SIRT1 Suppresses Pituitary Tumor Progression by Downregulating PTTG1 Expression

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Research Article

Keywords: pituitary tumor, PTTG1, SIRT1, proliferation

Posted Date: June 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-560632/v1

Abstract

Pituitary tumor is among the most common types of brain tumor, and the underlying molecular mechanism of this disease is still obscure. Here we found downregulation of SIRT1 in tumor tissues of pituitary tumor patients by real-time PCR and immunohistochemistry staining. In vitro studies reveal that upregulation of SIRT1 suppresses pituitary tumor cell line growth, and vice versa. We also find that enzymatic activity of SIRT1 is required for its cellular function. Mechanism study uncovers that SIRT1 negatively regulates PTTG1 expression by deacetylation of H3K9ac at promoter region of PTTG1. Moreover, H3K9ac level at PTTG1 promoter is an essential regulatory element for PTTG1 expression. Thus SIRT1/H3K9ac/PTTG1 axis contributes to pituitary tumor formation and may provide further potential therapeutic opportunity.

Introduction

Pituitary tumor ranks the third most common diagnosed intracranial tumor after meningioma and glioma, accounting for about 10-15% of all brain tumors [1]. Adenoma consists most of the cases, and only <1% cases will develop to cancer. However, attributed to the position and function of pituitary gland, pituitary tumor affects life quality of patients [2]. Although with good prognosis for pituitary patients, there's still a lack of knowledge about the underlying molecular mechanism which leads to this disease.

Genetic mutations recurred in pituitary tumor patients include GNAS, PKC, PI3K, HRAS and USP8, which are known regulators of signaling pathways controlling cellular proliferation, survival and motility [3]. Of note, these genes stand in the upstream of cellular signals which means heavy toxic effects when they are inhibited [4]. The most known differentiated expressed gene (DEG) between normal pituitary tissue and pituitary tumor tissue is PTTG1 [5]. It is first found expressed in rat pituitary tumor cell line GH4 but not in normal pituitary gland [6]. Functional study indicates that PTTG1 overexpression in mouse drives pituitary adenoma formation [7], while its knockout significantly inhibits pituitary tumor formation in Rb-deficiency background [8]. All the results demonstrate that PTTG1 is targetable in pituitary tumor.

SIRT1 is a protein deacetylation enzyme which removes acetyl moiety from proteins [9]. The activity of this enzyme is regulated by nicotinamide adenosine dinucleotide (NAD+), level of which represents low energy available in cells [10]. SIRT1 is reported as a key bridge linking metabolism and age [11]. As metabolism remodeling plays a prominent role in tumor cells, SIRT1 has gained tremendous attention in cancer research. However, the function of SIRT1 in cancer is controversial due to different cancer types and heterogeneity of cancer tissues. Few papers reported function and mechanism of SIRT1 in pituitary tumor.

Here we show that SIRT1 is downregulated in pituitary tumor tissues, and SIRT1 expression inhibited tumor growth. We also find that SIRT1 downregulates PTTG1 by deacetylation of H3K9ac. More importantly, resveratrol which activates SIRT1 enzymatic activity inhibits pituitary tumor cell growth. These results provide evidence that targeting SIRT1/PTTG1 axis may be potential treatment option.

Methods

Clinical samples Clinical pituitary tumor and paired normal tissues were collected after surgery with informed consents from all patients and in compliance with Medical Ethics Committee of Shanghai Changzheng Hospital.

Cell culture AtT-20 and 293T were purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. GT1-1 was purchased from National Infrastructure of Cell Line Resource. AtT-20 was cultured in 1640 supplied with 10% FBS, while 293T and GT1-1 were cultured in DMEM supplied with 10% FBS. All culture medium contains penicillin and streptomycin (both were 100 U/ml), and was conditioned at 37 °C with 5% C02.

Real-time PCR Total RNA was extracted from pituitary tumor and adjacent normal tissues by TRIzol reagent (Invitrogen), and quantified by Nanodrop 2000c (Thermo Fisher). 2 gg RNA was reverse transcribed according to the manual (Promega). For real-time PCR, we used 2x SYBR premix taq system (Yeasen Biotech) and detected with PikoReal Real-Time PCR system (Thermo Fisher). Primers used to detect SIRT1 were as follows, Forward primer: 5'- GGCGGCTTGATGGTAATCAG-3'; Reverse primer: 5'-AGTCCCAAATCCAGCTCCTC-3'. Primers used to detect GAPDH were as follows, Forward primer: 5'-CCATGGGGAAGGTGAAGGTC-3'; Reverse primer: 5'-GGGATCTCGCTCCTGGAAGA-3'.

