DAB2IP inhibits p53 ubiquitin-mediated degradation by competitively binding to GRP75 and suppresses tumor malignancy in colon cancer

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21

Abstract:

Increasing evidence has shown that DAB2IP acts as a tumor suppressor and plays an inhibition role in many tumors. However, the underlying mechanism is still uncertain. Our study shows that DAB2IP is positively associated with a better prognosis in colon cancer patients with wild-type TP53 expression. In vitro assay shows that DAB2IP elicits potent tumor-suppressive effects on inhibiting cell invasiveness, colony formation and promoting cell apoptosis in wild-type TP53 colon cancer cell lines. Subsequently, DAB2IP is demonstrated to up-regulate the stability of wild-type TP53 by inhibiting its degradation in a ubiquitin-proteasome-dependent manner. Using mass spectrometry profiling, we unveil that DAB2IP and p53 could both interact with the ubiquitin ligase-related protein, GRP75. Mechanistically, DAB2IP could competitively bind with GRP75, thus reducing GRP75-mediated p53 ubiquitination and degradation. Finally, animal experiments also reveal that DAB2IP inhibits the tumor progression in vivo. In conclusion, our study presents a novel function of DAB2IP in GRP75-driven wild-type p53 degradation, which provides a new insight in DAB2IP-induced tumor suppression and provides a novel molecular aspect of the p53 pathway.

Introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths worldwide (1). Most CRCs develop from the sequential inactivation of tumor suppressor genes, including APC, TP53, and SMAD4, as well as the activation of

1 oncogenes, including RAS and BRAF (2). TP53 was first discovered and classified as
2 a cellular SV40 large T antigen-binding protein (3). As a classical tumor suppressor,
3 mutations in p53 are frequently observed in multiple cancer types and are critical
4 factors in tumorigenesis (4). Previous studies have revealed that missense mutations
5 in p53 proteins are common in human cancer, and these mutant proteins acquire
6 oncogenic properties that promote proliferation, cell survival and metastasis (5). In
7 colorectal cancer, p53 mutations have been reported to occur in approximately
8 40%-50% of patients (2, 6). Although the oncological function of mutant p53 has
9 been widely explored, there is still a lack of understanding of the remaining CRCs
10 with wild-type p53.

11 Wild-type p53 mediates cell cycle arrest and provides a cell death checkpoint, and
12 wild-type p53 can be activated by multiple cellular stresses (7). Functionally, p53 is
13 stabilized and binds to DNA as a tetramer, and results the transcriptional regulation of
14 genes, such as PUMA, BAX, and CDKN1A, that are involved in DNA repair,
15 cell-cycle arrest, senescence and apoptosis in a sequence-specific manner (7, 8). In
16 addition to genetic mutations, emerging studies have also shown that the protein level
17 or activity of endogenous wild-type p53 could be downregulated in a considerable
18 proportion of cancer patients through epigenetic or posttranslational mechanisms (9).
19 Due to the significant role of p53 inactivation in tumorigenesis and therapeutic
20 responses (10-12), it is critical to explore the molecular mechanism by which
21 wild-type p53 is dysregulated in tumors.

22 DAB2IP, also called ASK1-interacting protein (AIP1), is often reported to act as a

1 tumor suppressor and frequently silenced by epigenetic modifications in many tumors
 2 (13-18). As a protein with GTPase activity, DAB2IP is involved in many signaling
 3 pathways associated with tumorigenesis, including the Ras-Raf, PI3K-Akt and
 4 ASK1-JNK (19-21). Additionally, DAB2IP also presents a function of scaffold protein
 5 in modulating nuclear b-catenin/T-cell factor activity, enhancing PROX1 mediated
 6 HIF1a ubiquitination, down-regulating NF-κB signaling, thereby inhibit
 7 epithelial-mesenchymal transition, cancer stem cell signatures and suppress tumor
 8 progression (22-24). A recent study found that mutant p53 potentiates the oncogenic
 9 effects of insulin and TNF-α by inhibiting DAB2IP (25, 26). Whether DAB2IP could,
 10 in reverse, regulate wild-type p53 in CRC is still not clear.

11 In this research, we provided evidences that DAB2IP exerted tumor-suppressive
 12 effects in wild-type p53 colon cancer, and involved in TP53 degradation in a
 13 ubiquitin-proteasome-dependent manner. Furthermore, we unveiled that DAB2IP and
 14 p53 could both interact with the ubiquitin ligase-related protein, GRP75.
 15 Mechanistically, DAB2IP could competitively bind with GRP75, thus reducing
 16 GRP75-mediated p53 ubiquitin-mediated degradation, and finally inhibit tumor
 17 progression in colon cancer. Collectively, our study presents a novel function of
 18 DAB2IP in GRP75-driven wild-type p53 degradation and reveals a new signaling axis
 19 that is involved in DAB2IP-induced tumor suppression in CRC.

20

21 **Materials/Subjects and Methods:**

22 **Reagents and antibodies**

1 Cell culture medium and other supplements were purchased from KeyGEN Biotech
2 (Nanjing, China). Fetal bovine serum was obtained from MULTICELL (Canada).
3 Basic chemical reagents for molecular biology were purchased from Sigma-Aldrich
4 (St. Louis, MO). Antibodies used in this research were described as follow.

