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Molecular mechanism of CCDC106 regulating the p53-Mdm2/MdmX signal axis

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Molecular mechanism of CCDC106 regulating the p53-Mdm2/MdmX signal axis

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Abstract

The tumor suppressor p53 (p53) is regulated by murine double minute 2 (Mdm2) and its homologous MdmX in maintaining the p53 basal level. The overexpressed Mdm2/MdmX inhibit the cellular p53 activity, highly relevant to cancer occurrence. The coiled-coil domain-containing protein 106 (CCDC106) has been identified as a p53-interacting partner. However, its molecular mechanism is still elusive. Here we show that CCDC106 functions as a signaling regulator of the p53-Mdm2/MdmX axis mediated by cellular p53. We identified that CCDC106 directly interacts with the p53 transactivation domain by competing with Mdm2 and MdmX. The CCDC106 overexpression downregulates the cellular level of p53 and Mdm2/MdmX, and the decreased p53 reversibly downregulates the cellular level of CCDC106. Our work not only provides a molecular mechanism of CCDC106 regulating the cellular levels of p53 andMdm2/MdmX, but also suggests that the CCDC106-p53 interaction as a novel target for cancer therapy.

Introduction

The endogenous CCDC106 and p53 can be colocalized in nuclei and interact each other in vivo, promoting the degradation of p53 protein and inhibit its transactivation activity[1], suggesting that CCDC106 is a negative regulator of p53 to promote cell proliferation in cancers. In ovarian cancers, the overexpression of CCDC106 promotes cell proliferation and invasion by suppressing p21 transcription through a p53-independent pathway, while the CCDC106 knockdown inhibits the expression of proliferation, invasion and EMT signaling markers in mutant p53 cells but not in wild-type p53 cells[2]. During cancer progression, the phosphorylation of CCDC106 by protein kinase CK2 is essential for p53 degradation and CK2 inhibitor can block the translocation of CCDC106 into the nuclei of mutant p53 cells[3]. In non-small cell lung cancers (NSCLC), the overexpression of CCDC106 significantly correlates with advanced TNM stage[4]. In two typical NSCLC cell lines that H1299 overexpresses CCDC106 but A549 expresses less CCDC106, the expression of CCDC106 upregulates the expression of Cyclin A2 and Cyclin B1, promoting cell proliferation via Akt-dependent signaling pathway[4].

The dysfunction of p53 is a key cause of cancer development[5], while CCDC106 can reduce p53 stability[3], suggesting that CCDC106 may be a novel target for cancer treatment. On the other hand, the phosphorylation of Mdm2 allows its entry into the nucleus where it targets p53 for degradation[6,7]. Thus, it is likely that the interaction of CCDC106 with p53-Mdm2/MdmX signaling pathway play an important role in cancer cell survive and drug resistance. However, it is elusive how the CCDC106 signaling regulates p53 signaling pathway. Here we identified that CCDC106 competes with Mdm2 and MdmX and directly interacts with the transactivation domain of p53. Such interaction downregulates p53 and Mdm2, promoting cell proliferation. Thus, our work

not only reveals a structural mechanism of CCDC106 interacting with p53, but also suggests that the CCDC106-p53 interaction is a novel target for cancer therapy.

Results

Characterization of CCDC106 protein structure bioinformatically

CCDC106 is a single polypeptide with 280 amino acid residues (**Fig. 1a**). We predicted its putative functional domains using the Simple Modular Architecture Research Tool (SMART)[8]. As shown in **Fig. 1b**, it contains a coiled coil domain predicted with confidence (**Table S1**). However, it is also likely that CCDC106 may have potential to constitute many other functional domains (**Table S2**), although their e-values are over threshed. As predicted with alphaFold2 program, the CCDC106 structure contains a long α-helix flanked by two coils at the two ends of the α-helix in the N-terminal region of the CCDC106 protein, while its C-terminal region forms a compact helix-rich structure. Therefore, we arbitrarily defined these two regions as the N-terminal domain (NTD) and the C-terminal domain (CTD) of CCDC106, respectively (**Fig. 1c**).

To understand how CCDC106 interacts with p53 protein, we recombinantly expressed the full-length CCDC106 with FLAG and GFP tags in mammalian cells, while its NTD and CTD proteins were expressed in GST fusion form using *E. coli* cells (**Supplementary Fig. 1**). These two GST fusion proteins were used as baits to pulldown p53 in investigating the interaction between CCDC106 and p53.

Expression of cellular CCDC106 is correlated with the cellular p53 level.

As shown in **Fig. 2a**, CCDC106 was overexpressed in H1299, 293T and HCT116 cells, while CCDC106 was marginally expressed in HepG2 cells. However, no

significant CCDC106 protein was detected in MCF-7 and A549 cells. We noted that both p53 and CCDC106 were overexpressed in 293T and HCT116 cells, while p53 expressed at low level in MCF-7, A549 and HepG2 cells. Thus, in these cell lines, the expression of cellular p53 protein was indispensably correlated with the cellular level of CCDC106 except the H1299 cells that was supposed to have a null p53 gene. These results also suggested that the high cellular level of CCDC106 was inversely associated with the high level of cellular p53 protein. To further confirm this possibility, we knocked down the CCDC106 expression in 293T cells using siRNA approach and found that the cellular level of p53 protein significantly decreased with decreasing CCDC106 (**Fig. 2b**).

To examine how CCDC106 interacts with p53 protein, we conducted in vitro GST pulldown assays using GST-NTD and GST-CTD fusion proteins. As shown in **Fig. 2c,** in the presence or absence of double strand DNAs for p53 protein, the CTD domain of CCD106 interacted with p53 protein, while the NTD domain of CCDC106 had no interaction with p53 protein (**Fig. 2d**).

CCDC106 interacts with the p53 TAD domain.

Next, we would like to employ p53-null H1299 cells to investigate how CCDC106 interacts p53-Mdm2/MdmX signaling pathway *in vivo*. In this work, we have firstly ensured that the full-length p53 gene is null in our H1299 cells (**Supplementary Fig. 2 & 3, and Supplementary Data 1-3**) as current literatures have not given a detailed information on its default p53 gene[9,10]. In an engineered H1299^{p53+} cells that can express the full-length wild-type p53 protein and a truncated form of p53 protein (p53$^{1-212}$), we used the GST-CTD fusion protein as a bait to conduct GST-pulldown assay. As indicated in **Fig. 2e**, both full-length p53 and p53$^{1-212}$ were pulled down from the

H1299^{p53+} cells, indicating that the CCDC106 binding site on p53 is located within the N-terminal region of p53. We further distinguished whether CCDC106 interacts with the p53-TAD/PRD domain (i.e., p53$^{1-93}$) or the p53-DBD (i.e., p53$^{94-312}$). As shown in **Fig. 2f**, GST pulldown assay indicated that the CTD domain of CCDC106 interacted with the p53-TAD/PRD region but not the p53 DBD domain. To further narrow down the binding site of CCD106 on p53 protein, we did GST-pulldown assay with the p53-TAD domain (p53$^{15-29}$) fused with the N-terminal domain of MdmX (N-MdmX). As shown in **Fig. 2g**, the GST-CTD fusion protein could in vitro pull down the His6-p53$^{15-29}$-MdmX fusion protein that was detected by anti-His6 and anti-p53 antibodies. A similar interaction between GST-CTD and His6-p53$^{15-29}$-Mdm2 was also observed (**Fig. 2h**). To exclude the interaction between CCDC106-CTDand N-MdmX or N-Mdm2, we used the GST-N-MdmX fusion protein to pulldown a His6-tagged CTD protein (i.e., His6-CTD), as shown in **Supplementary Fig. 4**, the CTD domain of CCDC106 had no interaction with N-MdmX. Finally, we quantitively determined the binding affinity of p53$^{15-29}$ for the CTD domain with a K_d value of 0.32 μM (**Supplementary Fig. 5**).

CCDC106 downregulates p53 and Mdm2.

In the H1299^{p53+} cells that the wild type p53 gene is fused with GFP gene, the G418 induces the overexpression of the cellular p53-GFP fusion protein that is detectable with anti-p53 and anti-GFP antibodies (**Fig. 3**). As shown in **Fig. 3**, the cellular level of the p53-GFP fusion protein is downregulated with the expression of exogenous CCDC106 protein that is detected by anti-GFP and anti-FLAG antibodies. It is notable that in the H1299^{p53+} cells the cellular level of the exogenous CCDC106 protein has no significant difference from that in the p53-null H1299 cells, indicating that the CCDC106 overexpression promotes the p53 degradation whereas p53 has no effect on

150 the cellular level of the exogenous CCDC106 protein.

151 However, we found that the endogenous CCDC106 level significantly decreased
152 with the overexpression of exogenous CCDC106, in comparison with these of the p53-
153 null H1299 cells and the H1299^{p53+} cells that contained no CCDC106-expression
154 plasmids (**Fig. 3**), although its detailed reason was unknown. Nevertheless, the cellular
155 level of p21 protein remained in an upregulated level independent of the cellular level
156 of CCDC106 protein. In this study, we found that the H1299 cells are typical Mdm2-
157 overexpressing cancer cell lines (**Fig. 3)**. We also noticed that the current concentration
158 of G418 downregulated the cellular level of Mdm2, although the mechanism was
159 unclear. Nevertheless, this biological effect was independent of the CCDC106
160 expression (**Fig. 3**). However, when both p53 and the exogenous CCDC106 were
161 overexpressed, the endogenous Mdm2 protein was significantly downregulated, and the
162 endogenous CCDC106 was also downregulated (**Fig. 3**).