Immunohistochemistry Immunostaining of SIRT1 (Cat. No. 13161-1-A P, Proteintech; diluted at 1:100) and IL6 (Cat. No. 21865-1-A P, Proteintech; diluted at 1:100) were performed with pituitary tumor sections. Essentially, sections were first deparaffinized at 65 °C for 30 min, and incubated with antibodies overnight in cold room, followed by incubation with secondary antibody (Cat. No. 7074 of Cell Signaling Technology; diluted at 1:200). Signal was obtained with DAB Substrate Kit (Cat. No. 8059 of Cell Signaling Technology). Photographs were taken at 40X and 200X with a light microscope (BX61, Olympus).

Western Blot Western blot was performed traditionally with little modifications. Briefly, cells were seeded at $3x10^5$ per well of 6-well plate, and were harvested 48 h later, followed by lysing in RIPA buffer supplied with protease inhibitor (Biomake). Protein concentration was determined with Bradford reagent (Bio-Rad). Unless other referred we added 30 gg protein samples per lane. 5% BSA dissolved in TBST was used to block the membrane, and to dilute primary and secondary antibodies. Incubation of the primary antibodies was undertaken in cold room overnight, and secondary antibody at room temperature for 1.5 h. ECL (Cat. No. sb-wb012 of Share-Bio) was used to detect HRP signal intensity. Chemiluminescent signal was recorded by CCD camera (Tannon system).

Stable cell line construction Stable cell lines were constructed via lenti-virus mediated gene delivery. We used pEF1a-MCS-IRES-GFP and pLKO.1-TRC to overexpress or knockdown targeted genes respectively. To produce the lentiviruses, 293T cells were transfected with the aforementioned core plasmids, along with the packaging plasmids psPAX2 and pMD2.G (12260 and 12259, Addgene) at a ratio of 12:8:4 jig and the Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Viruses were harvested at 72 h

post transfection (medium was refreshed every 24 h). Transfection of GT1-1 and AtT20 cells was performed in 6-well plates with polybrene at a final concentration of 2.5 ig/ml for 24 h. Cells were sorted for GFP signals by FACS using FACSAria (Beckman Coulter, Inc.) or selected with puromycin at 4 ug/mL for 3 days and longer. Overexpression and knockdown efficacies were demonstrated by western blot analysis. shRNA primers used were as follows: shSirt1-1: forward primer:

5'-CCGGAGTGAGACCAGTAGCACTAATCTCGAGATTAGTGCTACTGGTCTCACTTTTTT G-3', reverse primer: 5'-AATTCAAAAAAGTGAGACCAGTAGCACTAATCTCGAGATTAGTGCTACTGGTCTCACT-3'. shSirt1-2: forward primer: 5'-CCGGCCTCGAACAATTCTTAAAGAT CTCGAGATCTTTAAGAATTGTTCGAGGTTTTTG-3', reverse primer: 5'-AATTCAAAAACCTCGAACAATTCTTAAAGATCTCGAGATCTTTAAGAATTGTTCGAGG-3'. shGcn5: forward primer: 5'-
CCGGGCTACCTACAAAGTCAATTATCTCGAGATAATTGACTTTGTAGGTAGCTTTTTG-3', reverse primer: 5'-AATTCAAAAAGCTACCTACAAAGTCAATTATCTCGAGATAATTGACTTTGTAGGTAGC-3'. shBrd4-1: forward primer: 5'-CCGGTGAACCTCCCTGATTAC TATACTCGAGTATAGTAATCAGGGAGGTTCATTTTTG-3', reverse primer:

5'-AATTCAAAAATGAACCTCCCTGATTACTATACTCGAGTATAGTAATCAGGGAGGTTCA-3'. shBrd4-2: forward primer: 5'-CCGGGCGGCAGCTAAGTCTAGATATCTCGAGATATCTAGACTTAGCTGCCGCTTTTTG-3', reverse primer: 5'-AATTCAAAAAGCGGCAGCTAAGTCTA GATATCTCGAGATATCTAGACTTAGCTGCCGC-3'

MTT assay MTT assay was used to determine cell proliferation rate. GT1-1 Cells were dissociated from 10-cm plate, AtT-20 cells were pelleted down, and the number was determined by autonomous cell counter. $1x10^3$ Cells were added into each well of 96-well plate. 24 h post seeding, MTT was added into 96 well for 4 h, followed by aspirating the media away and using DMSO to dissolve cellular debris, OD570 was measured and recorded. Repeat for the next 4 days. All data was combined and pictured in GraphPad Prism 6.0 software.

