

CacyBP/SIP protein reduces p53 stability by enhancing Mdm2 activity in p53 mutant glioma cells

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Our previous studies have illustrated that CacyBP/SIP (Calcyclin-binding protein or Siah-1-interacting protein) promoted the proliferation of glioma cells. However, the possible mechanism still needs to be clarified. In the current study, we aimed to uncover the potential mechanism of CacyBP/SIP in regulating glioma cell proliferation. We found that CacyBP/SIP decreased the protein level of p53, but not the mRNA level of p53 in p53 mutant U251 cell line, whereas, in p53 wild-type U87 cell line, CacyBP/SIP neither promoted its proliferation nor regulated the changes of p53 protein. Further investigation indicated that CacyBP/SIP interacted with p53 and Mdm2 (Mouse double minute 2) to promote p53 ubiquitination and subsequent proteasome-mediated degradation in U251. Moreover, in the presence of Mdm2, CacyBP/SIP boosted the ubiquitination of p53 in a dose-dependent manner. On the contrary, inhibition of Mdm2 activity significantly increased the stability of p53. Finally, we found that the protein level of CacyBP/SIP and p53 is inversely correlated in p53 mutant human glioma tissues. These observations suggest an underlying mechanism that CacyBP/SIP promotes the degradation of p53 by enhancing Mdm2 E3 ligase activity, which reveals a novel pathway for the regulation of mutant p53 and provides a new therapeutic approach to target the CacyBP/SIP-induced glioma cell proliferation.

Key words: CacyBP/SIP, p53, Mdm2, glioma, tumorigenesis

Glioma is the most common type of brain tumor with the median survival time of only 12 to 15 months, despite the significant improvements in neurosurgery, radiotherapy, and chemotherapy [1, 2]. The poor prognosis of glioma is largely attributed to the rapid growth and invasive/migratory nature of glioma cells. Therefore, an understanding of the mechanisms underlying glioma development and progression is critical to discover specific molecular targets that could serve as effective methods for glioma treatment.

The p53 is a key modulator of tumorigenesis and a central target in cancer therapy [3]. Commonly referred to as a master regulator, p53 has the ability to initiate diverse cellular processes, including cell cycle arrest, senescence, and apoptosis [4]. The function of p53 could be limited by ubiquitin ligase Mdm2, the primary negative regulator of p53. Mdm2 is a member of the RING-finger-containing proteins family, which primarily functions as a ubiquitin ligase [5]. An important role of Mdm2 is to regulate the ubiquitination and proteasomal-dependent degradation of p53 [6, 7]. The capacity of Mdm2 to promote p53 degradation

mainly depends on the integrity of the p53-binding domain at N-terminal and RING-finger domain at C-terminal [8].

CacyBP/SIP was firstly identified in Ehrlich ascites tumor cells owing to its capability to interact with S100A6 in a Ca²⁺-dependent manner [9], while human homolog of CacyBP/SIP was discovered as a Siah1 (Seven in absentia homolog 1) interacting protein [10]. Siah1 is an E3 ubiquitin ligase whose expression could be induced by p53 in response to DNA damage [11, 12]. It has been reported that is CacyBP/SIP involved in the ubiquitination and degradation of β -catenin through assembling multiprotein E3 ubiquitin ligase complex containing Siah-1, Skp1, and F-box protein Ebi [10, 13]. Based on this, CacyBP/SIP has been reported to inhibit the proliferation, tumorigenicity, and invasion of gastric cancer [14]. Instead, we showed that CacyBP/SIP promoted the proliferation of glioma cells through regulating the cell cycle and inhibiting cell apoptosis in our previous study [15, 16]. However, the specific mechanism of CacyBP/SIP promoting glioma cell proliferation remains unclear.

In this study, we aimed to explore the interactions between CacyBP/SIP, p53, and Mdm2, and to elaborate on the role of CacyBP/SIP on the Mdm2/p53 pathway. Our results suggest a novel biological role for CacyBP/SIP in glioma cells.