163

164 **Overexpression of Mdm2 and MdmX downregulate p53 and CCDC106.**

165 In the H1299 cells, the expression levels of the exogenous MdmX and Mdm2 genes
166 were distinct difference. The MdmX level was much higher than that of the Mdm2
167 protein (**Fig. 4a**). When the exogenous MdmX and Mdm2 genes were co-expressed,
168 the exogenous MdmX protein was further downregulated. Nevertheless, the expression
169 of the exogenous MdmX or Mdm2 genes in the H1299 cells exhibited no significant
170 effects on the levels of the endogenous Mdm2 protein (**Fig. 4a**). It is notable that in the
171 H1299 cells the overexpression of the exogenous MdmX exhibited no effect on the
172 cellular level of the endogenous CCDC106, while the expression of the exogenous
173 Mdm2 slightly downregulated the cellular level of the endogenous CCDC106. When
174 both exogenous MdmX and Mdm2 were co-expressed, the cellular level of the

endogenous CCDC106 was significantly downregulated (**Fig. 4a**).

By comparison, in the H1299^{p53+} cells that the overexpression of the cellular p53 protein was induced by G418 resulted in the upregulation of the cellular p21 and CCDC106 levels (**Fig. 4b**). We noticed that G418 caused the downregulation of the cellular level of the endogenous Mdm2 as also observed in **Fig. 3**. The overexpression of the exogenous MdmX gene significantly downregulated the level of cellular p53, p21 and CCDC106 proteins, accompanied by the Mdm2 downregulation. The overexpression of the exogenous Mdm2 gene could maintain a high cellular level of the endogenous Mdm2 protein. However, compared with the exogenous MdmX gene, the overexpression of the exogenous Mdm2 gene further downregulated the level of cellular p53 protein, causing a low level of the cellular p21 proteins, while the cellular level of the CCDC106 protein was maintained at a low level independent of the expression of the exogenous and endogenous Mdm2 (**Fig. 4b**). On the other hand, the co-expression of the exogenous MdmX and Mdm2 significantly downregulated the cellular level of the endogenous Mdm2, while the cellular levels of the p53, p21 and CCDC106 proteins were maintained at their low levels. Therefore, it is likely that the overexpression of either MdmX or Mdm2 enables to downregulate the cellular level of CCDC106 only mediated by p53. The overexpression of the exogenous Mdm2 significantly downregulates the cellular level of p53 more than the exogenous MdmX expression. The presence of the exogenous MdmX protein significantly reduced the cellular level of the endogenous Mdm2 where this regulation was also mediated by p53. Taken together, Mdm2 and MdmX play important and different roles in regulating the cellular level of the CCDC106 protein.

CCDC106 attenuates the p53 inhibition of the H1299 cell growth.

To explore how CCDC106 affects the expression of the p53 gene and thereby controls cell cycle arrest, we treated the H1299^{p53+} cells and their negative control cells (i.e., the H1299 cells) with G418 for 48 h in the presence or absence of the CCDC106 expression vector. As shown in **Fig. 5a**, our flow cytometric assay indicated that the overexpression of p53 in the H1299^{p53+} cells significantly affected cell cycle, causing cell arrest at the G2 stage (*the bottom middle panel*), while the H1299 cell cycles arrested at the S-stage (*the top middle panel*). When both p53 and CCDC106 were overexpressed in the H1299^{p53+} cells, the cell amounts in the G1 and G2 stages were significantly reduced (*the bottom right panel*), compared with the H1299 cells (*the top right panel*), indicating that the CCDC106 overexpression attenuated the p53 function in regulating cell cycle.

Next, we evaluated the viabilities of the H1299 and H1299^{p53+} cells in the presence of the CCDC106 siRNA and the Mdm2/MdmX inhibitor by use of MTT experiments. As shown in **Fig. 5b**, insignificant decrease in the H1299 cell viability was observed when the cells were transfected with the MdmX and Mdm2 expression vectors. However, the viability of the H1299^{p53+} cells dropped much faster than that of H1299 cells when p53 was overexpressed (**Fig. 5c**). When the MdmX expression vector was transfected into the H1299^{p53+} cells, the cell viability exhibited further decreasing while the H1299^{p53+}cells transfected with the Mdm2 expression vector started to recover their cell viability (**Fig. 5c**). The co-transfection of the MdmX and Mdm2 expression vectors significantly recovered cell viability. Furthermore, we examined the effect of the CCDC106 overexpression on the viability of the H1299^{p53+} cells in the presence of the overexpressed p53 proteins. As shown in **Fig. 5d**, the transfection of the CCDC106 expression vector with or without the transfection of MdmX expression vector exhibited no significant effect on cell viability. However, the transfection of CCDC106

expression vector with the transfection of Mdm2 expression vector significantly recovered cell viability (**Fig. 5d**), comparable to that of the H1299 cells (**Fig. 5b**). A similar enhancement was also observed for the H1299^{p53+} cells that were transfected with bothMdm2 and MdmX expression vectors (**Fig. 5d**).

Thus, we considered examining how the inhibition of the cellular Mdm2/MdmX and CCDC106 proteins affect cell viability. As shown in **Fig. 5e**, the use of the Mdm2/MdmX dual peptide inhibitor, PMI[11], significantly attenuated the effects caused by the overexpression of MdmX and Mdm2, resulting in lower cell viability. Importantly, the cell viability could be further reduced when these were treated with the CCDC106 siRNA in combination with PMI (**Fig. 5f**).

Discussion

To date, many studies have found that CCDC106 interacts with p53, resulting in p53 degradation in cancer cells and promoting cell proliferation[1,3,4,12]. In this work, we provide evidence that the C-terminal domain of the CCDC106 protein (CCDC106-CTD) directly interacts with the N-terminal domain of the p53 protein. More precisely, the CCDC106-CTD domain interacts with the TAD domain of the p53 protein. Identification of such protein-protein interaction immediately raises critical questions how CCDC106 interferes Mdm2/MdmX to bind p53 and how CCDC106 regulates the p53 signaling pathway, as Mdm2/MdmX are specific to the p53 TAD domain[13,14].

Despite their profound relationship to p53, Mdm2 and MdmX may have quite rich and complex lives outside of p53[15]. In this work, it has been found that the CCDC106 overexpression can significantly downregulate p53 and Mdm2. we also found that the MdmX overexpression can downregulate Mdm2 and CCDC106. Importantly, these signaling processes are solely mediated through p53. Thus, a concise interactive

network between CCDC106 and the p53-Mdm2/MdmX signal axis is summarized in **Fig. 6**, depicting that CCDC106 promotes the p53 and Mdm2 degradation.

Mdm2/MdmX are overexpressed in many cancers to impair p53 activity[16,17], serving as a hot drug target for cancer therapy[18-21]. Considering that CCDC106 competes Mdm2/MdmX for binding p53 identified from this work, therefore, we reason that the CCDC106-p53 interaction should be a promising drug target for cancer-killing in synergetic combination with drugs targeting the aberrant p53-Mdm2/MdmX interactions.

Methods

Cell lines and cell cultures

NCI-H1299, HCT116, MCF-7, A549, 293T and HepG2 cells were purchased from ATCC, and NCI-H1299^{p53+} cell line was constructed in our group[22,23]. All kinds of cells were generally cultured in a 37 °C incubator with 5% CO_2 according to ATCC protocols in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), supplemented with 10% serum, penicillin and streptomycin. After the cell confluency reached 80%, an individual plasmid harboring a gene encoding the Mdm2, MdmX or CCDC106 tagged with RFP protein was individually or in combination transfected for overexpressing exogenous the Mdm2-GFP, MdmX-GFP and FLAG-CCDC106-GFP fusion proteins.

Transfection of siRNA

The siRNAs were designed with the following two pairs of sequences. siRNA_CCDC106-1: 5′-GCGUCAAGACCCAGCUGCACA-3′ and 5′-

UGCAGCUGGGUCUUGACGCUG-3′; siRNA_CCDC106-2: 5′-GGACAAUGAAGGACGAUGAGA-3′ and 5′-UCAUCGUCCUUCAUUGUCCGC -3′; siRNA_negative control: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′. 293T, H1299 and H1299^{p53+} cells were cultured in serum-free OPTI-DMEM medium and transfected with siRNAs using lipofectamine-RNAi MAX. The cells were collected after cultured for 72 hrs later analysis.

GST pulldown experiments

Pulldown experiments were carried out with GST-NTD or GST-CTD beads to pulldown p53 in cancer cell lines. GST-agarose beads were also used to examine the interaction of CTD with different domains of p53 protein, i.e., p53$^{15-29}$ (the p53 binding peptide, p53p), p53$^{1-93}$ (transactivation domain, TAD), p53$^{94-312}$ (DNA binding domain, DBD) and full-length p53. The GST pulldown samples were subjected to western blotting assay.