Cell viability Cell viability was determined by CCK8 assay. Cells were seeded at $1x10^4$ per well of 96-well. 24 h after seeding, drugs were added. After 24 h, 20 CCK8 was added, and incubating for 3 h. After incubation, absorbance at 450 nm was determined in a microplate reader (PerkinElmer Enspire). Data was processed in GraphPad Prism 6.0 software.

Invasion assay Invasion assay was performed using transwell (3422, Corning). $1x10^6$ cells resuspended in DMEM or 1640 were seeded into every transwell with 50 jxL matrigel (diluted at 1:2) at the bottom. After incubation for 48 h, cells on the outer membrane were stained with crystal violet containing 20% methanol. Pictures were taken under microscopy (RVL-100-G, ECHO).

Chromatin immunoprecipitation (ChIP) ChIP-qPCR assay was used to determine SIRT1 and H3K9ac binding to PTTG1 promoter. Cells were first cross-linked with 1% formaldehyde, followed by scraping into ice-cold PBS with protease inhibitors. Then cells were resuspended in lysis buffer (20 mM HEPES, pH = 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.5% NP-40, 25% glycerol, 1.5 mM MgCl2), followed by several brief periods of sonication. One-third of the cell extract was kept as the input sample, and two-thirds of the cell

extract was used as the substrate for immunoprecipitation with anti-SIRT1 and anti-H3K9ac antibodies. 5 M NaCl was used to reverse the cross-linking after which the eluted DNA was extracted for PCR analysis. The primer sequences for the PTTG1 promoter are listed as follows: Forward primer: 5'-ttcgtgaaagagtaatatgg3'; Reverse primer: 5'- atcctcaatacttcaggctaag-3'.

Result

SIRT1 expression reduces pituitary tumor cell growth and invasion

We first explored the expression pattern of SIRT1 in 20-paired clinical pituitary tumors by real-time PCR, and the result demonstrated lower SIRT1 expression in tumor tissues than paired normal tissues in 15 (75% of total samples examined) patients (Figure 1A). Consistently, immunohistochemistry staining of SIRT1 in paired tissues also showed decreased SIRT1 expression in tumor region (Figure 1B). To uncover the function of SIRT1 in pituitary tumors, we knocked down the endogenous expression of SIRT1 in AtT20 cells (a mouse pituitary tumor cell line) (Figure 1C), and examined cellular growth rate and invasive ability. We found accelerated growth rate as well as enhanced invasive ability of AtT20 cells upon SIRT1 knockdown (KD) (Figure 1C & D). Above results were also observed in GT1-1 cell line which showed increased growth rate and invasive ability after KD of SIRT1 expression. We next exogenously expressed SIRT1 in both AtT20 cells and GT1-1 cells (Figure 1 G & I). To this end, overexpression of SIRT1 in both cells inhibited cell growth and invasion (Figure 1 G-J). Taken together, SIRT1 is downregulated in pituitary tumor and its expression reduces pituitary tumor cell growth and invasion.

SIRT1 activity regulates pituitary tumor cell growth and invasion

Considering that resveratrol is an activator of SIRT1, we subsequently asked whether resveratrol mimicked the tumor suppressor effect of SIRT1 overexpression in pituitary tumor cells. To this end, resveratrol induced cell death in both AtT20 and GT1-1 in a concentration-dependent pattern (Figure 2A), and it also inhibited invasion of AtT20 and GT1-1 cells (Figure 2B). Above result indicated that promoting SIRT1 activity would inhibit pituitary tumor cell growth and invasion, the question remained whether inhibition of SIRT1 activity could increase tumor cell growth and invasion. As nicotinamide was known as an antagonist for SIRT1, we next treated pituitary cells with nicotinamide. Consistently, nicotinamide promoted cell growth (Figure 2C) and cell invasion (Figure 2D).