Materials and methods

Antibodies and reagents. CacyBP/SIP and Mdm2 antibodies were bought from Santa Cruz (Santa Cruz, CA). Antibodies for p53, GAPDH, Myc, and ubiquitin were from Cell Signaling Technology (Danvers, MA). Goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Millipore (Billerica, MA). Etoposide was bought from Sigma (Louis, MO) and Mdm2 inhibitor Nutlin-3 was purchased from Beyotime (Nantong, China).

Tissue samples. Fourteen specimens of human glioma tissues (surgical resection) were collected at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China). None of the patients were treated with such preoperative therapies as immunotherapy, radiation, or chemotherapy. All selected glioma specimens were definitely diagnosed as p53 mutant glioma. Written informed consent was obtained from each patient, and the study was approved by the Research Ethics Committee of Xuzhou Medical University.

Cell culture. Human glioma cell line U251, U87 were purchased from the Shanghai Cell bank, Type Culture Collection Committee, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (TranGen, Beijing, China) and were grown in a humidified incubator with 5% CO₂ at 37°C.

Plasmids. Lentiviral plasmids for silencing or overexpressing of CacyBP/SIP have been previously described [15]. The pLL3.7-CacyBP/SIP and pWPXLd-CacyBP/SIP plasmids were used to produce lentivirus to generate the CacyBP/SIP knockdown (KD) and overexpressing (OE) cell lines, respectively. The transient expression plasmid Myc-CacyBP/SIP was cloned by inserting the CacyBP/SIP cDNA into the pcDNA6A plasmid using *Hind* III and *Xho* I sites. The pcDNA3-Mdm2 plasmid was purchased from Addgene.

Establishment of stable cell lines. The stable CacyBP/SIP knockdown (KD) and overexpressing (OE) U251 cells have been previously described. CacyBP/SIP KD and OE U87 cells were generated by a similar procedure as U251. Briefly, for CacyBP/SIP KD U87 cells, virus-infected U87 cells were sorted by flow cytometry. GFP-positive cells were cultured for stable lines. For CacyBP/SIP OE U87 cells, the U87 cells were infected by GFP or GFP-CacyBP/SIP viruses, respectively. Forty-eight hours after infection, the cells were continuously cultured in the medium containing 2.5 µg/ml puromycin. The surviving cells were cultured into cell lines stably expressing GFP (Vector) or GFP-CacyBP/SIP (CacyBP/SIP OE).

RNA extraction, cDNA synthesis, and quantitative RT-PCR. RNA was extracted from stable lines and the

cDNA was synthesized using reverse transcription reagents (Roche, Basel, Switzerland) according to the manufacturer's protocol. Quantitative RT-PCR was performed by an ABI 7300 real-time PCR instrument (Applied Biosystems, Carlsbad, CA) using SYBR Green. Primers for the amplification of Jab1 and GAPDH were as follows: GAPDH forward: 5'-TGGAGTCCACTGGCGTCTTC-3', GAPDH reverse: 5'-CATTGCTGATGATCTTGAGGCT-3'; p53 forward: 5'-ACCTATGGAACTACTTCCTGAAA-3', p53 reverse: 5'-GCTGCCCTGGTAGGTTTTCT-3'. The products were 257 bp and 162 bp, respectively.

Western blotting. At the designated time, the cells were lysed and the total lysates were collected. Equal amounts of protein lysates were subjected to 10% SDS-PAGE and then transferred to 0.45 µm pore size PVDF membrane (Millipore, Billerica, MA). After blocking with 5% nonfat milk, the membrane was incubated with primary antibodies against CacyBP/SIP (sc-98356, 1:1000) and GAPDH (sc-25778, 1:3000) from Santa Cruz Biotechnology, Mdm2 (86934, 1:500), Myc (2276, 1:1000), p53 (2524, 1:1000) and ubiquitin (3936, 1:1000) from Cell Signaling Technology at 4°C overnight. On the following day, membranes were incubated in a horseradish peroxidase-labeled goat anti-rabbit/mouse IgG (AP132P or AP124P, Sigma) and detected by an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA). The band densities were quantified by ImageJ Software. The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the loading control.