Western blotting assay

Total cellular proteins were extracted with RPMI lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Trition-X100, 0.1% sodium deoxycholate, 0.1% SDS and 0.1% PMSF. The concentration of the protein extracts was firstly measured with Nanodrop-2000c at OD_{280nm}, and the cellular β-actin contents then calibrated with mouse anti-β-actin monoclonal antibody from ProteinTech (Wuhan, China; Cat#: HRP-60008; Gene ID 60, 100 μg/ml) with a dilution ratio of 1:10000. Calibrated samples were separated by SDS-PAGE, while a normal molecular marker was used. Each SDS-PAGE gel was electrically transferred to a polyvinylidene difluoride (PVDF) membrane

(Millipore, USA). To minimize the usage of antibodies, each PVDF membrane was cut into different sections, based on the molecular weights of cellular p53, Mdm2, MdmX, p21, CCDC106 and β-actin and the features of their corresponding antibodies provided by their manufacturers. Each section of PVDF membranes was blocked with a 5% skimmed milk powder dissolved in TBST buffer for 1 hr at room temperature, followed by incubation individually with their corresponding primary antibodies for 2 hrs in a sealed plastic bag. After washed 3 times with TBST, the membranes were incubated with HRP-conjugated secondary antibody for 1 hr in a sealed plastic bag. Multiple sections of PVDF membranes collected and the protein bands were visualized using enhanced chemiluminescence (ECL) reagents from BioSharp (Beijing, China) on a Tanon 5200Chemiliminescent Imager (Shanghai, China). Each blotting was repeated until a good quality image was achieved. Cellular p53 protein was blotted with a rabbit anti-TP53 polyclonal antibody IgG from CUSABIO (TX, USA; Cat#: CSB-PA15509AORB, Lot#: F0912A) with a dilution ratio of 1:4000; Cellular Mdm2 and MdmX proteins tagged with RFP were assayed with a mouse anti-RFP monoclonal antibody from Solarbio (Beijing, China; Cat#: K20016M) with a dilution ratio of 1:10000. Cellular p21 protein was detected with a rabbit anti-p21 polyclonal antibody from Elabscience (Wuhan, China; Cat#: E-AB-40097) with a dilution ratio of 1:500. Cellular CCDC106 protein was detected with a rabbit anti-human CCDC106 polyclonal antibody from Abnova (Wuhan, China; Cat#: PAB19286; protein ID NP_037433) with a dilution ratio of 1:1000; Primary antibodies were detected with either HRP-conjugated goat anti-rabbit IgG(H+L) which was from ProteinTech (Wuhan, China; Cat#: SA00001-2) with a dilution ratio of 1:10000 or HRP-conjugated goat anti-mouse IgG(H+L) which was form Biosharp (Guangzhou, China; Cat#: BL001A, 0.8 mg/ml) with a dilution ratio of 1:10000, respectively.

Flow cytometric assay

Cells were counted and plated in 6-well plate at 1×10^5 cells/ml in RPMI 1640 culture medium supplemented with 10% FBS. After being incubated for 3 days, Cells were washed twice with cold PBS buffer and resuspended in 1x PBS buffer at $1x10^6$ cells/mL. The cell samples were incubated with Alexa Fluor488 Annexin V and PI work solution (Dead Cell Apoptosis Kit) at room temperature for 15 min. The cell cycles were analyzed by the propidium iodide (PI) reagent with a BD FACSMelody flow cytometer.

Assessment of cell viability

MTT assay was performed on a Synergy H1 multiplate reader (Biotek, USA) using H1299 cells and the $H1299^{p53+}$ cells. Cells were grown at 37°C with 5% CO_2 in RPMI 1640 culture medium supplemented with 10% FBS until the cell density reached 90% confluency. Cells were counted and plated in 96-well plate at 5×10^3 cells per well. After being incubated for 3 days, the medium was refreshed with RPMI 1640 supplemented with 3% FBS. G418 was added in the final concentration of 50 μg/ml, and the cells continued to culture for 48 h before PMI was added in the final concentration of 1 μM in the absence or presence of siRNAs.

Then the MTT (5 mg/ml) solution was added in 10 μl/well. The cells were incubated for another 4 h at 37°C. After discarding culture media, the cells were washed with PBS, followed by adding 100 μl DMSO to each well. The plates were shaken on a plate shaker for 10 min. The plates were then read with the plate reader at $OD_{490\ nm}$. Data were processed with MicroCal Origin software (v2017, MicroCal, USA).

Data availability

The nucleotide sequence of p53 genome in H1299 cell line generated in this study have been deposited GenBank under Submission #2667019 (will be updated upon available).

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Contributions

T. Zhou, X.Y. Cheng and Z.Q. Ke carried out cell biological assays; T. Zhou, X. Y.

Cheng, Q.Q. Ma, and J.N. Xiang performed protein preparation; X.Y. Cheng and Z.Q. Ke performed fluorescence polarization assay; M. Gao carried out the structure predication; X.Y. Cheng, Y.Q. Huang and Z.D. Su designed the experiments; Z.D. Su, X.Y. Cheng and Y.Q. Huang wrote the paper. Z.D. Su conceived of the project.

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Conflict of interest

We declare that we have no conflicts of interest.

Supporting information available

Supplementary tables and figures are available in the online version of this paper.

Figure legends

Fig. 1. Prediction of the structure domains of CCDC106 protein. a. Amine acid sequence of human CCDC106. **b**. Potential domains in CCDC106 predicted by SMART program. Putative domains are described in *Supplementary Information*. **c**. Putative three-dimensional structure of CCDC106 was predicted with alphaFold2 program. Source data for **c** are provided as a Source Data file.

Fig. 2. Correlation between CCDC106 and p53. GST: GST protein; Input: the cell extracts; GST-CTD: the GST-CTD fusion protein; Beads: GST agarose beads; p53: the full-length p53 protein, $p53^{1-212}$: the N-terminal segment of p53 containing TAD, PRD and partial DBD domain; $p53^{1-93}$: the TAD and PRD domains; $p53^{94-312}$: the DBD domain of p53, $p53^{15-29}$: the p53 TAD domain, N-MdmX and N-Mdm2: the N-terminal domains of MdmX and Mdm2, respectively. **a**. The cellular levels of endogenous CCDC106 and p53 proteins in H1299, 293T, HCT116, MCF-7, A549 and HepG2 cells were assayed by western blotting. **b**. Knockdown of the endogenous CCDC106 in 293T cells downregulates the cellular level of endogenous p53. **c**. In vitro pulldown of p53 protein with the GST-CTD fusion protein was detected with anti-p53 polyclonal antibody. **d**. No obvious interaction was detected in vitro between p53 and the GST-NTD with GST-pulldown assay that was detected with anti-p53 polyclonal antibody. **e.** Pulldown of the full-length and a N-terminal segment of p53 with the GST-CTD fusion protein. **f.** Pulldown of $p53^{1-93}$ but not $p53^{94-312}$ with the GST-CTD fusion protein. Inputs were the cell extracts of *E. coli* cells expressing $p53^{1-93}$ or $p53^{94-312}$. **g** & **h.** Pulldown of $p53^{15-29}$ in the N-MdmX or N-Mdm2 fusion forms with the GST-CTD fusion protein. Blots in each subgroup were made from either the same gel or different gels with similar

exposure time. Source data for **a – h** are provided as a Source Data file.

Fig. 3. CCDC106 downregulates cellular p53 and Mdm2 in H1299^{p53+} cells. Assays were done with western blotting in the presence or absence of G418. G418: inducer of the p53 expression in the H1299^{p53+} cells; PL(CCDC106): the expression plasmid of CCDC106; GFP(CCDC106) and FLAG(CCDC106): CCDC106 fused with FLAG and GFP tags; The p53 protein was detected with p53 polyclonal antibody and anti-GFP tag monoclonal antibody. CCDC106 was detected with CCDC106 polyclonal antibody, anti-GFP tag monoclonal antibody and anti-FLAG monoclonal antibody. p21 and Mdm2 were detected with their polyclonal antibodies. Actin was used a control. All blots were made from the same gel. Source data are provided as a Source Data file.

Fig. 4. Mdm2 and MdmX downregulate cellular CCDC106 via p53. PL-MdmX and PL-Mdm2: plasmids harboring MdmX or Mdm2 genes, respectively; RFP(MdmX/Mdm2): MdmX-RFP and Mdm2-RFP fusion proteins, respectively. Exogenous MdmX and Mdm2 were detected with their polyclonal antibodies. Endogenous Mdm2 was detected with its polyclonal antibody. The p53 was detected with p53 polyclonal antibody. Endogenous p21 and CCDC106 proteins were detected with p21 and CCDC106 polyclonal antibodies, respectively. Actin was used a control. Blots in **a** and **b** were made from two different gel, respectively. Source data for **a – b** are provided as a Source Data file.

