to determine the mechanisms responsible for COMMD7-induced CXCL10 expression. It was previously reported that COMMD7 is required for tumor necrosis factor (TNF)- $\alpha$ -induced NF- $\kappa$ B activation (5). As a pilot result, it was observed that overexpression of COMMD7 augmented p65 phosphorylation, suggesting that overexpression of COMMD7 alone may be sufficient to induce NF- $\kappa$ B activation (Fig. 2A). Considering that NF- $\kappa$ B was able to directly bind to the CXCL10 promoter and initiate CXCL10 expression, it was thus examined whether NF- $\kappa$ B was involved in COMMD7-induced CXCL10 expression. To this end, two different NF- $\kappa$ B inhibitors, celastrol and Bay 11-7085, were used (7,8). In line with previous data, the overexpression of COMMD7 induced a significant upregulation of CXCL10 expression in Huh7 cells. Notably, inhibition of NF- $\kappa$ B by either celastrol or Bay 11-7085 impeded COMMD7-mediated CXCL10 expression (Fig. 2B). Furthermore, the pro-proliferative effects of CM derived from COMMD7-overexpressing cells were abolished when cells were pre-incubated with either celastrol or Bay 11-7085 (Fig. 2C). These results suggested that COMMD7 may activate CXCL10 by modulating NF- $\kappa$ B.

*COMMD7 activates NF- $\kappa$ B by modulating intracellular ROS.* It has been reported that oxidative stress is involved in interferon- or TNF-induced NF- $\kappa$ B expression (9). To examine the potential signaling cascades underlying COMMD7-mediated NF- $\kappa$ B activation, of particular interest, the impact of COMMD7 expression on the level of intracellular ROS was examined. It was demonstrated that overexpression of COMMD7 in Huh7 cells augmented intracellular ROS level

by >3-fold, as demonstrated by the use of the specific ROS probe DCF (Fig. 3A). Similarly, overexpression of COMMD7 reduced the GSH/GSSG ratio, an additional hallmark of oxidative stress (10) (Fig. 3B). To determine whether ROS are involved in COMMD7-mediated NF- $\kappa$ B activation, NAC, a pan inhibitor of ROS production, was used (11). As presented in Fig. 3C, COMMD7-mediated p65 phosphorylation was attenuated when cells were treated with NAC, suggesting that oxidative stress was required for COMMD7-mediated NF- $\kappa$ B activation. Accordingly, the elimination of ROS by NAC reduced COMMD7-mediated CXCL10 expression (Fig. 3D). These results indicated that COMMD7 activates NF- $\kappa$ B by modulating intracellular ROS production.

## Discussion

HCC is considered to be one of the most important life-threatening tumors worldwide, and remains a notable problem for public health (12). Multiple genetic and environmental factors, including mutations of oncogenes and tumor suppressor genes, infection with oncogenic microbes and metabolic imbalances, are associated with the development of HCC (13). Therefore, the identification of novel proteins with aberrant expression or function in HCC is required for early diagnosis and discovery of novel drug targets.

Chemokines refer a family of structurally similar extracellular proteins which govern leukocyte trafficking. It is well accepted that chemokines are involved in tumorigenesis by regulating immune cell and tumor cells (14). CXCL10 initiates its downstream signaling cascade by interacting with and activating CXCR3 (15). Aberrant regulation of



CXCL10 was demonstrated to be associated with metastasis of colorectal cancer (16). Previously, it was reported that CXCL10 expression was positively correlated with COMMD7 expression in a multitude of HCC cell lines. In addition, previous studies (17,18) also reported that CXCR3 was predominately expressed and upregulated significantly in HCC cancer tissues compared with adjacent non-cancerous tissues. Meanwhile, the expression of CXCR3 was significantly increased in certain hepatic cell lines, including HepG2 (19), Huh-7 (20) and SMMC-7721 (21). Therefore, it was confirmed that CXCR3 was highly expressed in HCC cell lines and HCC human samples (17,18). Overexpression of COMMD7 induced CXCL10 production in the culture medium. Furthermore, inhibiting CXCL10 signaling via treatment with a neutralizing antibody markedly inhibited CM-mediated HCC cell proliferation (17). These results suggested that CXCL10 served an essential role in COMMD7-mediated HCC cell proliferation. The present study further tested whether CXCL10 signaling was a potential target for treating COMMD7-positive tumors. It was demonstrated that treatment with the CXCR3 inhibitor NBI-74330, markedly reduced HCC cell proliferation *in vitro* and *in vivo* model. Moreover, it was additionally demonstrated that NF- $\kappa$ B is a prerequisite for CXCL10 expression.

Oxidative stress is characterized as an imbalance between producing and scavenging free radicals and reactive metabolites, termed ROS. ROS were historically considered to be toxic byproducts from metabolism, which lead to damage to important macromolecules in cells (22). A recent study, however, indicated that ROS may function as essential physiological regulators of diverse biological processes, including gene expression (23). In the present study, it was demonstrated that ROS served an important role in COMMD7-mediated CXCL10 expression. Overexpression of COMMD7 induced severe oxidative stress in HCC cells, illustrated by the increased DCF signal and reduced GSH/GSSG ratio. In addition, phosphorylation of p65 and expression of CXCL10 were markedly reduced in COMMD7-expressing HCC cells following the inhibition of ROS. Further work is required to examine the primary source of COMMD7-induced ROS production.

In conclusion, the present data suggest a COMMD7-initiated pro-tumor pathway. COMMD7 is upregulated in HCC and triggers ROS production. As a consequence of accumulated cellular ROS, NF- $\kappa$ B is activated and, in turn, activates CXCL10 expression. CXCL10 finally promotes HCC cell proliferation in an autocrine manner. The present study highlighted the role of COMMD7 in the development of HCC, and provides new options for anticancer drug design.

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