Immunoprecipitation and ubiquitination. Cells were transiently transfected with the indicated plasmids. 24 h after transfection, cells were treated with MG132 for an additional 6 h. Then, the cells were harvested and lysed in a Triton-X-100-based lysis buffer (1% Triton-X-100, 150 mM NaCl, 20 mM HEPES, pH7.4, 2 mM EDTA, 5 mM MgCl₂) for 1 h on ice. The lysates were reacted with indicated antibodies for 16 h and then bounded to protein A/G beads for 6 h at 4°C with gentle agitate. The immunoprecipitants were washed three times with lysis buffer, and the bounded proteins were recovered by boiling the beads in 2×SDS sample buffer and then were subjected to western blotting. For protein interaction, the blots were probed by indicated antibodies, respectively. For ubiquitination, the cell lysates were immunoprecipitated with p53 antibody, and the ubiquitin-conjugated p53 was detected by immunoblotting with ubiquitin antibody.

Statistical analysis. The results are representative of experiments repeated at least three times and quantitative data were presented as means ± SEM. Statistical analyses were performed using SPSS Version 13.0. Differences in multiple groups were determined by a one-way analysis of variance (ANOVA) followed by a post-hoc test. Comparison between two groups was performed by Student's t test. The p-values less than 0.05 were considered statistically significant (*p<0.05).

Results

CacyBP/SIP regulates the p53 protein level in U251 cells, but not in U87 cells. In our previous study, we have demonstrated that CacyBP/SIP promoted the proliferation of glioma cell line U251, and discovered that CacyBP/SIP KD significantly increased the protein levels of p53 and CacyBP/SIP OE decreased it [15, 16]. However, in p53 wild-type U87 cells, CacyBP/SIP neither promoted its proliferation (data not shown) nor regulated p53 changes (Figures 1A, 1B). To address how CacyBP/SIP affects p53 expression, we analyzed the effect of CacyBP/SIP on p53 mRNA. We found that there was no obvious difference in p53 mRNA level upon CacyBP/SIP knockdown or overexpression (Figures 1C, 1D).

CacyBP/SIP interacts with p53 and Mdm2 to promote the ubiquitination and degradation of p53. Next, we investigated the mechanism of CacyBP/SIP-induced p53 reduction in U251 cells. As seen in Figure 1, we speculate that CacyBP/SIP may regulate p53 at the post-transcriptional level. Thus, we then inspected whether decreasing of p53 protein by CacyBP/SIP was caused by the increased ubiquitination. As shown in Figure 2A, CacyBP/SIP KD caused a remarkable

increase in p53 stability, whereas CacyBP/SIP OE led to an obvious reduction in p53 stability. Additionally, the effect of CacyBP/SIP on p53 stability could be attenuated by proteasome inhibitor MG132 (Figure 2B). As expected, CacyBP/SIP also inhibited the increase of p53 induced by the genotoxic agent, etoposide (Eto) (Figure 2C). Importantly, we found that CacyBP/SIP OE aggravated the ubiquitination of p53 (Figure 2D), and CacyBP/SIP-induced p53 reduction could be rescued by proteasome inhibitor MG132 (Figures 2E, 2F). These results indicate that CacyBP/SIP reduces p53 level by enhancing its ubiquitination and subsequent proteasome-mediated degradation in U251 cells.

As Mdm2 acts as the chief E3 ubiquitin ligase for proteasomal degradation of p53, we decided to explore the ubiquitin ligase activity of Mdm2 regulated by CacyBP/SIP. As shown in Figures 3A and 3B, co-immunoprecipitation assays with either anti-CacyBP/SIP or anti-Myc antibodies showed that both endogenous and exogenous CacyBP/SIP associated with p53 and Mdm2 in p53 mutant U251 cells. Next, the ubiquitination of p53 was assayed in the presence of Mdm2 and CacyBP/SIP. As shown in Figure 3C, in the presence of Mdm2, CacyBP/SIP OE increased p53 ubiquitination and