Antibody	Manufacturer	Cat. #
DAB2IP	Abcam, Inc. (Cambridge, UK)	Ab87811
p53	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)	sc-1226
p21	Cell Signal Technology, Inc. (Beverly, MA)	2947
BAX	Cell Signal Technology, Inc. (Beverly, MA)	5023
GRP75	Cell Signal Technology, Inc. (Beverly, MA)	3593
GAPDH	Cell Signal Technology, Inc. (Beverly, MA)	97166
Myc	Cell Signal Technology, Inc. (Beverly, MA)	2276
HA	Cell Signal Technology, Inc. (Beverly, MA)	3724
FLAG	Cell Signal Technology, Inc. (Beverly, MA)	14793

5

6 **Tissue microarray and Immunohistochemistry (IHC)**

7 Human colon cancer tissue microarray of 78 cases primary lesion and 2 adjacent
8 normal colon tissue (US Biomax, Cat,# CO802c) was used evaluated the expression
9 of DAB2IP and P53. Slide was dewaxed, rehydrated and heated in sodium citrate
10 buffer (0.01 M, pH 6.0) for antigen retrieval. Subsequently, the endogenous
11 peroxidase was inhibited with 3% hydrogen peroxide with 0.1% sodium azide for 30

1 min and non-specific staining was blocked through incubation in 5% bovine serum
2 albumin for 2 hours. Next, slide was incubated with 1:200 diluted DAB2IP antibodies
3 or 1:400 diluted P53 antibodies at 4°C overnight and with biotinylated secondary
4 antibody for 2 hours. DAB Horseradish Peroxidase Color Development Kit (Wuhan
5 BosterBio Co.Ltd, Cat. #AR1022) was used for Immuno-staining and counterstain
6 was performed by hematoxylin staining. The results were analyzed under a
7 microscope.

8 The expression level of DAB2IP and P53 was evaluated by IHC score, which was
9 calculated through multiplying proportion score and intensity score, and categorized
10 into level 1 (IHC score 0–3), level 2 (IHC score 4-6) and level 3 (IHC score more than
11 6). The proportion score reflected the fraction of positive staining cells (0, none; 1,
12 ≤10%; 2, 10% to ≥25%; 3, >25% to 50%; 4, >50%), and the intensity score revealed
13 the staining intensity (0, no staining; 1, weak; 2, intermediate; 3, strong).

14

15 **Cell lines and cell culture**

16 Colon cancer cell lines, Caco-2, HT29, HCT116, KM12, Lovo, SW48, SW480,
17 SW620 and human embryonic kidney cell line HEK293T were purchased from
18 American Type Culture Collection (ATCC). All the cell lines were cultured routinely
19 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum
20 and 1% penicillin/streptomycin at 37 °C and in 5% CO₂.

21

22 **Small interfering RNAs, Plasmids and vectors constructing**

1 Small interfering RNAs (siRNAs) targeting DAB2IP and GRP75 were designed and
2 synthesized by Guangzhou RiboBio Co. Ltd. The sequences of siRNAs were
3 described as follow.

siRNAs	Sequence
siDAB2IP-1#	GGTGAAGGACTTCCTGACA
siDAB2IP-2#	GGAGCGCAACAGTTACCTG
siGRP75-1#	GCGATATGATGATCCTGAA
siGRP75-2#	GAGTCAGATTGGAGCATTT

4 pcDNA3.1-Flag-tagged-DAB2IP, pcDNA3.1-Myc-tagged-DAB2IP overexpressing
5 plasmids were gifts from Jer-Tsong Hsieh. pcDNA3.1-TP53 overexpressing plasmid
6 was generated before and were verified by performing sequencing. GRP75 DNA
7 fragment was obtained by PCR and cloned into pcDNA3.1 vector containing Green
8 fluorescent protein (GFP) tag. HA-ubiquitin plasmid was purchased from Addgene
9 (Cat.#17608). pLKO-DAB2IP-lentiviral-shRNA was constructed previously and used
10 for knocking down DAB2IP expression. The sequences of shDAB2IP were described
11 as follow.

shDAB2IP	Sequence
1#	CCGGTTCGAGGTGACGACGTCATCACTCGAGTGATGACGTC GTCACCTCGAATTTTTTG
2#	ACCGGTGGAGCGCAACAGTTACCTGTTCAAGAGCAGGTAA CTGTTGCGCTCCTTTTTTGGTACCGAATTC

12 All the plasmids were verified by performing sequencing.

1

2 **Transfection and establishment of stable-expressing cell line**

3 Small interfering RNAs transfections were performed using Lipofectamine®2000
4 transfection reagent (Thermofisher, Cat. #11668019) according to the manufacture`s
5 protocol. Non-targeting siRNA (siNC) were used for negative control. Plasmid
6 transfections were performed using Lipofectamine®3000 transfection reagent
7 (Thermofisher, Cat. # L3000015) according to the manufacture`s manual. After 48
8 hours, cell biological and biochemical experiments were performed.

9 For establishment of stable-expressing cell lines, recombinant lentivirus of
10 sh-scramble, sh-DAB2IP (purchased from Origene) were transfected into HCT116
11 and SW48 according to the manufacturer`s suggested protocol. After transfection,
12 cells were treated with 10µg/ml puromycin (Thermofisher, Cat. # A1113802) for 4
13 weeks to get stable cell lines. DAB2IP expression of the stable cell lines was
14 confirmed via western blotting.

15

16 **Transwell assay, colony formation assay and apoptosis assay**

17 24-well Transwell plates with pore size 8µm (Corning, Cat. #3422) were used for
18 Transwell assay. 1×10^5 cells were harvested in 100µl of serum free culture-medium
19 and added into the upper chamber. Then 600µl 30% fetal bovine serum medium was
20 placed into the bottom compartment of the chamber as a source of chemo-attractant.
21 After 24 hours culture, cells that crossed the inserts were fixed and stained with
22 Crystal-Violet. Migrated cells were photographed and counted via an inverted

1 microscope (100X magnification).
 2 For the colony-formation assay, the cells were plated in six-well plates at a density of
 3 500 cells/well and were cultured for 2 weeks. Then culture medium was removed. The
 4 colonies were fixed in methanol, stained with Crystal-Violet and photographed.
 5 For apoptosis analysis, 1×10^4 cells were seeded on six-well plates and cultured to
 6 reach 70% confluence. Then cells were treated with 10ug/ml 5-fluorouracil (5-FU) for
 7 24 hours. Next, cells were collected by 0.02% trypsin without ethylene diamine tetra
 8 acetic acid (EDTA) and stained with annexin V-FITC/ propidium iodide kit (BD Cat.
 9 #559763) according to the manufacturer`s manual, and analyzed by flow cytometer.