Fig. 5. Overexpression of CCDC106 attenuates the p53 function on cell proliferation. All the cells were cultured in the presence of 50 μg/mL G418 for inducing the p53 overexpression. **a**. A flow cytometric assay of H1299^{p53+} cells

overexpressing CCDC106 in comparison with the H1299 cells. Control: no G418 and PL-CCDC106 plasmid. **b-f**. Effects of the Mdm2/MdmX dual inhibitors (PMI) and the CCDC106 siRNA on the H1299^{p53+} cell viability in comparison with that of the H1299 and H1299^{p53+} cells. *Dark*: negative control; *light grey*: transiently transfected with the MdmX expression vector; *Dark grey*: transiently transfected with the Mdm2 expression vector; *Blank*: transiently transfected with both MdmX and Mdm2 expression vectors. n = 3 independent experiments, $p>0.05$ compared with the control group. **b**. H1299 cells. **c**. H1299^{p53+} cells. **d**. The H1299^{p53+} cells were transfected with the CCDC106 expression plasmid. **e** The H1299^{p53+} cells were treated with 1 μM of PMI. **f**. The H1299^{p53+} cells were treated with the CCDC106 siRNA and 1 μM of PMI. Source data for **b – f** are provided as a Source Data file.

Fig. 6. A signaling map depicting the CCDC106-p53-Mdm2 interactions regulating cell proliferation. This signaling map was constructed by combining the data from this work with previously published data [6,7]. IR: irradiation; DSB: DNA double strand break; ROS: reactive oxygen species; AKT: Akt serine/threonine kinase; ATM: ataxia telangiectasia mutated kinase; NBS1: Nijmegen breakage syndrome 1; Ac: acetyl group; T: threonine; S: serine; K: lysine; U: ubiquitin. p27: Cyclin dependent kinase inhibitor p27.