Mutation of histidine of SIRT1 protein at position 363 to tyrosine abrogated the enzymatic activity of SIRT1. To further verify that SIRT1 activity regulated pituitary tumor cell growth and invasion, we expressed wildtype (WT) SIRT1 and H363Y mutant SIRT1 in AtT20 respectively (Figure 2E). While WT SIRT1 inhibited cell growth, H363Y mutant SIRT1 did not affect cell growth (Figure 2F). Further treatment of resveratrol induced robust inhibition of cell growth in AtT20 cells expressed with WT SIRT1 but not in cells with H363Y SIRT1 expression (Figure 2G). Endogenous activity of SIRT1 was mainly regulated by NAD+ levels, thus upregulating cellular NAD+ levels would promote SIRT1 activity. PJ34 and DPQ were two chemical compounds known to inhibit PARP function and upregulating cellular NAD+ levels. Treatment of control AtT20 cells with PJ34 and DPQ both inhibited cell growth, while SIRT1 knockdown

abolished this effect (Figure 2H). To lower cellular NAD+ levels, we overexpressed CD38, which was known as a NADase enzyme, in AtT20 cells, cell growth assay showed that CD38 overexpression led to promoted cell growth while this promotion was diminished in SIRT1 KD cells (Figure 2I). Thus enzymatic activity of SIRT1 regulates pituitary tumor cell growth and invasion.

SIRT1 regulates PTTG1 expression

PTTG1 was an oncogene expressed in pituitary tumor but not in normal pituitary gland. As SIRT1 was mainly localized in nucleus, we thus asked whether PTTG1 expression was regulated by SIRT1. We found that both PTTG1 mRNA and protein levels were increased upon SIRT1 inhibition (Figure 3A). SIRT1 overexpression in AtT20 cells led to decreased levels of mRNA and protein of PTTG1 (Figure 3B). Moreover, SIRT1 enzymatic activation by resveratrol decreased PTTG1 expression, while its inhibition by nicotinamide increased PTTG1 expression (Figure 3C & D). More importantly, this pattern could also be observed in clinical pituitary tumor tissues (Figure 3E). As insulin and IGF-1 activated PTTG1 mRNA transcription, we thus asked whether SIRT1 was involved in this process. As shown in Figure 3F, while insulin and IGF-1 treatment increased PTTG1 expression, resveratrol attenuated PTTG1 expression. Above results demonstrated that SIRT1 regulated PTTG1 expression.

SIRT1 deacetylates the histone H3K9 to regulate PTTG1 expression

To gain insights into the mechanism of how PTTG1 was regulated by SIRT1, we speculated that SIRT1 might deacetylate histone to suppress PTTG1 transcription. To this end, we found that SIRT1 localized to PTTG1 promoter region, and its inhibition enriched H3K9ac at PTTG1 promoter (Figure 4A). Furthermore, resveratrol treatment which activated SIRT1 decreased H3K9ac level at PTTG1 promoter (Figure 4B). To confirm that H3K9ac level regulated PTTG1 expression, we knocked down the expression of GCN5 which could modify H3K9 with acetyl moiety. ChIP result showed decreased level of H3K9ac at PTTG1 promoter (Figure 4C), and as a result downregulation of PTTG1 mRNA upon GCN5 inhibition (Figure 4D). As GCN5 inhibition decreased H3K9ac level in PTTG1 promoter, we asked whether SIRT1 regulated PTTG1 expression independent of H3K9ac level. As shown in Figure 4E, while SIRT1 KD upregulated PTTG1, this regulation disappeared in GCN5 KD cells. These results suggested that SIRT1 deacetylated the histone H3K9 to regulate PTTG1 expression.