10

11 **Western blot assay and immunoprecipitation**

12 Total protein was extracted via NP40 lysis buffer added with Phenylmethylsulfonyl
 13 fluoride (PMSF) and cocktail protease inhibitor (MedChemExpress, Cat. # HY-K0010)
 14 and separated on 10% SDS-PAGE gels. After electrophoresis, separated protein bands
 15 were transferred into polyvinylidene fluoride membranes (Millipore, Cat.
 16 #IPVH00010) and blocked in 5% nonfat milk for 1 hour at room temperature. The
 17 membranes were then incubated with the primary antibodies at a recommend diluted
 18 ratio according to the manufacture instruction overnight at 4°C. After 3 times washing,
 19 membranes were incubated in 1:5000 horseradish peroxidase-linked secondary
 20 antibodies at room temperature for 2 hours. Finally, the membranes were washed for
 21 three times and were visualized using ECL Kit (Thermofisher, Cat. #34096).
 22 For immunoprecipitation, cells were harvested and lysis in NP40 buffer,

1 supplemented with PMSF and cocktail protease inhibitor. After centrifugation,
2 supernatant was collected into fresh tubes. About 1mg of cell lysis was incubated with
3 1.0ug primary antibodies (anti-Flag, anti-DAB2IP, anti-p53, anti-HA) with rotation
4 overnight at 4°C. Then 25ul protein A agarose beads (Sigma) were added into the
5 lysates and a further incubation of 2 hours at 4°C with rotation was performed.
6 Subsequently, breads were collected via centrifugation and washed 3-4 times with
7 NP40 buffer. The immune-complex was released by boiling the breads in SDS loading
8 dye and analyzed by western blotting.

9

Quantitative real-time PCR (RT-qPCR)

11 Total RNA was extracted using TRIzol (Takara, Cat. #9108) and reverse-transcribed
12 using PrimeScript™ RT Master Mix reagent Kit (Takara, Cat. #RR036A) according
13 to the manufacturer`s manual. Quantitative real-time PCR was carried out using TB
14 Green™ Premix Ex Taq™ II Kit (Takara, Cat. #RR820A) in ABI 7300 Real-Time
15 PCR System (Applied Biosystems, Foster City, CA). GAPDH gene expression was
16 used as an endogenous control and the results from qRT-PCR were analyzed though
17 the comparative Ct method ($2^{-\Delta\Delta Ct}$). Primers` sequences used in this research were
18 provided as follow.

Primer	Forward	Reverse
DAB2IP	CTGAGCGGGATAAGTGGAT GG	AAACATTGTCCGTCTTGAGCT T
TP53	CAGCACATGACGGAGGTTG	TCATCCAAATACTCCACACGC

	T	
CDKN1A	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCT C
BAX	CCCGAGAGGTCTTTTCCG AG	CCAGCCCATGATGGTTCTGAT
BBC3	GACCTCAACGCACAGTACG AG	AGGAGTCCCATGATGAGATT GT
GAPDH	CTCCTGCACCACCAACTGC T	GGGCCATCCACAGTCTTCTG

1

2 Ubiquitination assay

3 DAB2IP or GRP75 was knocked down in SW48 cell lines via recombinant lentivirus
4 or small interfering RNAs. HEK293T cells were stable transfected with
5 pcDNA3.1-Myc-tagged-DAB2IP to overexpress DAB2IP expression. Then, these cell
6 lines were co-transfected with pcDNA3.1-Flag-tagged-TP53 and
7 HA-tagged-Ubiquitin plasmid. After 24-48 hours, cells were treated with MG-132 at a
8 dose of 10uM for 8h and were lysed in NP40 buffer and centrifuged. The supernatants
9 were collected and immunoprecipitated with anti-Flag agarose, and the
10 immunocomplexes were examined using anti-HA antibody via western blotting.

11

12 Mass spectrum analysis

13 The mass spectrum analysis was performed by Shanghai Lu-Ming Biotech Co., Ltd.

1 Briefly, protein was extracted via NP40 buffer added with cocktail protease inhibitor
2 and separated on 10% SDS-PAGE gels. After electrophoresis, separated protein bands
3 was stained with Coomassie brilliant blue. Next the protein bands were cut into
4 1mm² gel particles and transferred into low protein binding tubes and rinsed twice
5 with ultrapure water. Subsequently, gel was digested and the protein was purified
6 according to the Katayama et al. (27).

7 For mass spectrum (MS) analysis, samples were pre-processed and loaded on the
8 mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Finnigan) according to the
9 manufacturer`s protocol. Based on MS spectra, proteins were successfully identified
10 based on 95% or higher confidence interval of their scores in the Proteome Discoverer
11 2.3 search engine (Thermo Scientific) and Uniprot-Homo database.

13 **Animals**

14 All animal experiments were approved by Ethical Committee of Tongji Hospital.
15 Briefly, female BABL/c athymic nude mice (age 4 w) were purchased from Charles
16 River Inc., Beijing. The nude mice were subcutaneously injected 2×10^6 tumor cells
17 with Matrigel (Corning), 6 mice per group. After 4 weeks, the mice were euthanized,
18 and the tumors were removed. After measuring the size, tumors were embedded,
19 sliced and stained by hematoxylin-eosin (HE). The expression of DAB2IP and p53
20 was evaluated via immunohistochemistry. Ki-67 was used to evaluate the proliferation
21 of tumor. TdT-mediated dUTP Nick-End Labeling assay kit (Biosci, Wuhan) was used
22 to measured apoptosis rate of tumor cells.