Fig. 1, Zhou et al.,

a

```
          10         20         30         40         50
KNDRSSRRRT MKDDETFEIS IPFDEAPHLD PQIFYSLSPS RRNFEEPPEA

          60         70         80         90         100
ASSALALMNS VKTQLHMALE RNSWLQKRIE DLEEERDFLR CQLDKFISSA

          110        120        130        140        150
RMEAEDHCRM KPGPRRMEGD SRGGAGGEAS DPESAASSLS GASEEGSASE

          160        170        180        190        200
RRRQKQKGGA SRRRFGKPKA RERQRVKDAD GVLCRYKKIL GTFQKLKSMS

          210        220        230        240        250
RAFEHHRVDR NTVALTTPIA ELLIVAPEKL AEVGEFDPSK ERLLEYSRRC

          260        270        280
FLALDDETLK KVQALKKSKL LLPITYRFKR
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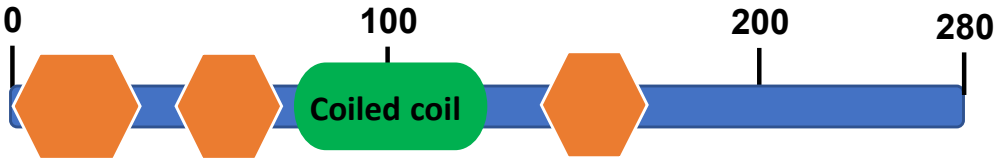
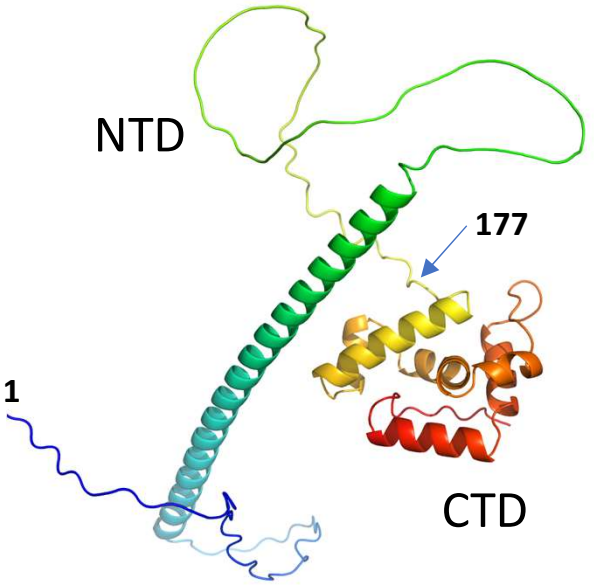
b



c



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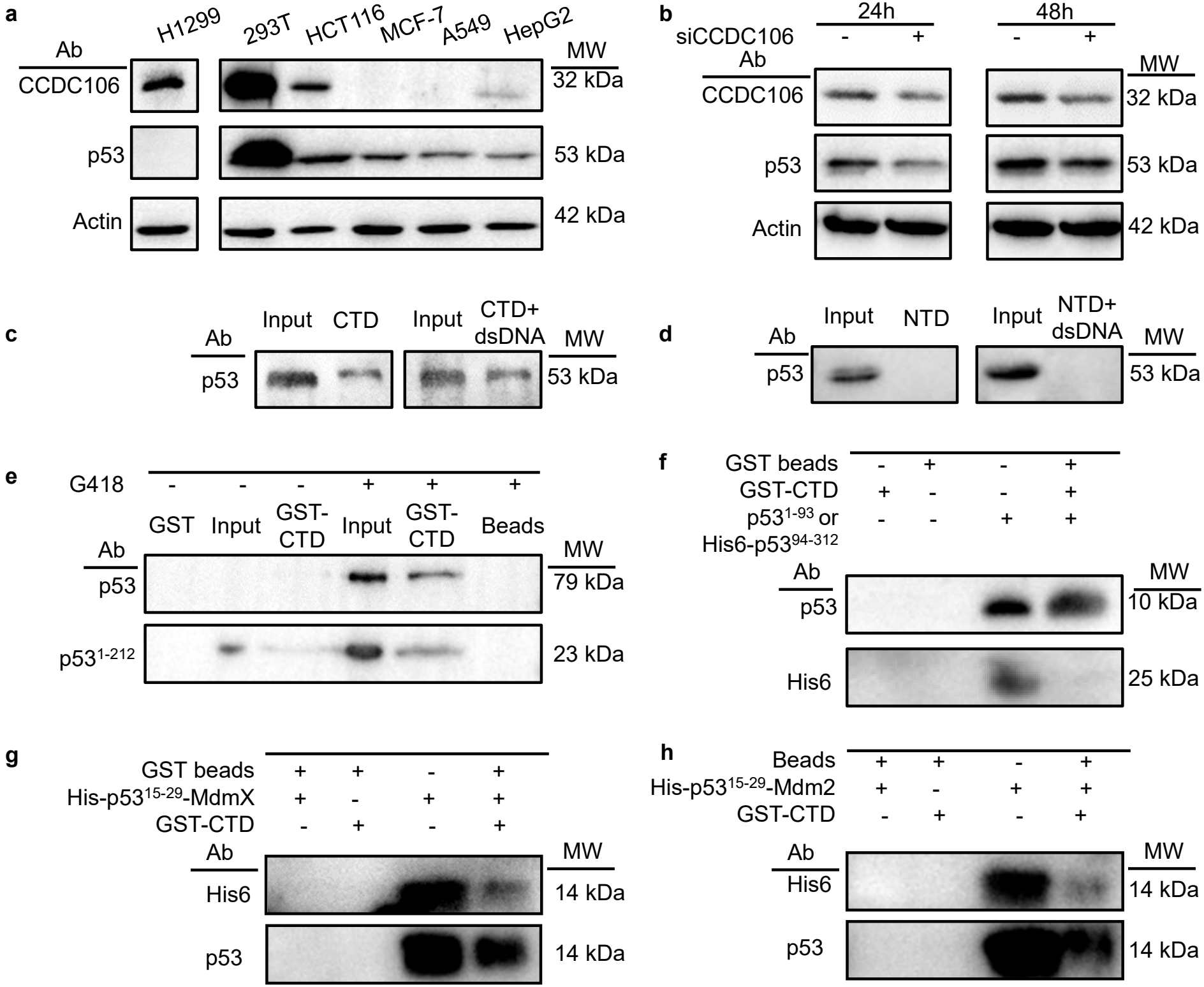

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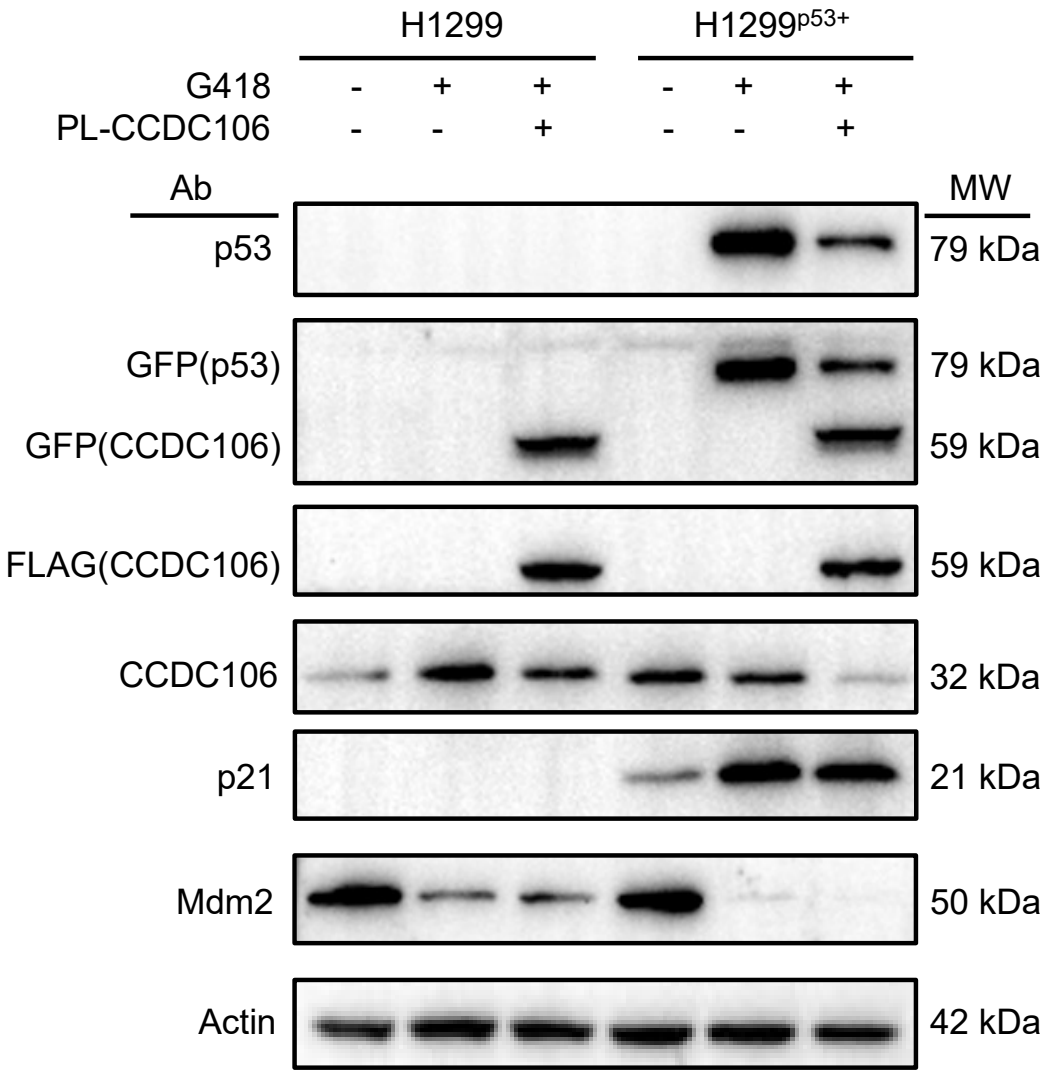


Fig. 4, Zhou et al.,

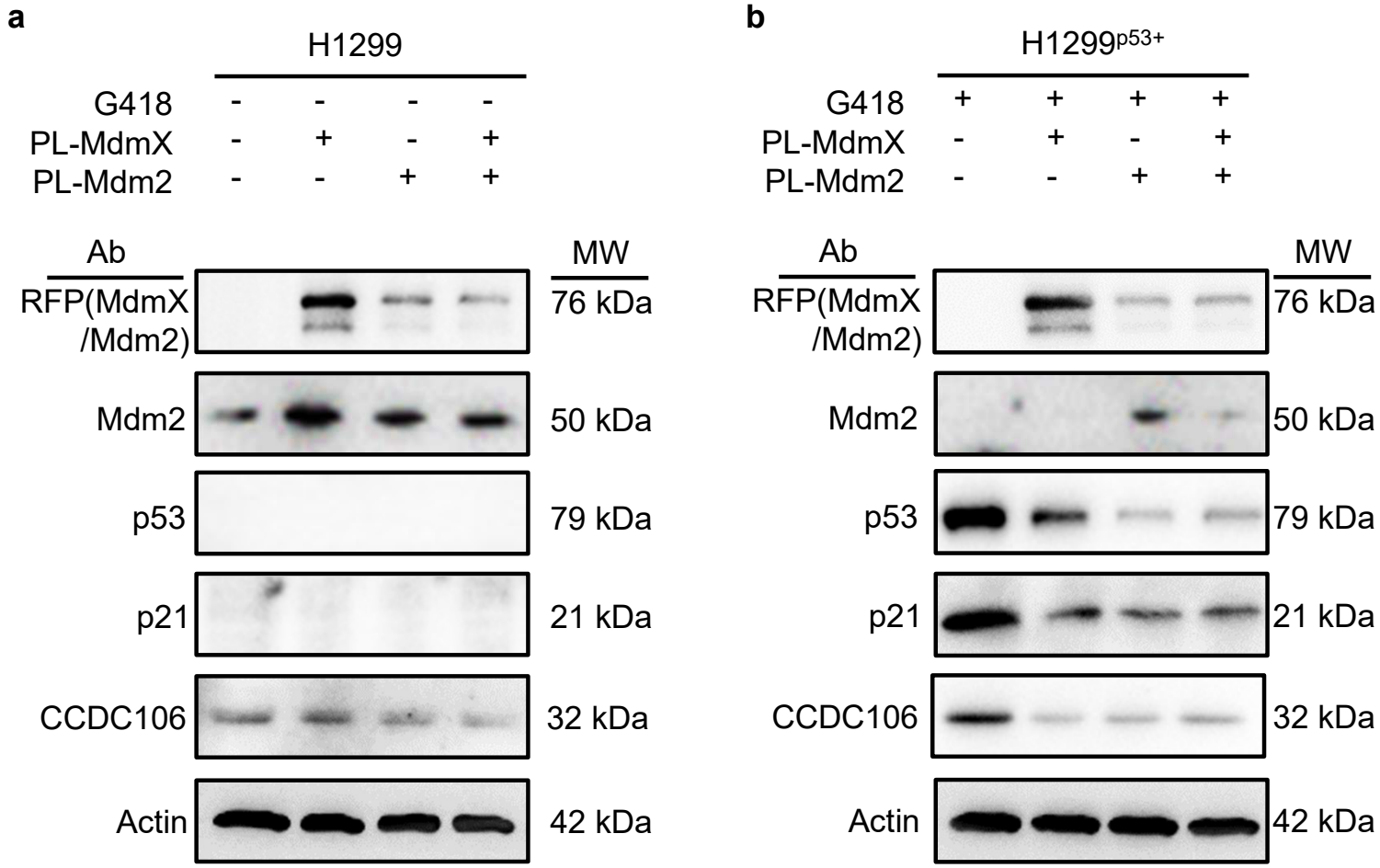


Fig. 5, Zhou et al.,

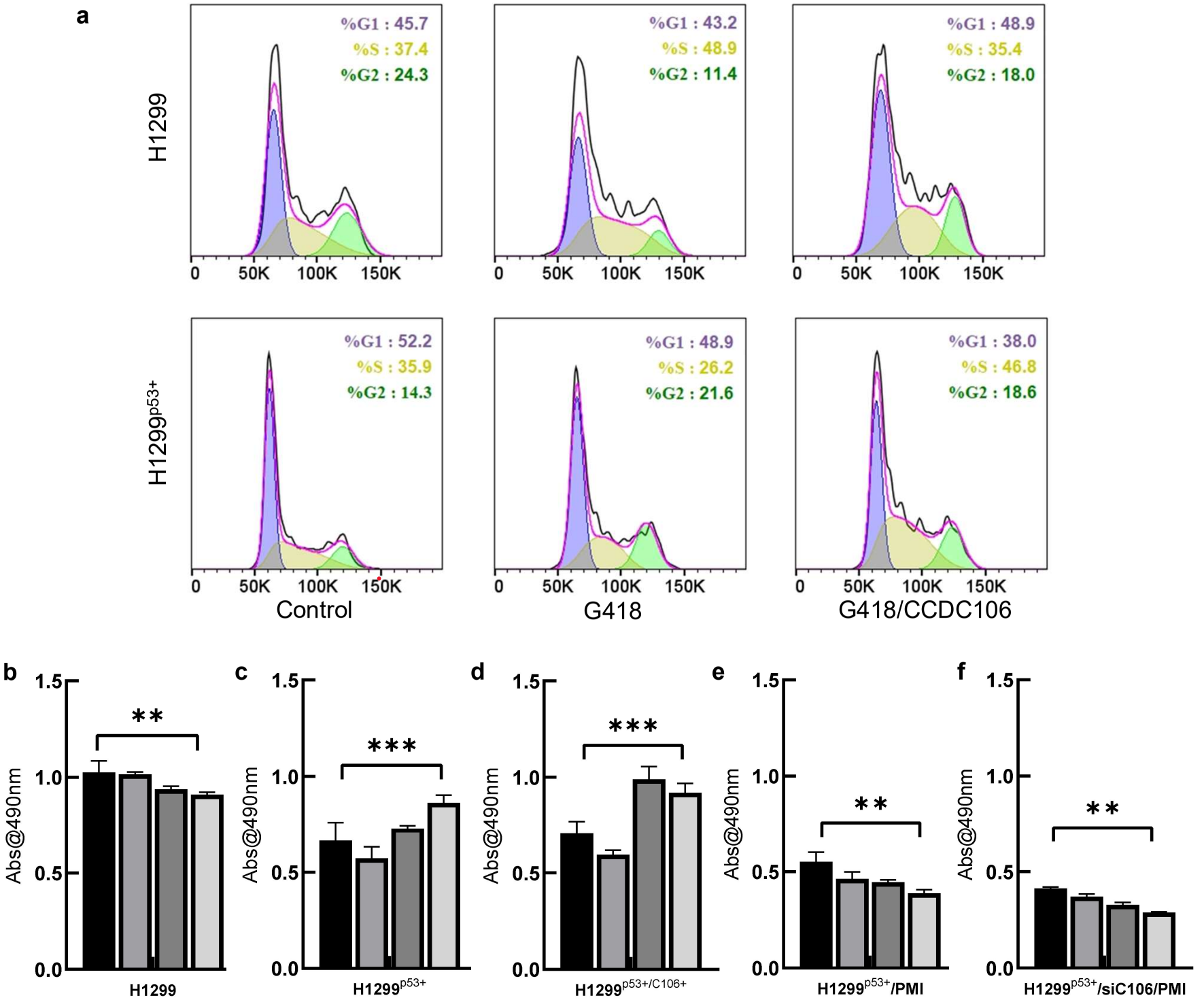
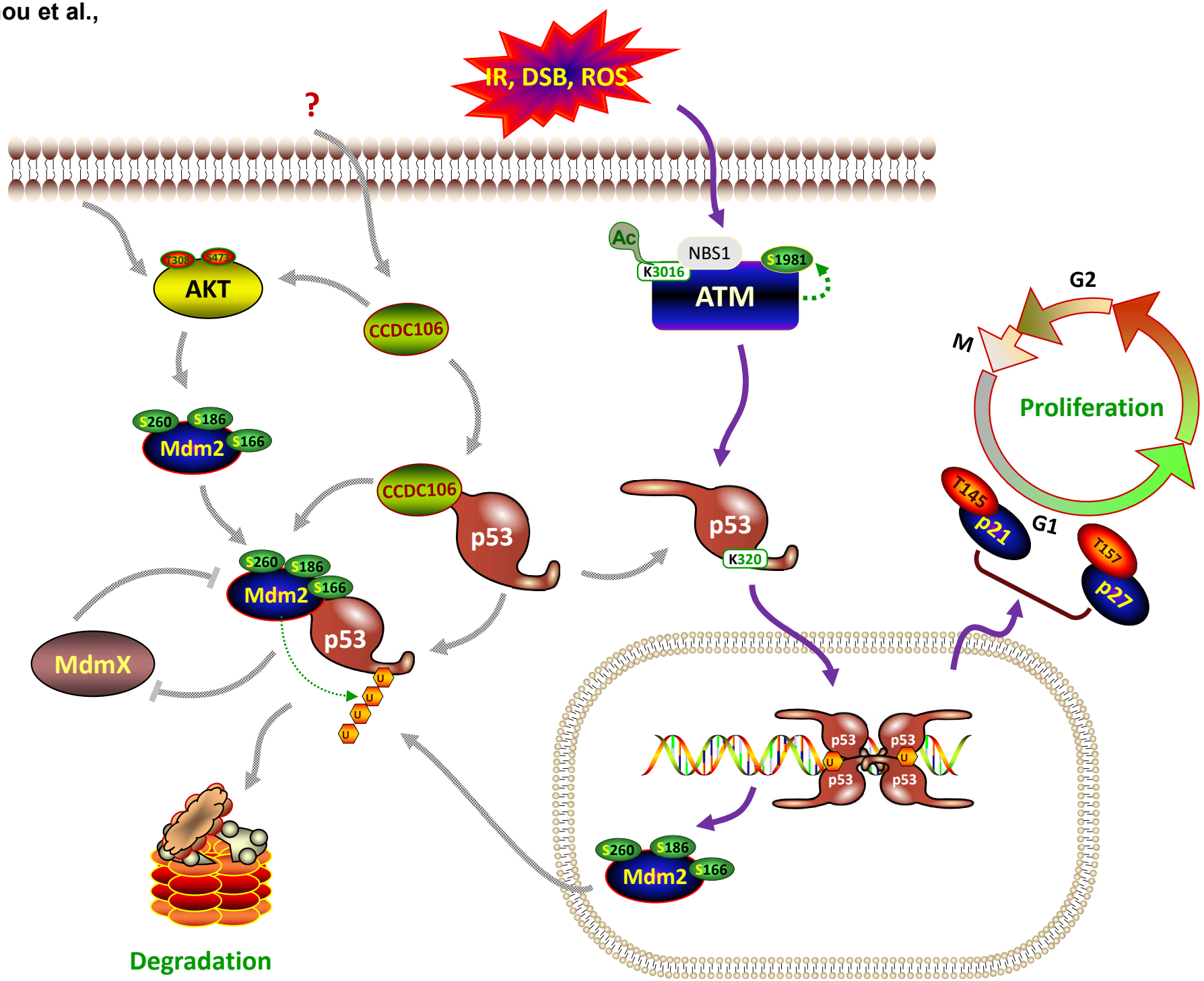


Fig. 6, Zhou et al.,



Supplementary Information for

Molecular mechanism of CCDC106 regulating the p53-Mdm2/MdmX signal axis

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Keywords: CCDC106; p53; Mdm2; MdmX; p21; cell cycle; apoptosis; cancer, NSCLC.

Supplementary Results

Bioinformatic characterization of CCDC106 protein

As its biological function has not been thoroughly investigated, we use the Simple Modular Architecture Research Tool (SMART) [1] to predict its putative domains. As shown in **Fig. 1b**, it contains a coiled coil domain predicted with confidence (**Supplementary Table 1**). However, it is also likely that CCDC106 may have potential to constitute other functional domains, such as Basic region leucin zipper (BRLZ), Parathyroid hormone (PTH), Repeats in fly CG4713, worm Y37H9A.3 and human FLJ20241 (DM14), Suppressor of glucose by autophagy (SOGA), Homeobox associated leucin zipper (HALZ), Worm-specific N-terminal domain (WSN), Helicase and RNaseD C-terminal (HRDC) domains, although their e-values are over threshed (**Supplementary Table 2**).

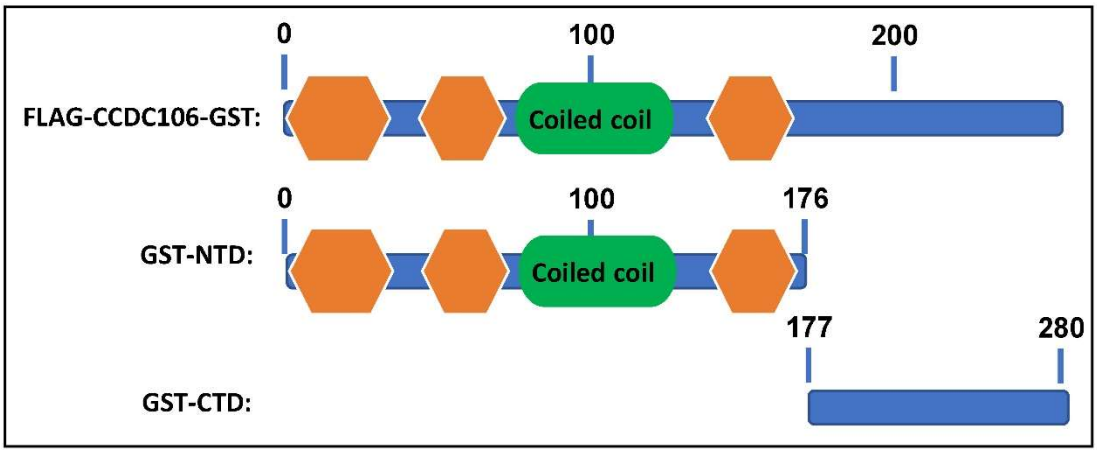
Supplementary Table 1. Confidently predicted of domains, repeats, motifs and features of CCDC106

Structure property	Sequence start	Sequence end	E-value
Low complexity	45	57	N/A
Coiled coil	63	101	3.9e-100
Low complexity	133	150	N/A

Supplementary Table 2. Possible structure features of CCDC106

Name	Start	End	E-value	Reason
SOGA	58	157	0.21	threshold
BRLZ	43	95	32.2	threshold
PTH	49	85	60.1	threshold
HALZ	241	278	583	threshold
WSN	187	249	683	threshold
HRDC	186	265	893	threshold
DM14	55	114	1140	threshold

As predicted using alphaFold2 program, the CCDC106 structure contains a long α-helix flanked by two coils at the two ends of the α-helix in the N-terminal region of the CCDC106 protein, while its C-terminal region forms a compact helix-rich structure. Therefore, we arbitrarily define these two regions as the N-terminal domain (NTD) and the C-terminal domain (CTD) of CCDC106, respectively (**Fig. S1**).

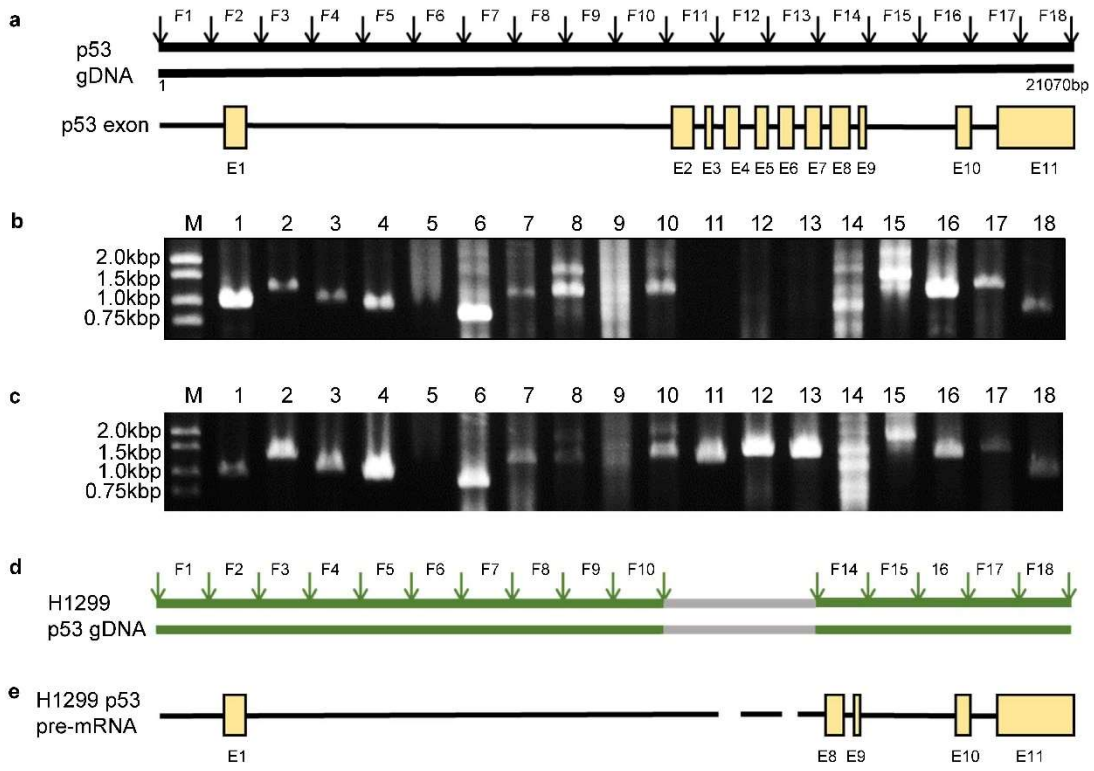


Supplementary Fig. 1. Dissection of CCDC106 structure for expression in GST fusion protein.

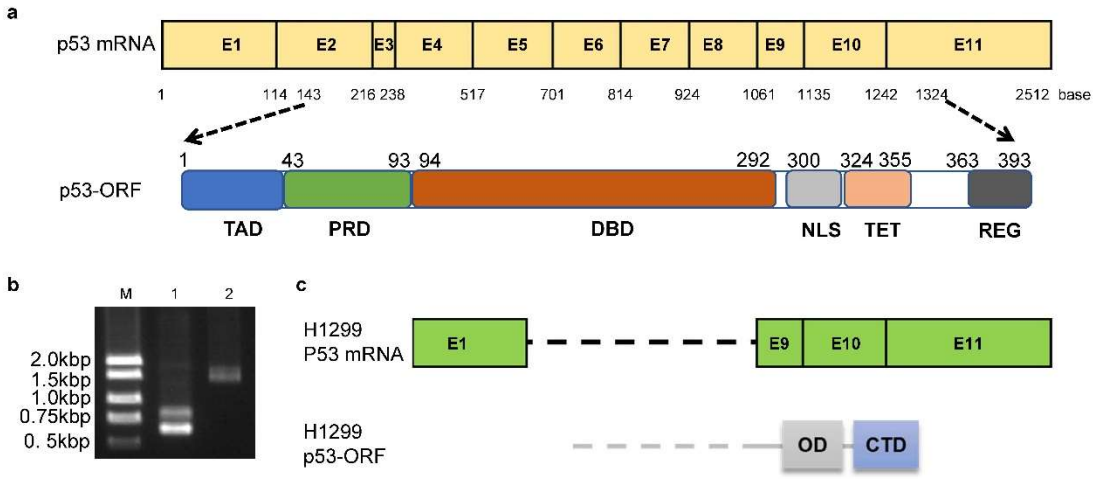
Examination of p53 gene in H1299 genome