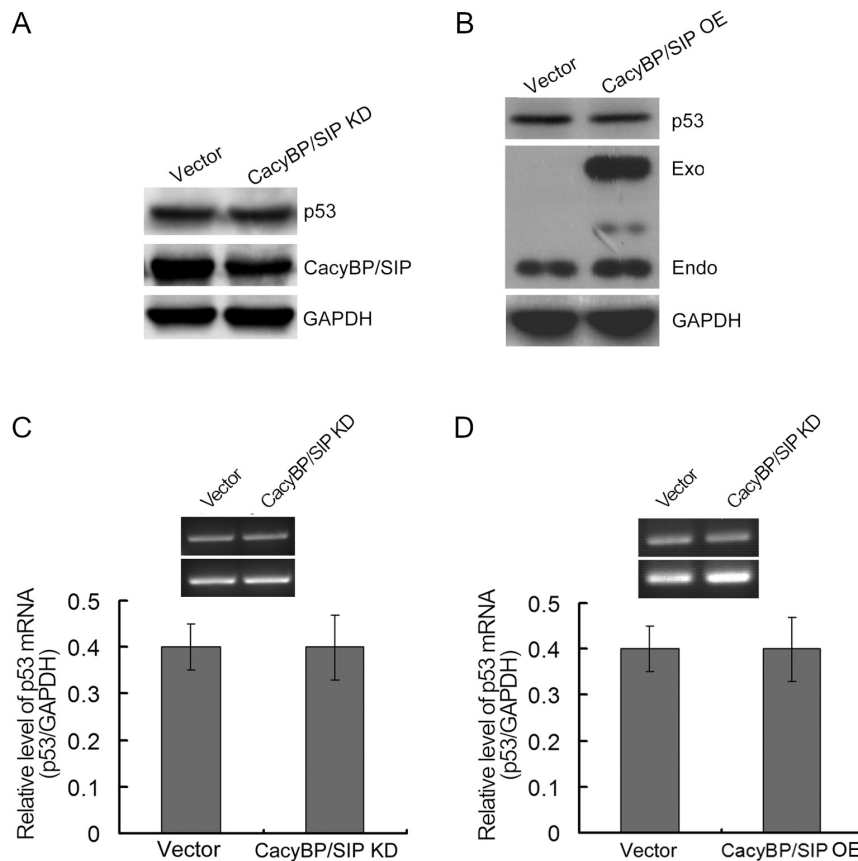


Figure 1. CacyBP/SIP decreases the p53 protein level in U251, but not in U87. A, B) Knockdown or overexpression of CacyBP/SIP had no effect on the protein level of p53 in p53 wild-type U87 cells. C, D) RT-PCR and histogram showed that CacyBP/SIP KD and OE had no effect on the mRNA level of p53. ** $p < 0.01$