1

2 **Public database and bioinformatics analysis**

3 The gene expression dataset (GSE21510, (28)) of purified cancer cells in 104 patients
 4 with colorectal cancer was obtained from Gene Expression Omnibus database and
 5 was used in Gene Set Enrichment Analysis (GSEA)
 6 (<http://software.broadinstitute.org/gsea/>) (29). Mutation data, gene expression profile
 7 and prognostic data of colon adenocarcinoma patients was obtained from
 8 TCGA-COAD dataset (<https://portal.gdc.cancer.gov>). String database
 9 (<http://www.string-db.org/>) (30) was used for protein interaction analysis. Gene
 10 expression correlation was revealed by 'R2: Genomics Analysis and Visualization
 11 Platform (<http://r2.amc.nl>)' using Sugihara dataset (28). Gene ontology enrichment
 12 were performed by DAVID software (<https://david.ncifcrf.gov/tools.jsp>). Mutation
 13 data of p53 in various colon cancer cell line was obtained from Cancer Cell Line
 14 Encyclopedia (CCLE, Broad Institute, <https://portals.broadinstitute.org/ccle>).

15

16 **Statistical analysis**

17 Statistical analysis was performed by SPSS software package (version 19.0 for
 18 Windows; IBM, USA) and Prism 5 (GraphPad). In all graph, continuous data were
 19 expressed as mean $\pm$ standard deviation (SD), and statistically analyzed with student t
 20 test (two-tailed) or Analysis of Variance (ANOVA). A p value less than 0.05 was
 21 considered statistically significant.

22

1 **Results**

2 **DAB2IP was positively associated with a better prognosis of colon cancer** 3 **patients expressing wild-type TP53**

4 To assess the potential correlation between the DAB2IP and TP53 protein levels in
5 colon cancer, we evaluated a tissue chip containing 78 clinical specimens of
6 malignant colon tumors via IHC staining. As shown in [Figure 1A](#), the IHC score of
7 TP53 was significantly increased in patients with high DAB2IP expression ([Figure](#)
8 [1A](#)). Additionally, gene set enrichment analysis (GSEA) of a public colorectal cancer
9 expression profile (GSE21510) also revealed that p53 signaling was positively
10 associated with DAB2IP expression ([Figure 1B](#)). R2 gene correlation analysis
11 (<http://r2.amc.nl>) showed that DAB2IP was positively associated with TP53 target
12 gene (CDKN1A, BAX, and PUMA) expression; however, no correlation was
13 observed between DAB2IP and TP53 expression in colon cancer specimens ([Figure](#)
14 [1C](#)). Considering the frequency of TP53 mutations in colon cancer, we also analyzed
15 the DAB2IP expression level in subgroups with wild-type TP53 and mutated TP53
16 from the TCGA-COAD dataset. As shown in [Figure 1D](#), no difference in DAB2IP
17 expression was observed between the two groups. These results indicated that
18 DAB2IP could positively regulate wild type TP53 signaling at the posttranscriptional
19 level.

20 To explore the effect of DAB2IP on colon cancer prognosis, we performed
21 Kaplan-Meier analysis on the TCGA-COAD dataset. Interestingly, although high
22 expression of DAB2IP did not result in significantly better survival in all patients,

1 patients with high DAB2IP expression had a better prognosis than those with low
2 DAB2IP expression in the wild-type TP53 subgroup (Figure 1E). These data
3 suggested that DAB2IP could be positively associated with a better prognosis in colon
4 cancer patients expressing wild-type TP53.

5

6 **DAB2IP exerted tumor-suppressive effects and positively regulated the TP53** 7 **protein levels in colon cancer cell lines expressing wild-type TP53**

8 To evaluate the effects of DAB2IP on wild-type TP53 colon cancer, we first examined
9 DAB2IP and p53 expression pattern in 8 colon cancer cell lines (CaCo2, HT29,
10 HCT116, KM12, LoVo, SW48, SW480 and SW620) (Figure 2A). The western blot
11 results showed that the DAB2IP protein was relatively absent in Lovo cells, while
12 TP53 was relative low expression in Lovo and absent in HT29 cell (Figure 2A, up).
13 Then, to assess the TP53 mutation type, the gene mutation data of the colorectal
14 cancer cell lines were obtained from the CCLE database and analyzed (Figure 2A,
15 down and Supplemental Figure 1). As shown in Figure 2A, HCT116 and SW48 cells
16 expressed both DAB2IP and wild-type TP53. Thus, these two cell lines were selected
17 for further studies.

18 According to previous results, the protein level of p53 was positively correlated with
19 DAB2IP in clinical colon cancer specimens. To further elucidate whether DAB2IP
20 could regulate p53 expression in vitro, we used siRNAs to knockdown DAB2IP in
21 both the HCT116 and SW48 cell lines. Western blot assays revealed that knockdown
22 of DAB2IP significantly reduced the protein level of p53 and its downstream targets

(p21 and BAX) (Figure 2B). In contrast, overexpressing DAB2IP via the pcDNA3.1-DAB2IP plasmid in these two cell lines significantly increased TP53 expression (Supplemental Figure 2A). Additionally, knockdown of DAB2IP significantly induced tumor cell colony formation, inhibited cell apoptosis and promoted invasiveness and in both the SW48 and HCT116 cell lines (Figure 2C, D, E). Interestingly, in colon cancer cell lines with mutated TP53 expression (SW480 and SW620, Figure 2A, down), neither knocking down nor overexpressing DAB2IP could affect the protein level of TP53 and its downstream (Supplemental Figure 2B, C). Thus, we speculated that DAB2IP could regulated wild-type TP53 expression. To further investigated whether DAB2IP exerted oncogenic effects in vivo, we stably transfected HCT116 and SW48 cell line with sh-scramble or sh-DAB2IP lentivirus and subcutaneously transplanted it into nude mice. After 4 weeks, the mice were euthanized, and the tumor size was measured. Xenografted tumors from mice injected with shDAB2IP-HCT116 and shDAB2IP-SW48 were larger than those of sh-scramble mice (Figure 2F and Supplemental Figure 2D). Additionally, tumors with shDAB2IP exhibited higher levels of proliferation and lower levels of apoptosis than the control tumors (Figure 2G and Supplemental Figure 2E). These results indicated that the tumor-suppressive effects of DAB2IP in colon cancer cell lines expressing wild-type p53 and revealed a potential positive mechanism of DAB2IP regulation at the wild-type p53 protein level.