H3K9ac reader BRD4 is required for PTTG1 expression

To corroborate that H3K9ac mediated regulation of PTTG1 expression by SIRT1, we knocked down the expression of BRD4, which was an H3K9ac reader that coupled this histone modification to RNA polymerization. We found that BRD4 KD reduced PTTG1 expression (Figure 5A& B). JQ1 was reported as an inhibitor of BRD protein family, thus JQ1 treatment would mimic the effect of BRD4 KD. As shown in Figure 5C and D, JQ1 treatment led to decreased expression of PTTG1 in a concentration-dependent manner. Furthermore, treatment of JQ1 abolished PTTG1 expression induced by SIRT1 KD (Figure 5E). Above results revealed important regulation of PTTG1 expression by H3K9ac, and this modification could be divided into two aspects: processes determining the abundance of H3K9ac and cellular ability to

decode H3K9ac. While SIRT1 reduced the abundance of H3K9ac, BRD4 KD attenuated cellular ability to decode H3K9ac signal. We asked whether SIRT1 activation and BRD4 inhibition would synergically inhibit PTTG1 expression. To achieve this, we treated AtT20 cells and GT1-1 cells resveratrol and JQ1. It was found that resveratrol inhibited cell growth, and simultaneous treatment of cells with resveratrol and JQ1 significantly reduced cell growth (Figure 5F & G). These results suggested that H3K9ac mediated PTTG1 expression by SIRT1.

Discussion

Gene regulation was lesser known in pituitary tumor than other tumor types. Although SIRT1 dysregulation was reported in many cancer types, there was no paper describing the role of SIRT1 in pituitary tumor. We found that SIRT1 was downregulated in pituitary tumor, and activated its expression or its enzymatic activity inhibited pituitary tumor cell growth. Since SIRT1 could be activated by small chemical molecule, resveratrol, it might provide potential treatment to pituitary tumor.

The present work also revealed regulation of PTTG1 mRNA expression by SIRT1 in pituitary tumor. The function and mechanism of PTTG1 protein in the development and progression of different cancer types had gained tremendous interest, and lots of papers demonstrated PTTG1 as an oncogene [12]. PTTG1 acted as a modulator of sister chromatid separation process [13]. Its upregulation led to genetic instability including aneuploidy, and thus might contribute to tumor cell evolution. Functional studies demonstrated that PTTG1 was an initiator and promoter of cancer cell. Nevertheless, regulation of PTTG1 mRNA expression was less reported. We found that SIRT1 expression inhibited PTTG1 expression. Further study demonstrated deacetylation activity of SIRT1 was responsible for its regulation of PTTG1. As SIRT1 belonged to class III histone deacetylase family, we focused on whether histone acetylation/deacetylation would mediate this regulation. Acetylated lysine 9 of Histone 3 (H3K9ac) was the classical substrate of SIRT1 [14], and it was well established that H3K9ac activated gene transcription [15]. Our data showed that SIRT1 bond to promoter of PTTG1, and deacetylated H3K9ac, and thus attenuated PTTG1 mRNA expression. Although SIRT1/H3K9ac/gene transcription pathway was well accepted, the specific regulation of PTTG1 by SIRT1 via deacetylation of H3K9ac was not reported.

Although we did not screen for acetyl transferases, GCN5 was reported to mediate H3K9 acetylation [16]. In an attempt to confirm the importance of the level of H3K9ac to regulate PTTG1 expression, we found GCN5 expression mediated acetylation of H3K9 at PTTG1 promoter region. This result not only validated H3K9ac as an essential regulatory element of PTTG1 expression, but also identified GCN5 as the main acetyl transferase of H3K9ac at PTTG1 promoter. Thus factors influencing GCN5 protein level or its enzymatic activity would regulate PTTG1 expression.

Declarations

Funding This work was funded by research grant from National Natural Science Foundation of China (No. 81672491).

Conflict of interest The authors declare no competing interests.

Availability of data and material All data are available from the corresponding author upon reasonable request.

Authors' contributions J. H. and F. Z. conceived the project and performed most experiment, H. G. helped in data analysis, Y. P. performed animal and immunohistochemistry experiment, W. S. performed ChIP assay, L. J., P. W. and J. Q. gave essential suggestions. X. D. supervised the project and provided funding support.

Acknowledgements

We thank the National Natural Science Foundation of China of its financial support to this study.