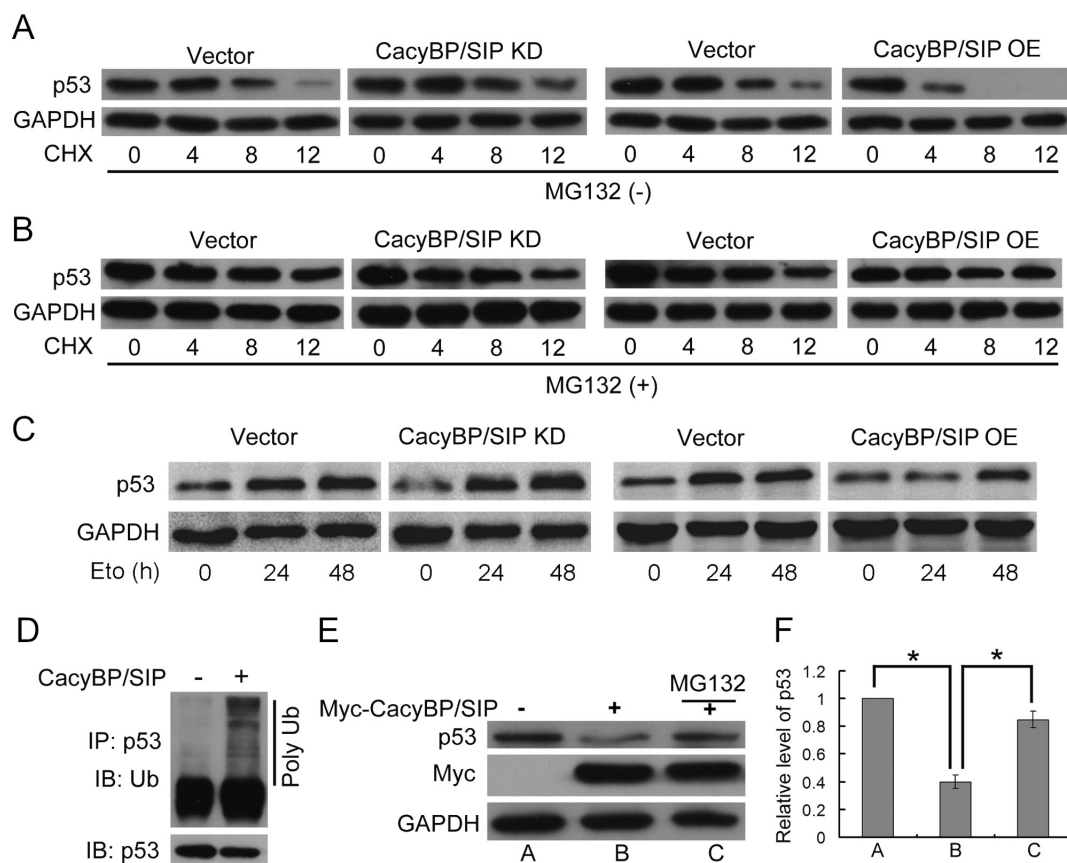


Figure 2. CacyBP/SIP decreases the p53 level by promoting its ubiquitination and subsequent proteasome-mediated degradation in U251 cells. **A)** CacyBP/SIP KD increased the stability of p53, whereas CacyBP/SIP OE decreased p53 stability. **B)** The effect of CacyBP/SIP on p53 stability was abolished in the presence of proteasome inhibitor MG132. **C)** CacyBP/SIP reduced etoposide-induced increase in p53. **D)** CacyBP/SIP OE aggravated the ubiquitination of p53. **E, F)** Western blotting and histogram showed that CacyBP/SIP-induced p53 reduction could be rescued by proteasome inhibitor MG132. * $p < 0.05$

degradation in a dose-dependent manner in p53 mutant U251 cells. However, in p53 wild-type U87 cells, CacyBP/SIP was not able to bind wild-type p53 at all and promote its ubiquitination by Mdm2 (Figures 3D, 3E). To answer how CacyBP/SIP promoted Mdm2 mediated p53 ubiquitination in U251 cells, we first detected the Mdm2 level. It was showed that CacyBP/SIP KD or OE had no effect on the protein level of Mdm2 (Figure 3F). Then, we consider whether this was attributed to the activity of Mdm2. Mdm2 inhibitor Nultin-3 was thus introduced to treat the U251 cells. It was found that blocking of Mdm2 activity with Nultin-3 significantly increased the level of p53 whatever CacyBP/SIP KD or OE (Figure 3G). Together these results indicate that CacyBP/SIP promotes p53 ubiquitination by increasing the ubiquitin ligase activity of Mdm2.

The protein level of CacyBP/SIP and p53 is inversely correlated in p53 mutant human glioma tissues. Finally, we studied the relationship of CacyBP/SIP and mutant p53 in clinical glioma samples. The total lysates of glioma tissues were subjected to western blotting with CacyBP/SIP and p53

antibodies. As shown in Figures 4A and 4B, CacyBP/SIP and p53 were inversely correlated in p53 mutant human glioma tissues ($p < 0.05$). These results show that CacyBP/SIP also regulates p53 stability *in vivo*.

Discussion

Our previous study showed that CacyBP/SIP could significantly promote glioma cell proliferation. But the mechanism was not clear. Here, we exhibited that CacyBP/SIP participated in the regulation of mutant p53, which may be achieved by enhancing the activity of Mdm2 thereby increasing the ubiquitination of p53.

Studies have discovered that CacyBP/SIP can interact with the member of the S100 family, as well as with protein Skp-1, tubulin [10, 17, 18]. It has been reported that CacyBP/SIP interacts with ERK1/2 and reduces the phosphorylation of ERK1/2 [19]. Besides, CacyBP/SIP associates with Siah-1, Skp1, and TBL1 to form a ubiquitin ligase complex called SCF^{TBL1}, which degrades β -catenin by ubiquitination [10].