21

22 **DAB2IP inhibited the degradation of the p53 protein in a**

ubiquitin-proteasome-dependent manner

To elucidate the regulatory mechanism of DAB2IP-induced p53 expression, we first stably knocked down DAB2IP in the SW48 cell line with an shDAB2IP plasmid (DAB2IP-KD) (Figure 3A) and validated the mRNA level of TP53 and its downstream genes (CDKN1A, BAX and BBC3) through RT-qPCR. As shown in Figure 3B, knocking down DAB2IP significantly reduced the expression of the downstream genes of TP53 (CDKN1A, BAX and BBC2); however, DAB2IP did not exert a significant effect on the TP53 mRNA levels. Thus, we speculated that the regulation of DAB2IP-induced p53 expression might occur at the posttranscriptional stage.

Based on the previous results, we hypothesized that DAB2IP could regulate p53 at the protein synthesis/degradation level. To further explore the mechanism underlying DAB2IP-induced p53 signaling, we first used MG132, a reversible proteasome inhibitor, to indirectly evaluate protein synthesis efficiency in vitro. As shown in Figure 3C, after inhibiting protein degradation, knockdown of DAB2IP did not decrease the accumulation of the TP53 protein in the SW48 cell line. Next, we detected the half-life of p53 when DAB2IP was knocked down. After knockdown of DAB2IP, SW48 cells were treated with cycloheximide (CHX) at a dose of 50 µg/ml. The half-life of endogenous p53 was significantly shorter in the DAB2IP-KD SW48 cells than in the control cells (Figure 3D). These results indicated that DAB2IP acted as a negative regulatory factor on TP53 degradation.

The ubiquitin-proteasome pathway has been proven to be a highly effective pathway

1 of p53 protein degradation in eukaryotic cells (31). To determine whether DAB2IP
2 could regulate the ubiquitin-mediated degradation of the p53 protein, we first used a
3 ubiquitination assay to detect the ubiquitination level of endogenous p53 protein in
4 the SW48 cell line. As shown in [Figure 3E](#), the ubiquitination level of endogenous
5 p53 protein was significantly increased in DAB2IP-KD SW48 cells compared with
6 control cells ([Figure 3E](#)). Subsequently, SW48 cells were coinfectd with shDAB2IP
7 lentivirus and Flag-tagged TP53 plasmid. The results showed that the ubiquitination
8 of exogenous wild-type p53 protein was stronger in the DAB2IP-knockdown group
9 than in the control group ([Figure 3F](#)). Similarly, the overexpression of DAB2IP also
10 significantly inhibited exogenous p53 ubiquitination in the HEK293T cell line ([Figure](#)
11 [3G](#)). These results revealed that DAB2IP could inhibit the degradation of the p53
12 protein in a ubiquitin-proteasome-dependent manner.

13

14 **DAB2IP suppressed p53 ubiquitination through competitive binding GRP75**

15 Previous results demonstrated that DAB2IP inhibited ubiquitin-proteasome-dependent
16 p53 degradation. How DAB2IP regulates p53 ubiquitination is still unclear. In this
17 section, we focused on exploring the detailed mechanism of DAB2IP-mediated TP53
18 ubiquitination.

19 First, a coimmunoprecipitation assay revealed that neither endogenous nor exogenous
20 DAB2IP protein interacted with p53 directly ([Supplemental Figure 3A, B](#)). To screen
21 for any potential mediator between DAB2IP and p53, Immunoprecipitation and mass
22 spectrometry were performed and 166 proteins that interacted with both DAB2IP and

1 p53 were identified in the SW48 cell line (Figure 4A, and Supplemental Figure 3C).

2 Among them, TUBB, HSP90AA1, HSP90AB1, HSPA1B, HSPA5, HSPA8, XRCC5,

3 GRP75 and RPL23 were classified into the “ubiquitin protein ligase binding”

4 category via gene ontology enrichment (Figure 4B). Through the String protein

5 interaction analysis (string-db.org), GRP75 (also known as HSPA9) and HSP90AA1

6 appeared to be a potential inhibitor of p53 protein (Figure 4C). Considering the

7 relative higher binding capacity between DAB2IP and TP53 (Figure 4B), GRP75

8 were selected for further studies.

9 To further elucidate the role of GRP75 in DAB2IP-regulated p53 degradation, we first

10 used coimmunoprecipitation assays to verified that both endogenous DAB2IP and p53

11 could bind to GRP75 in the SW48 cell line (Supplemental Figure 3D, E). Western

12 blotting assay revealed that GRP75 could negatively regulate p53 and its downstream

13 at the protein level in both SW48 and HCT116 cell lines (Supplemental Figure 3F).

14 Subsequently, we found that knocking down GRP75 could increase the p53 protein

15 levels in cells with low DAB2IP expression and significantly reversed

16 DAB2IP-regulated clone formation and cell apoptosis in both the SW48 and HCT116

17 (Figure 4D and Supplemental Figure 3G, H). Through a ubiquitination assay, we

18 observed that knockdown of GRP75 could significantly reverse DAB2IP-mediated

19 p53 ubiquitination in the SW48 cell line (Figure 4E). Similarly, overexpressing

20 GRP75 also increased the ubiquitination level of exogenous wild-type p53 in the

21 HEK293T cells transfected with the DAB2IP plasmid (Figure 4F). These findings

22 indicated that GRP75 acted as a positive regulator in DAB2IP-mediated p53

1 ubiquitination.