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Figures

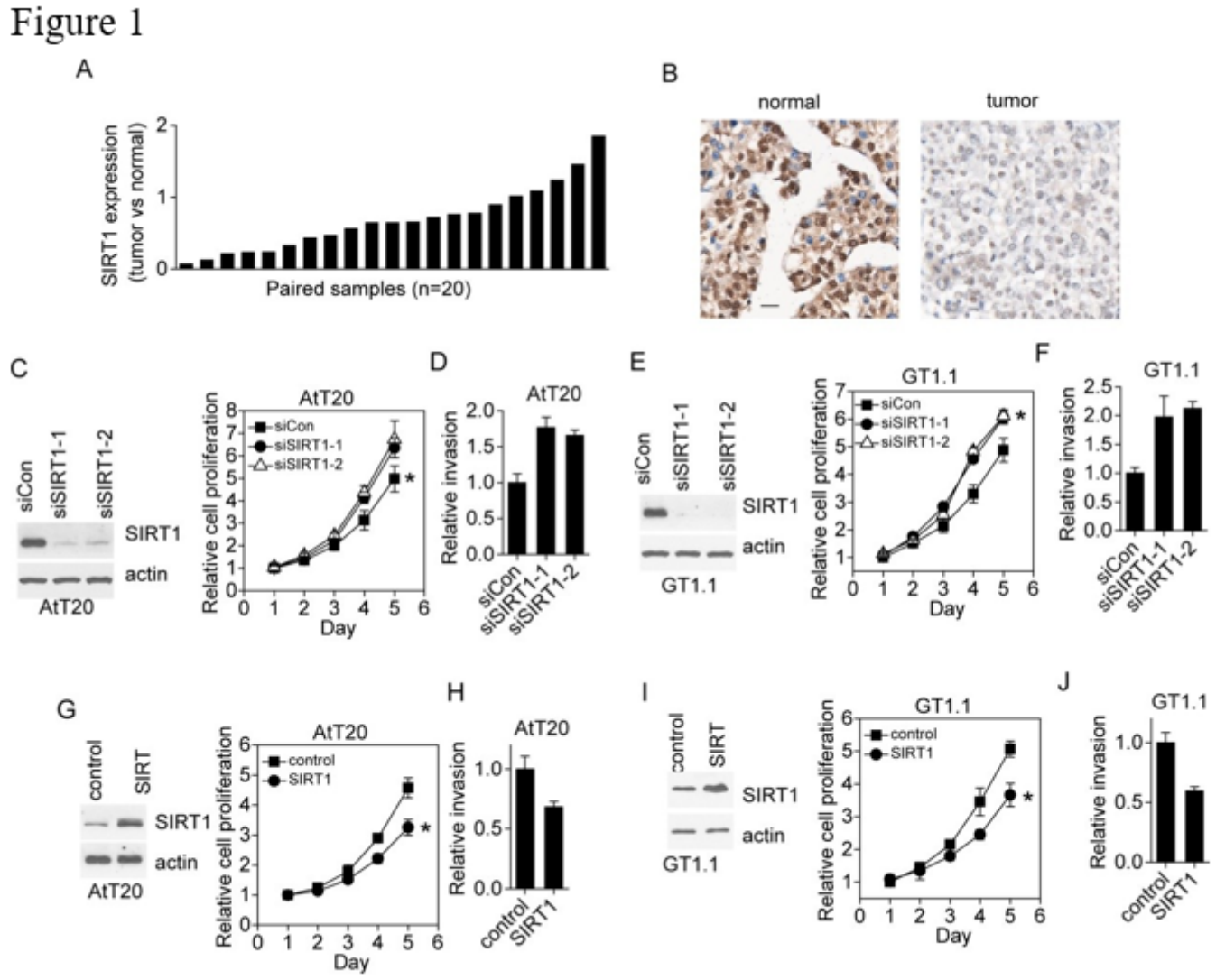

Figure 1

SIRT1 expression reduces pituitary tumor cell growth and invasion. (a) Real-time PCR result showing SIRT1 expression in human pituitary tumor tissues. (B) Immunohistochemistry staining of SIRT1 in human pituitary sections. Scale bar, 50 pm. (C) WB result showing protein expression in cells (left). Line plot of cell proliferation rate (right). *P<0.05 by paired student t-test. (D) Bar plot showing number of invaded cells. (E) WB result showing protein expression in cells (left). Line plot of cell proliferation rate (right). *P<0.05 by paired student t-test. (F) Bar plot showing number of invaded cells. (G) WB result showing protein expression in cells (left). Line plot of cell proliferation rate (right). *P<0.05 by paired student t-test. (H) Bar plot showing number of invaded cells. (I) WB result showing protein expression in cells (left). Line plot of cell proliferation rate (right). *P<0.05 by paired student t-test. (J) Bar plot showing number of invaded cells.