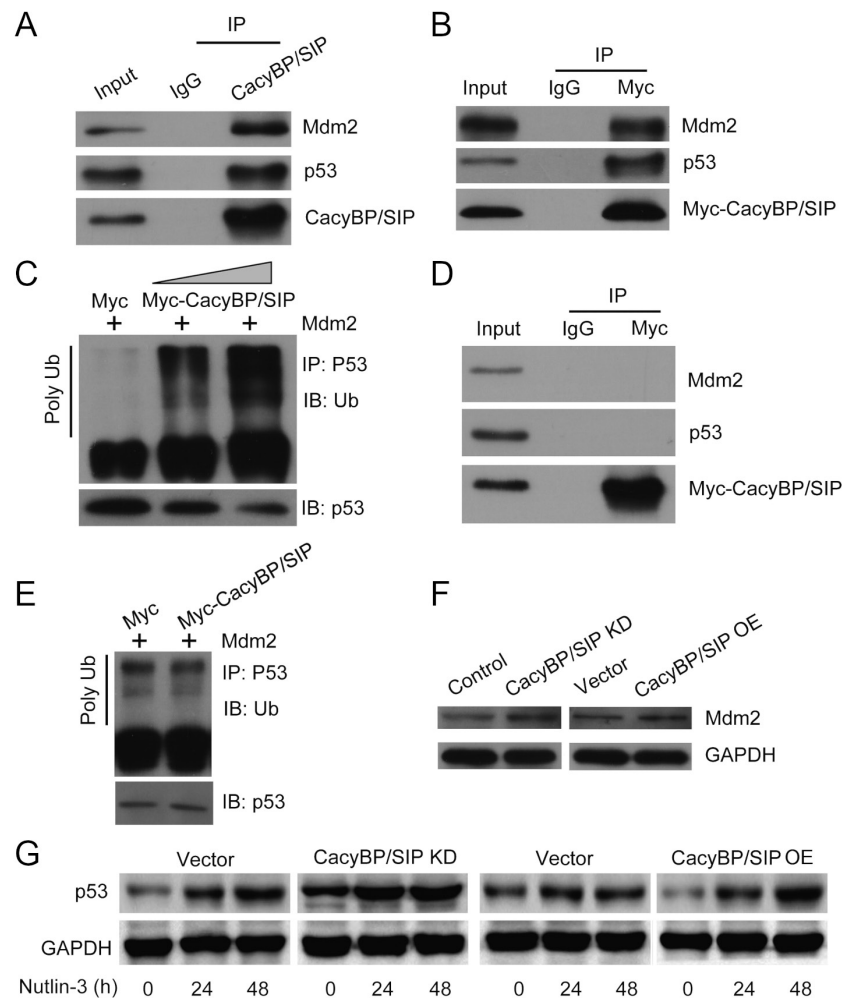


Figure 3. CacyBP/SIP interacts with p53 and Mdm2 to stimulate p53 ubiquitination in U251 cells, but not in U87 cells. A) Endogenous CacyBP/SIP interacted with p53 and Mdm2. B) Exogenous CacyBP/SIP interacted with p53 and Mdm2. C) In the presence of Mdm2, CacyBP/SIP enhanced p53 ubiquitination in a dose-dependent manner. D) CacyBP/SIP did not interact with p53 and Mdm2 in U87 cells. E) In the presence of Mdm2, CacyBP/SIP did not promote p53 ubiquitination. F) CacyBP/SIP KD or OE had no effect on the Mdm2 protein level. G) Inhibition of Mdm2 activity significantly increased the level of p53 protein whatever CacyBP/SIP KD or OE.

Recently, it was proposed that CacyBP/SIP could promote the interaction between Siah1 and cytoplasmic p27, which in turn increases the ubiquitination and degradation of cytoplasmic p27 [20]. In this study, we showed that CacyBP/SIP interacted with Mdm2 to promote the ubiquitination of mutant p53, but not wild-type p53. These indicate that CacyBP/SIP may usually function as an adaptor protein in the regulation of the ubiquitination of substrate protein. In this process, CacyBP/SIP did not affect the protein level of Mdm2 itself, but promoted the activity of Mdm2, because the Mdm2 inhibitor could rescue the decrease of p53 caused by overexpression of CacyBP/SIP. Whether there are other proteins that participate in the process needs to be investigated in further study.