2 Previous results revealed that both DAB2IP and p53 could bind to GRP75
3 ([Supplemental Figure 3D, E](#)). Considering the promoting effect of GRP75 on
4 DAB2IP-mediated p53 ubiquitination, we hypothesized that DAB2IP could suppress
5 ubiquitination-mediated p53 degradation by competitively binding to the GRP75
6 protein. To verify this hypothesis, we transfected siDAB2IP into SW48 cells at the
7 indicated concentrations. Immunoprecipitation revealed that with a decline in
8 DAB2IP protein levels, the binding capacity of GRP75 to p53 was gradually
9 increased ([Figure 4G](#)). Similarly, the amount of GRP75 bound to p53 was negatively
10 correlated with the DAB2IP protein levels in HEK293T cells ([Figure 4H](#)). These data
11 revealed that DAB2IP suppressed p53 ubiquitination by competitive binding to the
12 GRP75 protein.

13 In summary, by competitive binding to GRP75, DAB2IP inhibits GRP75-mediated
14 p53 ubiquitin-degradation, thus increasing the p53 protein levels, promoting p53
15 signaling, and finally exhibiting tumor-suppressive characteristics against colon
16 cancer ([Figure 4I](#)).

17

18 **Discussion**

19 The important roles of p53 in tumor suppression, including cell death, damage repair,
20 metabolism, proliferation and senescence, have been widely established in cancer
21 development (8, 12). Due to its frequent alterations in human cancer, the mutation
22 state of the TP53 gene has become a highly valuable predictive and prognostic

1 biomarker in multiple tumors (4, 32). The mutation incidence of TP53 is nearly 40%
 2 in colon cancer, while the remaining 60% of patients express the wild-type protein
 3 (33). Numerous studies have focused on exploring the oncological role of mutant p53;
 4 however, the mechanism of genesis and progression of tumors expressing wild-type
 5 p53 is still unclear. In the current study, through a combination of bioinformatics
 6 analysis and clinical specimens, we unexpectedly revealed that DAB2IP had a higher
 7 anti-oncologic effect in colon cancer patients expressing wild-type p53, and it
 8 positively regulated p53 signaling by influencing its protein level.

9 DAB2IP was first identified as a DOC-2/DAB2 interactive protein and was
 10 demonstrated to be a tumor suppressor of cell growth, metastasis and other aspects of
 11 cancer progression by our group (34). As a scaffold protein, DAB2IP contains
 12 multiple domain structures to interact with key components of various pathways, and
 13 then, it regulates tumor-related signaling, such as RAS/ERK (35), ASK1/JNK (20),
 14 PI3k/Akt (19), androgen receptor (36), NF-kb (24), and b-catenin/TCF signaling (22).

15 Previous studies have demonstrated that mutant p53 can bind to DAB2IP and interfere
 16 with its function in Akt activation and TNF signaling (25, 26). Additionally, Lunardi
 17 A. et al. also reported that DAB2IP can bind to wild-type p53, at least in vitro and in
 18 transient overexpression conditions, although whether such an interaction could occur
 19 physiologically is still unclear (37). Our study is the first to discover the positive
 20 regulatory effect of DAB2IP on wild-type p53 and its downstream protein levels in
 21 colon cancer cells. Contrary to earlier reports, DAB2IP may act upstream of the
 22 wild-type p53 protein and inhibit tumor progression by directly activating p53

1 signaling. Our findings present a new possibility regarding the mechanism of the
2 DAB2IP-induced tumor suppression effect in colon cancer.

3 Subsequently, our research elucidated how DAB2IP regulated wild-type p53
4 expression. One of the key mechanisms by which p53 is regulated is through rapid
5 degradation via the ubiquitin proteasomal pathway (31). Coincidentally, our present
6 study also revealed that DAB2IP could regulate p53 abundance at the
7 posttranscriptional levels. Mechanistically, DAB2IP significantly inhibited p53
8 ubiquitination, prolonged the half-life of wild-type p53, and activated p53 signaling.
9 These findings revealed the function of DAB2IP in p53
10 ubiquitin-proteasome-dependent degradation and increased our understanding of
11 DAB2IP-mediated tumor suppression.

12 Through an in vitro assay, we found that DAB2IP could not directly interact with the
13 p53 protein. Additionally, DAB2IP did not exhibit E3 ligase activity, which is
14 necessary for the selection of proteins for ubiquitination-dependent degradation (34,
15 38). Thus, we suggested that an intermediate should exist between the two proteins
16 and be involved in the regulation of DAB2IP-mediated p53 ubiquitination.

17 To date, nearly 20 E3 ligases (including MDM2, CHIP, TRIMs, etc.) and 8 non-E3
18 ligases that target p53 for proteasomal degradation have been identified (31). Among
19 them, MDM2 has been regarded as the canonical regulator of p53 degradation, while
20 CHIP, an E3 ligase able to target its substrates for proteasomal degradation by binding
21 to the C termini of Hsc70 and Hsp90 and mediating the ubiquitination of
22 chaperone-bound proteins, has been widely reported to drive p53 ubiquitination in the

1 noncanonical pathway (39). In mass spectrometry analysis, MDM2 was not detected
2 among the DAB2IP binding proteins. Interestingly, GRP75, a chaperone protein that
3 interacts with the CHIP E3 ligase (40), has been verified to bind to both endogenous
4 and exogenous DAB2IP proteins and to play an important regulatory role in
5 DAB2IP-mediated p53 ubiquitination. Thus, we assumed that DAB2IP regulated p53
6 protein levels via ChIP-dependent ubiquitination.

7 GRP75, also known as mortalin and HSPA9, is a member of the family of
8 glucose-regulated proteins, which belong to the stress-inducible molecular chaperones
9 of the heat shock protein (HSP) family and play an important role in the regulation of
10 protein quality control and degradation (41). Early studies showed that GRP75 could
11 induce abrogated cytoplasmic retention of wild-type p53 (42) and facilitated
12 proteasome-mediated degradation of p53 by the ChIP-GRP75 complex (43).
13 Additionally, disrupting the formation of the GRP75-CHIP complex could
14 consequently suppress the CHIP-mediated destabilization of p53 (43). In our study,
15 we first revealed that DAB2IP could suppress p53 ubiquitination by competitively
16 binding to the GRP75 protein. These findings revealed a new component of
17 CHIP-mediated p53 degradation and might increase our understanding of the dynamic
18 regulation of p53 stabilization.