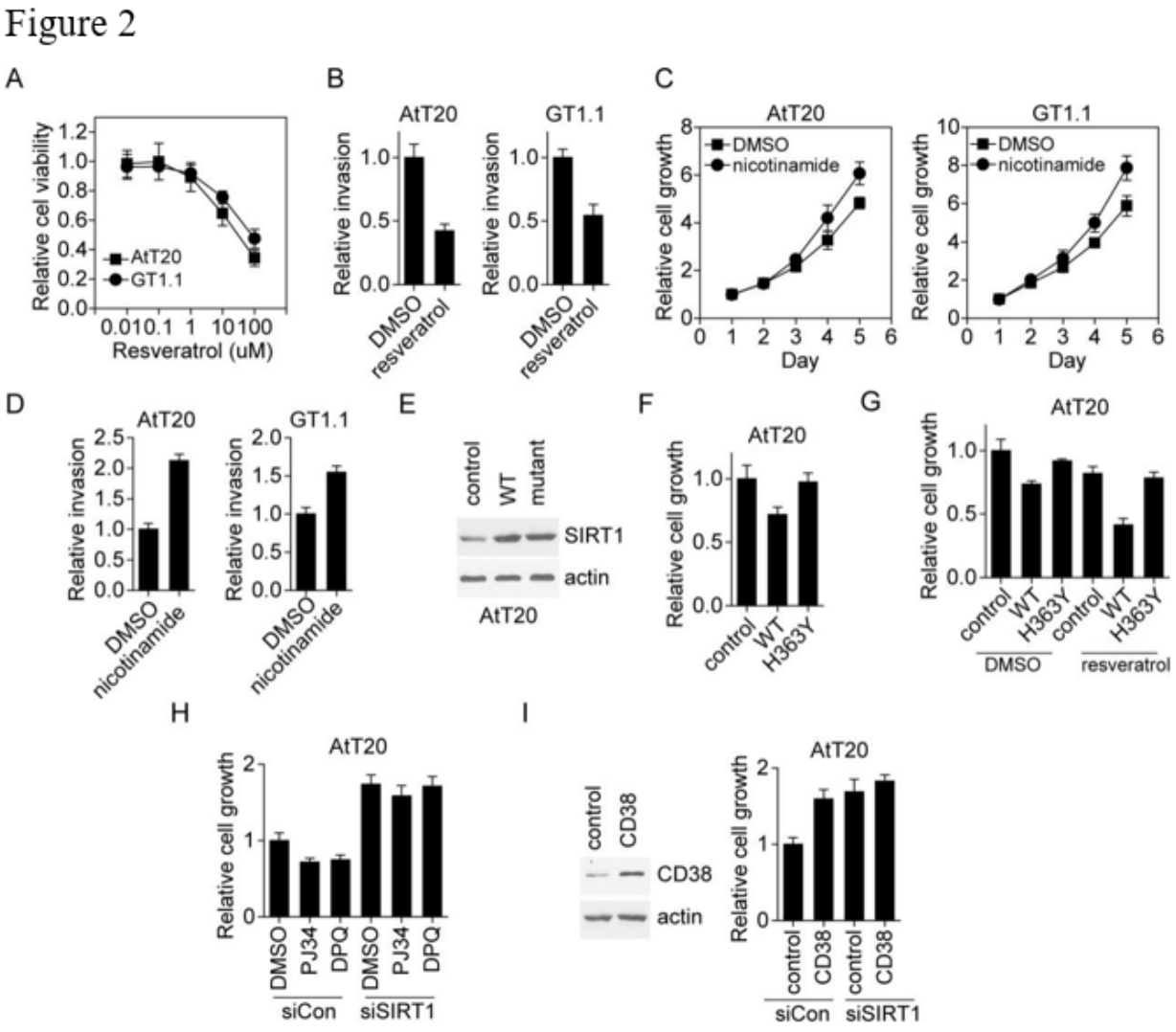

Figure 2

SIRT1 activity regulates pituitary tumor cell growth and invasion (A) Line plot showing cell viability. (B) Bar plot demonstrating relative invaded cells. (C) Line plot showing cell growth rate. (D) Bar plot demonstrating relative invaded cells. (E) WB result showing protein expression in cells. (F-H) Bar plot

showing cell growth. (I) WB result showing the protein expression levels in cells (left). Bar plot showing cell growth (right). SIRT1 inhibitor nicotinamide promotes tumor cell growth.