Transcription factor p53 is reported to be activated by diverse genotoxic and cytotoxic stresses [21]. It could

inhibit the proliferation of cells by regulating the expression of genes that participate in cell cycle arrest, apoptosis, and DNA repair [22]. The p53 also can combine with the p53-response elements located in the Mdm2 gene to increase its transcription [23, 24]. Mdm2 can degrade p53 by ubiquitination, while p53 transcriptionally activates Mdm2, which in turn Mdm2 targets p53 for degradation. The feedback can restrain p53 activity in normal cells [25]. Thus, p53 always maintains at a low level in normal cells, whereas mutant p53 is generally more stable in cancer cells. That is because mutant p53 is unable to transcriptionally induce Mdm2. Besides, when p53 is phosphorylated at Ser20 and Thr18, it will disrupt its binding with Mdm2 [26]. In this study, we discovered that CacyBP/SIP interacted with both Mdm2 and p53 in p53 mutant cell line, and this interaction could increase Mdm2-mediated p53 ubiquitination and degrada-

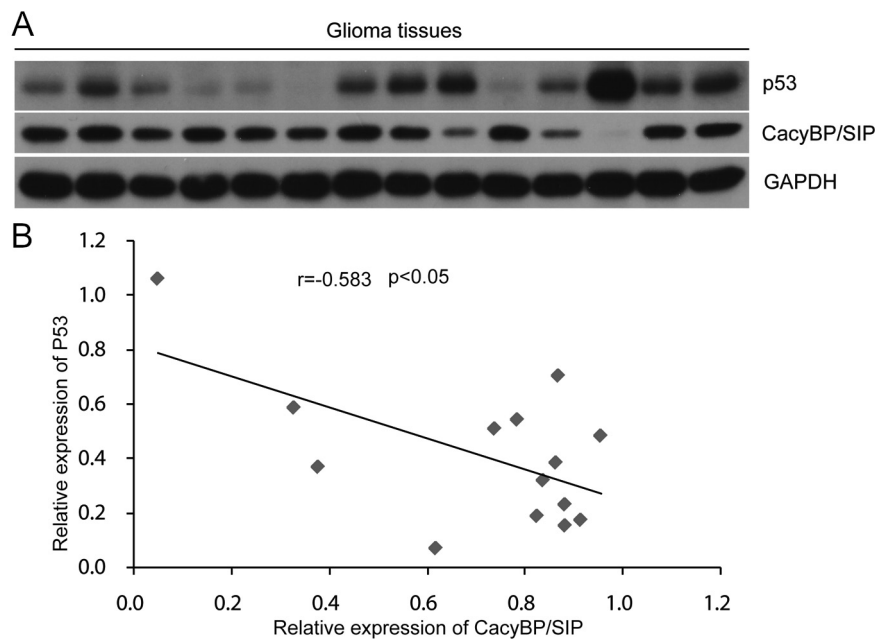


Figure 4. The protein level of CacyBP/SIP and p53 is inversely correlated in p53 mutant human glioma tissues. A) Representative immunoblots showed the protein level of CacyBP/SIP or p53 in each human glioma tissues. B) Correlation analysis showed that the protein level of CacyBP/SIP and p53 is inversely correlated in human glioma tissues. $r = -0.583$, $*p < 0.05$

tion. However, the exact role of CacyBP/SIP in this process still awaits further study.

In addition, it is interesting to investigate how CacyBP/SIP regulates p53. It has been reported that CacyBP/SIP can interact with ERK1/2 and reduce the phosphorylation of ERK1/2 [19]. Whether CacyBP/SIP can also dephosphorylate p53, which causes p53 to be more easily degraded by Mdm2. These should also be elaborated in further investigation.

In summary, we revealed a novel molecular mechanism regulating the stability of mutant p53. We demonstrated that CacyBP/SIP enhanced the ubiquitination and degradation of p53 by affecting the activity of Mdm2 associating with p53. These results have revealed a novel pathway for the regulation of mutant p53 and provided a new therapeutic strategy for glioma treatment.

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