In normal human cells, the loco of the wild type p53 gene is in the 17th chromosome with a length of 21070 bp embedded in a long promotor region and the p53 genome. The p53 genome contains 11 exons and 10 introns (**Supplementary Fig. 2**). We used 18 pairs of specific primers to amplifyp53 DNA fragments from the H1299 genome using the p53 genome of the 293T cells as template. As shown in **Supplementary Fig. 2b**, the fragments 11, 12 and13 were not detected from the H1299 genome, while these three fragments could be amplified from the HCT116 genome (**Supplementary Fig. 2c**) that contains a wild type p53 gene [2]. Thus, the p53 genomic DNA in H1299 cells was missing the entire DNA sequence covering the region from exon 2 to exon 7

(**Supplementary Fig. 2d** & **2e**). After amplified DNA fragments were evaluated by DNA sequencing, we found that all the amplified fragments matched the DNA sequences expected from human genomics and the p53 genome of HCT116 cells (see **Supplementary Data 1**).



Supplementary Fig. 2. DNA sequencing of the p53 gene in H1299 cell line. a. A schematic structure of p53 genome from human genomics. F1-F18 represent 18 fragments for designing primers for sequencing the p53 gDNA. E1-E11 represent 11 annotated exons in native p53 gDNA. **b**. Agarose gel imaging of the PCR fragments for F1-F18 using the H1299 gDNA as template. **c**. Agarose gel imaging of the PCR fragments for F1-F18 using the HCT116 gDNA as template (positive control). **d**. A cartoon summarizes DNA sequencing results. *Green*: detected segments and *grey*: undetected segments in the p53 gDNA from the H1299 genome. **e**. A cartoon represents the primary mRNA of p53 in H1299 cells. Boxes represent existed exons.



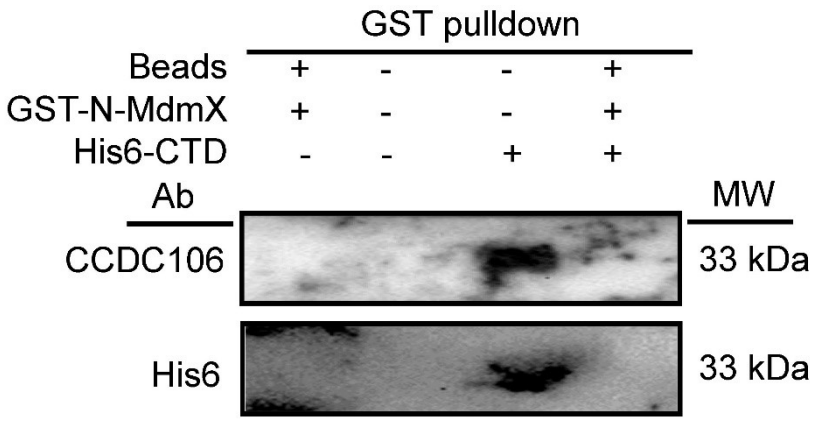
Supplementary Fig. 3. Determination of p53 mRNA in H1299 cells. a. The wild type p53 mRNA from human genomics is composed of 11 exons. An open reading frame (ORF) of matured p53 protein is deduced based on the p53 mRNA. TAD: Transactivation domain; PRD: Proline-rich domain; DBD: DNA binding domain; NLS: Nuclear localization sequence; TET: Tetramerization domain; REG: Regulation domain. **b**. The p53 cDNA reversely-transcripted from the H1299 mRNA sample was visualized by agarose gel.

The wild type p53 gene is composed of exons 2-11 (**Supplementary Fig. 3a**). And a matured p53 mRNA encodes a full-length polypeptide of 393 residues, consisting of multiple functional domains including a transactivation domain (TAD), an proline-rich domain (PRD), DNA-binding domain (DBD), nuclear localization sequence (NLS), Tetramerization domain (TET) and C-terminal regulatory domain (REG) [3] (**Supplementary Fig. 3a**). To examine whether an p53 fragment exist in H1299 cells, we isolated the total RNAs from the H1299 cell extracts and amplified p53 cDNA with two pairs of primers designed based on native p53 mRNA sequence. One pair of primers was used to amplify the region from promotor to stop codon and other pair was used to

amplify whole region from promotor to terminator. As shown in **Supplementary Fig. 3b**, the resultant PCR products was shorter than expected length. These PCR bands were further subcloned with a TOPO-Blunt vector for DNA sequencing. The DNA sequencing results revealed a shorter p53 mRNA that contains only exons 1, 9, 10 and 11 (**Supplementary Fig. 3c** and **Supplementary data 1-3**). This short mRNA can be only translated into a short peptide containing TET and REG domains (**Supplementary Fig. 3c**). Thus, our data revealed that H1299 cells cannot express a full-length p53 protein.