19 In summary, our study presents a novel function of DAB2IP in GRP75-driven
20 wild-type p53 ubiquitination-degradation. This finding not only reveals a new
21 signaling axis that is involved in DAB2IP-induced tumor suppression but also
22 provides a novel molecular aspect of the p53 pathway.

1

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8

9 **Competing Interests:** The authors declare that they have no conflict of interest.

10

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- 15

Figure legends:

Figure 1. DAB2IP was positively associated with a better prognosis in colon cancer patients with wild-type TP53 expression.

A) DAB2IP and TP53 expression in a tissue chip containing 78 clinical specimens of malignant colon tumor. IHC score of TP53 was significantly increased in tumor with DAB2IP high expression. (H&E: hematoxylin-eosin staining, p value was calculated via Students` t test, Scale bar: 50 um).

B) Gene set enrichment analysis (GSEA) of public colorectal cancer expression profile (GSE21510). Representative GSEA plots indicated that the p53 pathway was positively associated with DAB2IP high expression among 50 hallmark of cancer gene sets (NES=1.51, NOM p-value < 0.01).

C) R2 gene correlation analysis of public clinical colorectal cancer dataset (Sugihara, n=148) revealed that DAB2IP was positively associated with TP53 target genes CDKN1A (R=0.723, p<0.001), BAX (R=0.477, p<0.001, PUMA(R=0.468, p<0.001), however, no correlation was observed between DAB2IP and TP53 expression (R=-0.046, p=0.582).

D) TP53 mutation data and DAB2IP expression profile of colon cancer was obtained from TCGA-COAD dataset (n=419). Among them, 211 cases represented wild type TP53, while TP53 mutation was detected in the remaining 208 cases. Missense was the most common type in TP53 mutation. No difference of DAB2IP expression was observed between TP53 wild type and TP53 mutation subgroup (WT: wild type; Del: deletion; Ins: insertion; ns: no significance; p value was calculated via Student`s t

1 test).

2 **E)** Kaplan-Meier overall survival analysis based on DAB2IP mRNA expression in
3 419 colon cancer patients from TCGA-COAD dataset (211 with TP53 wild type and
4 208 with TP53 mutation). Cut-off value of DA2IP expression was lower quartile.
5 Patients with high DAB2IP expression had significantly better prognosis than those
6 with low DAB2IP expression in TP53 wild type subgroup ($p < 0.05$).

7

8 **Figure 2. DAB2IP represented tumor-suppressive characteristics and positively**
9 **regulated TP53 protein level in wild type TP53 colon cancer cell lines.**

10 **A)** Up: DAB2IP and p53 protein level was evaluated 8 colorectal cancer cell lines
11 (Caco-2, HT29, HCT116, KM12, Lovo, SW48, SW480 and SW620) via western blot
12 assay. Down: TP53 mutation data of the 8 colorectal cancer cell lines (Caco-2, HT29,
13 HCT116, KM12, Lovo, SW48, SW480 and SW620) was obtained of CCLE database.
14 TP53 in HCT116, Lovo and SW48 maintained wild type.

15 **B)** DAB2IP was knocked down via siRNAs in both HCT116 and SW48 cell lines.
16 Western blot assay showed that knockdown DAB2IP could significantly reduce the
17 protein level of p53 and its downstream (p21 and BAX).

18 **C)** Knockdown DAB2IP could significantly induce tumor cell clone formation in both
19 HCT116 and SW48 cell lines.

20 **D)** Knockdown DAB2IP could significantly suppress cell apoptosis in both HCT116
21 and SW48 cell lines.

22 **E)** Knockdown DAB2IP could significantly induce tumor cell migration in both

1 HCT116 and SW48 cell lines (scale bar: 100um).

2 **F)** sh-scramble-HCT116 and sh-DAB2IP-HCT116 cells were subcutaneously injected
3 into the Nude Mice. After 4 weeks, the mice were euthanized and the tumors were
4 removed and measured. Representative images were shown. Tumors of mice injected
5 with shDAB2IP-HCT116 were significantly larger than that of the sh-scramble mice.
6 *** $p < 0.01$ with Student's t test.

7 **G)** HCT116 xenografted with shDAB2IP exhibited higher levels of proliferation and
8 lower levels of apoptosis than the control tumors. DAB2IP and p53 expression were
9 detected via immunohistochemistry, cell proliferation and apoptosis were detected via
10 Ki-67 staining and TUNEL assay. Representative images were shown. Scale bar:
11 50um.

12
13 **Figure 3. DAB2IP inhibited the degradation of p53 protein in a**
14 **ubiquitin-proteasome-dependent manner.**

15 **A)** DAB2IP protein expression was stably knocked down in SW48 cells (labelled as
16 DAB2IP-KD). Western blot assay showed that p53 protein level was significantly
17 down-regulated in DAB2IP-KD group.

18 **B)** RT-qPCR showed that knocking down DAB2IP could significantly reduce the
19 expression of TP53 downstream genes (CDKN1A, BAX and BBC3), however,
20 DAB2IP did not exhibit a significant influence on TP53 mRNA level. * $p < 0.05$ by
21 student's t test; n.s. no significant difference.

22 **C)** SW48 (control or DAB2IP-KD) was treated with MG132 at 10uM for the

1 indicated time points. DAB2IP and p53 expressions were detected using western blot
2 assay.

3 **D)** SW48 (control or DAB2IP-KD) was treated with CHX at 150ug/ml for the
4 indicated time points. DAB2IP and p53 expressions were detected using western blot
5 assay.

6 **E)** SW48 (control or DAB2IP-KD) was transfected with HA-ubiquitin plasmid and
7 treated with MG132 at 10uM. HA antibody was used to immunoprecipitated
8 ubiquitination proteins, and endogenous p53 in the immunocomplexes were detected
9 via western blot assay.