No interaction between CCDC106-CTD and N-MdmX

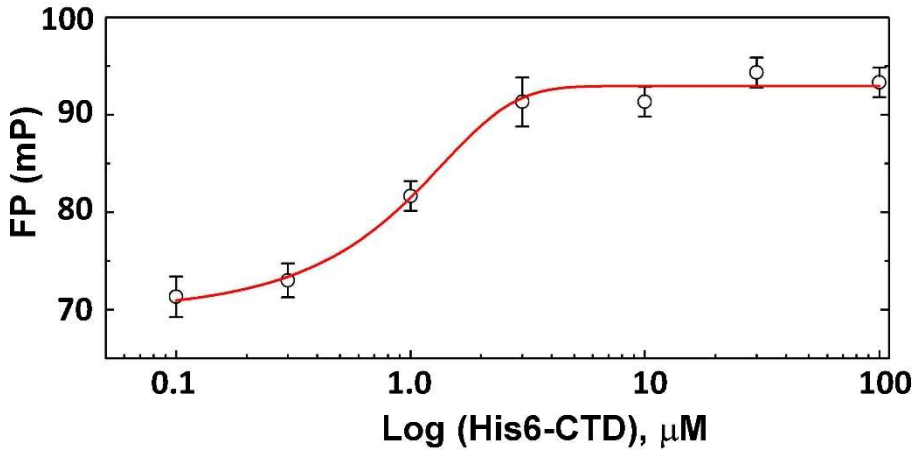
To exclude the interaction between CTD and N-MdmX or N-Mdm2, we used the GST-N-MdmX fusion protein to pulldown a His-tagged CTD protein (i.e., His6-CTD), as shown in **Supplementary Fig. 4**, the CTD domain of CCDC106 had no interaction with MdmX.



Supplementary Fig. 4. GST pulldown assay of CCDC106-CTD. Beads: GST agarose beads; GST-N-MdmX: the GST and N-MdmX fusion protein; His6-CTD: His6-tagged CTD protein. CCDC106 protein was detected CCDC106 polyclonal antibodies. Source data are provided as a Source Data file.

Quantitation of the interaction of CCDC106-CTD with p53$^{15-29}$

We quantitively determined the binding affinity of p53$^{15-29}$ for the CTD domain with a K_d value of 0.32 μM (**Supplementary Fig. 5**).



Supplementary Fig. 5. Determination of the binding affinity of p53$^{15-29}$ with the CTD domain of CCDC106 using fluorescence polarization (FP) assay. Source data are provided as a Source Data file.

Supplementary methods

Materials

The cDNA of the human CCDC106 gene was synthesized by GenScript (Wuxi, China) and sub-cloned to the pcDNA3.0 plasmid where the FLAG tag and the RFP tag were inserted at its 5-terminal and at its 3'-terminal ends, respectively. Lipofectamine 2000 was purchased from Invitrogen (Shanghai, China). The peptides including PMI and fluorescein-labeled p53p (Flu-p53p) were synthesized by TOPE Biotech (Shanghai, China). NCI-H1299, HCT116, MCF-7, A549, 293T and HepG cells were purchased

from ATCC, and NCI-H1299^{p53+} cell line was constructed in our group[4,5].

The primers used in this study were synthesized by GenScript (Nanjing, China). The siRNAs were designed and synthesized by Genepharma (Suzhou, China). Genomic DNA extraction kit, plasmid mini-prep kit and PCR cleanup kit were purchased from Tiangen Biotech (Beijing, China). RNA extraction kit RNeasy® Mini Kit was purchased from QIAGEN China (Shanghai, China). RT-PCR kits and Taq Mix were purchased from Vazyme Biotech (Nanjing, China). TOPO-Blunt Simple Cloning Kit was purchased from Yisheng Biotech (Shanghai, China). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was obtained from Biofroxx (Guangzhou, China).

Protein expression and purification from *E. coli* cells

The DNA sequences of the N-terminal domain (1-176) and C-terminal domain (177-280) of CCDC106 were optimized with *E. coli* bias codons and synthesized by GenScript (Wuxi, China) and sub-cloned to the pGEX-6P-1 plasmid. The wild-type p53 and its TAD domain (1-93) and DBD domain (94-312) were subcloned in a modified pET28b plasmid that its thrombin cleavage site was substituted with the Tev protease cleavage site[6].

Recombinant proteins were prepared in *E. coli* BL21 (*DE3*) cells. Cells were grown in LB medium containing kanamycin (34 μg/mL) for modified pET28b vector or ampicillin (50 μg/mL) for pGEX-6P-1 vector and induced with 0.4 mM IPTG at 18°C for 12 h.

To purify His-tagged protein, cells were harvested by centrifugation at 5,000 ×*g* for 30 min, resuspended in a buffer containing 10 mM Tris-HCl, 40 mM NaCl, 2 mM β-mercaptoethanol, 2 mM imidazole, pH 8.0 (Buffer A), and lysed by sonication and

homogenization, followed by spinning at 18,000 ×*g* for 30 min. The supernatant was loaded onto a 5 mL Ni-NTA agarose column (Qiagen, USA) and His-tagged protein was competitively eluted using a gradient of Buffer A mixed with Buffer B containing 10 mM Tris-HCl, 40 mM NaCl, 2 mM β-mercaptoethanol, 300 mM imidazole, pH 8.0. The eluate was diluted in 20 times with a buffer containing 20 mM sodium citrate (pH 6.5), 10% glycerol, 2 mM β-mercaptoethanol.

To purify GST-tagged protein, cells were harvested by centrifugation at 5,000 ×*g* for 30 min, resuspended in 1x PBS buffer and lysed by sonication and homogenization, followed by spinning at 18,000 ×*g* for 30 min. The supernatant was loaded onto a 5 mL GST agarose column (GE, USA) and GST-tagged protein was competitively eluted using 1x PBS buffer containing10 mM glutathione. The eluate was desalted with a 1xPBS buffer. All purified protein samples were freshly frozen in liquid nitrogen, and kept at -80 °C.

DNA sequencing of p53 genomic gene in H1299 cells

The genomic DNA of H1299 and HCT116 cells was extracted with the cell genome extraction kit, and the 53 genome was divided into 18 fragments according to the genome data on NCBI (Genebank ID: 7157). PCR was used to identify whether each fragment existed in H1299, and HCT116 was used as a control. PCR was carried out with *Taq* DNA polymerase in 50 μL reaction mixture, which contained 25 μL of 2 × Taq mix, 2 nM primers and 10 ng template DNA.

The total RNA of H1299 cells was extracted using the RNA extraction kit, and immediately reverse transcription PCR (RT-PCR) was performed to obtain the cDNA library of H1299, and then each putative fragment of p53 gene were amplified with designed primers (**Supplementary Table 3**). PCR products were evaluated by agarose

gel electrophoresis. The fragments obtained by the above PCR were purified and ligated with TOPO-Blunt vector. The ligation mixture was transformed into *E. coli* DH5α competent cells for selecting correct colonies. After cultured for 12 hours, a single colony was picked and identified using colony PCR with primers M13-F: 5'-TGTAAAACGACGGCCAGT-3' and M13-R: 5'-CAGGAAACAGCTATGACC-3'. PCP products were directly used for DNA sequencing.

Supplementary Table 3. Primers for sequencing p53 genomic DNA and cDNAs

Primer	Sequence (5′-3′)
1	TGCTCAAGACTGGCGCTA
2	AAAAAGAAATGCAGGCGGAGAATAG
3	CGATGAGAGGGGAGGAGAGAGA
4	ATATATACAACATGAACGAAT
5	GGAATCATACATTATGTG
6	CAAAGAAAAAAAGAAAATAGC
7	CCTTTCTCTACTGAATGCTTT
8	CTGGCCTATTTATCCTTTTT
9	ATGCAACAGCTAACCAATTTT
10	TAGGCCTCCCAAAGTGCTGGCAT
11	GTCGGAGTTCCACTAGCAGCA
12	GCGTGAGACATCGGGCCACTAA
13	GCCTGGGCGACAGAGCAAGACTGT
14	ATTACAGGCGCCCACCACTACA
15	TGAGACCAACCTAACATGGTG
16	TACCTAGTACTCTGTGTATTA
17	GCAGAAAGAGCTAACCTTTGTT
18	CGGAGTCTCGCCCTGTCACC
19	AATCCCAGCTACTCAGGAAGT
21	GGGCTGAGGAGTGTCCGAAGA
22	TGGGTCTTCAGTGAACCATTG
23	CTTTTCACCCATCTACAGTCC
24	GCAACCAGCCCTGTCGTCTCT
25	GCACATGACGGAGGTTGTGAG
26	CTACCTGTCCCATTTAAAAAA
27	TCCTCCACCTACCTGGAGCTG
28	GCTATGATCACATCACTGTAA
29	GCCTGCCTAGCCTACTTTTAT
30	TGAGCCAGTGCGCCTGGCCTTTT
31	AGCATGGTTGCATGAAAGGAG
32	TCAACCGGAGGAAGACTAAAA
33	CCATTCTCATCCTGCCTTCAT
34	TGGTTAGTACGGTGAAGTGGG

35	GGAGATGTAAGAAATGTTCTT
36	TGGCAGCAAAGTTTTATTGTA
p53-promoter-F	CTCAAAAGTCTAGAGCCACCG

Fluorescence polarization (FP) assay

Fluorescence polarization assay was done using black, low-protein-binding 96-well plates (Corning, NY) in a total volume of 100 μL per well of 20 mM phosphate (pH 6.8), 200 mM NaCl, and 1 mM DTT. One nM off luorescein-p53p (fluorescein-GSGSSQETFSDLWKLLPEN, Flu-p53p) titrated with GST-CTD and FP readings were taken with a 555 nm excitation filter and a 632 nm static and polarized filter on a BioTek H1 multiplate reader with Gen5 software. All FP data were fitted with Origin 2017 for obtaining K_d values.

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