10 **F)** SW48 was co-transfected with HA-ubiquitin, Flag-TP53 plasmids, and infected
11 with shDAB2IPs lentivirus, then treated with MG132 at 10uM for 8h. Flag antibody
12 was used to immunoprecipitated exogenous p53 proteins, and ubiquitination p53 in
13 the immunocomplexes were detected by HA antibody via western blot assay.

14 **G)** HEK293T was co-transfected with HA-ubiquitin, Flag-TP53, and myc-DAB2IP
15 plasmids, and treated with MG132 at 10uM for 8h. Flag antibody was used to
16 immunoprecipitated exogenous p53 proteins, and ubiquitination p53 in the
17 immunocomplexes were detected by Flag antibody via western blot assay.

18

19 **Figure 4. DAB2IP suppressed p53 ubiquitination through competitive binding**
20 **GRP75.**

21 **A)** 245 endogenous DAB2IP-binding proteins and 286 endogenous p53-binding
22 protein were detected via mass spectrum in SW48 cell line, among them, 166 proteins

1 interacted with both DAB2IP and p53 protein.

2 **B)** Proteins that interacted with both DAB2IP and p53 from (A) were used in gene
3 ontology enrichment. Ten proteins involved in “ubiquitin protein ligase binding” were
4 shown. Bar plot represented the Sequest score of these proteins.

5 **C)** Using STRING analysis to identify the interaction between p53 and the ubiquitin
6 protein ligase binding proteins from (B). GRP75 (also known as HSPA8) presented
7 the potential inhibitor of p53.

8 **D)** Knocking down GRP75 could significantly increase p53 and its downstream, p21,
9 in SW48 and HCT116 cells with DAB2IP low expression. As determined by western
10 blot assay.

11 **E)** SW48 was co-transfected with HA-ubiquitin and Flag-TP53 plasmids, then
12 knocked down DAB2IP and/or GRP75 with relevant siRNAs. After treated with
13 MG132 at 10uM for 8h, Flag antibody was used to immunoprecipitated exogenous
14 p53 proteins, and ubiquitination p53 in the immunocomplexes were detected by HA
15 antibody via western blot assay.

16 **F)** HEK293T was co-transfected with HA-ubiquitin and Flag-TP53 plasmids, then
17 overexpressed DAB2IP and/or GRP75 with relevant plasmids. After treated with
18 MG132 at 10uM for 8h, Flag antibody was used to immunoprecipitated exogenous
19 p53 proteins, and ubiquitination p53 in the immunocomplexes were detected by HA
20 antibody via western blot assay.

21 **G)** After transfecting siDAB2IP into SW48 at the indicated concentrations, p53
22 antibody was used to immunoprecipitated endogenous p53 and its binding proteins.

1 GRP75 in the immunocomplexes were detected by relevant antibody via western blot
2 assay.

3 **H)** After transfecting pcDNA3.1-DAB2IP and pcDNA3.1-FLAG-TP53 plasmids into
4 HEK293T at the indicated concentrations, FLAG antibody was used to
5 immunoprecipitated exogenous p53 and its binding proteins. GRP75 in the
6 immunocomplexes were detected by relevant antibody via western blot assay.

7 **I)** Model for how the DAB2IP regulates p53 expression and suppresses tumor
8 progression.

9

Supplementary Information:

Supplemental Figure 1. TP53 mRNA expression level and mutation type in colorectal cancer cell lines from CCLE database.

Supplemental Figure 2. DAB2IP represented tumor-suppressive characteristics and positively regulated TP53 protein level in wild type TP53 colon cancer cell lines.

A) Western blot showed that overexpression of DAB2IP could significantly increase the protein level of p53 and its downstream (p21 and BAX) in both SW48 and HCT116 cell lines.

B) Western blot showed that neither knocking down nor overexpressing DAB2IP could affect the protein level of TP53 and its downstream in SW620 cell line.

C) Western blot showed that neither knocking down nor overexpressing DAB2IP could affect the protein level of TP53 and its downstream in SW480 cell line.

D) sh-scramble-SW48 and sh-DAB2IP-SW48 cells were subcutaneously injected into the Nude Mice. After 4 weeks, the mice were euthanized and the tumors were removed and measured. Representative images were shown. Tumors of mice injected with shDAB2IP-SW48 were significantly larger than that of the sh-scramble mice.

*** $p < 0.01$ with Student's t test.

E) SW48 xenografted with shDAB2IP exhibited higher levels of proliferation and lower levels of apoptosis than the control tumors. Representative images were shown.

Scale bar: 50um.

Supplemental Figure 3. Screening the potential proteins that interacted with DAB2IP

and TP53 protein.

A) Endogenous DAB2IP could not immuno-precipitate p53 in SW48 cell line.

B) Exogenous p53 could not bind with DAB2IP in HEK293T cell line.

C) 245 endogenous DAB2IP-binding proteins and 286 endogenous p53-binding protein were detected via mass spectrum in SW48 cell line. Through intersecting the binding protein, 166 proteins that interacted with both DAB2IP and p53 protein were detected in SW48 cell lines.

D) Coimmunoprecipitation assay revealed that endogenous DAB2IP could bind to GRP75 in SW48 cell lines.

E) Coimmunoprecipitation assay revealed that endogenous p53 could bind to GRP75 in SW48 cell lines.

F) Western blot assay showed that knockdown GRP75 could significantly increase the protein level of p53 and its downstream (p21 and BAX) in both SW48 and HCT116 cell lines.

G) Knocking down GRP75 could significantly reduce clonal formation in both SW48 and HCT116 cells with DAB2IP low expression.

H) Knocking down GRP75 could significantly induce cell apoptosis in both SW48 and HCT116 cells with DAB2IP low expression.

B

C

D

E

A

Mass spectrum analysis

B

Intersected proteins in "ubiquitin protein ligase binding" category

C

String analysis of intersected proteins in "ubiquitin protein ligase binding" category

D

E

F

G

H

I

TP53 mutation and RNA expression in colorectal cancer

Data obtain from CCLE database