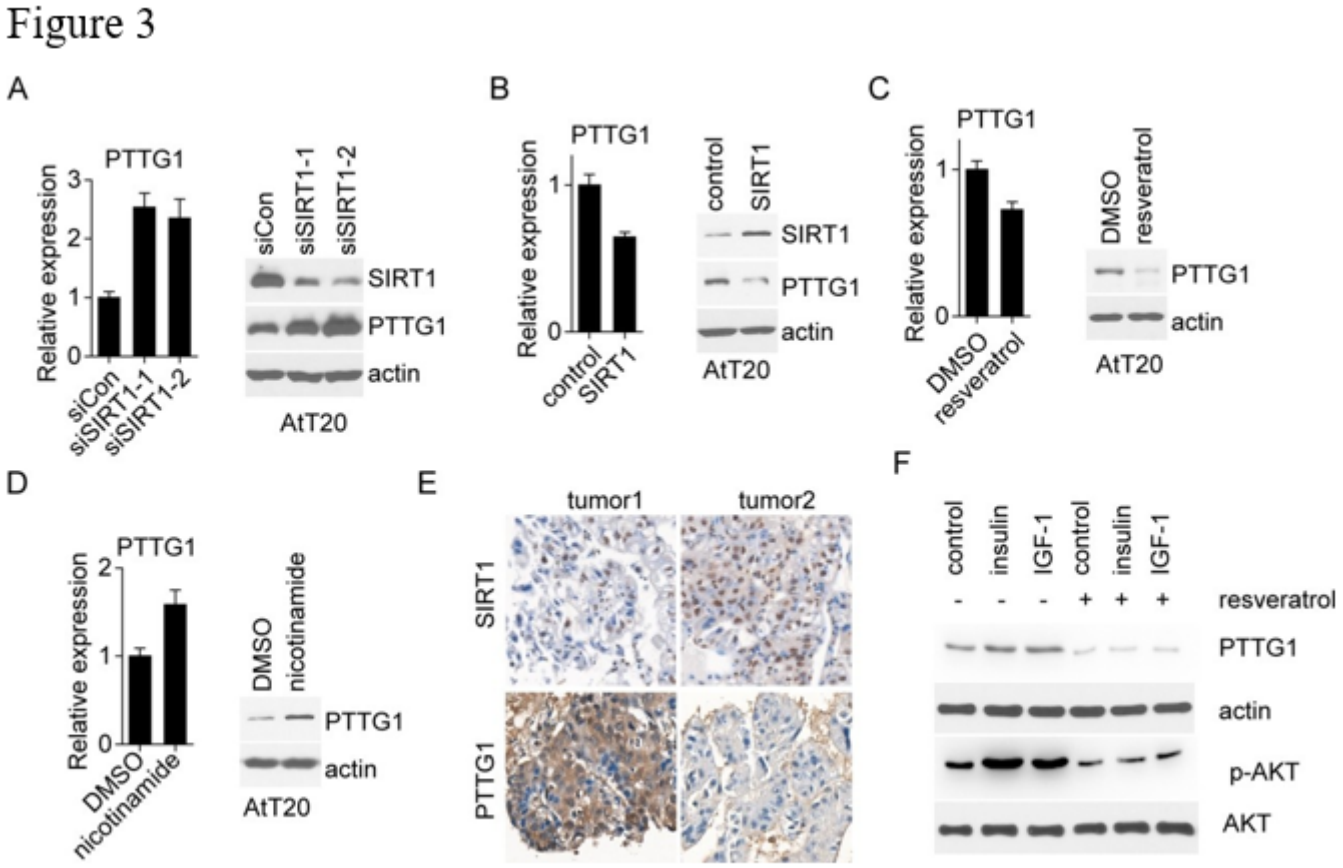

Figure 3

SIRT1 regulates PTTG1 expression (A) Bar plot showing relative mRNA expression (left). WB result showing protein expression levels in cells. (B-D) Bar plot showing relative mRNA expression (left). WB result showing protein expression levels in cells. (E) Representative images showing immunohistochemistry staining result. (F) WB result showing protein expression in cells.

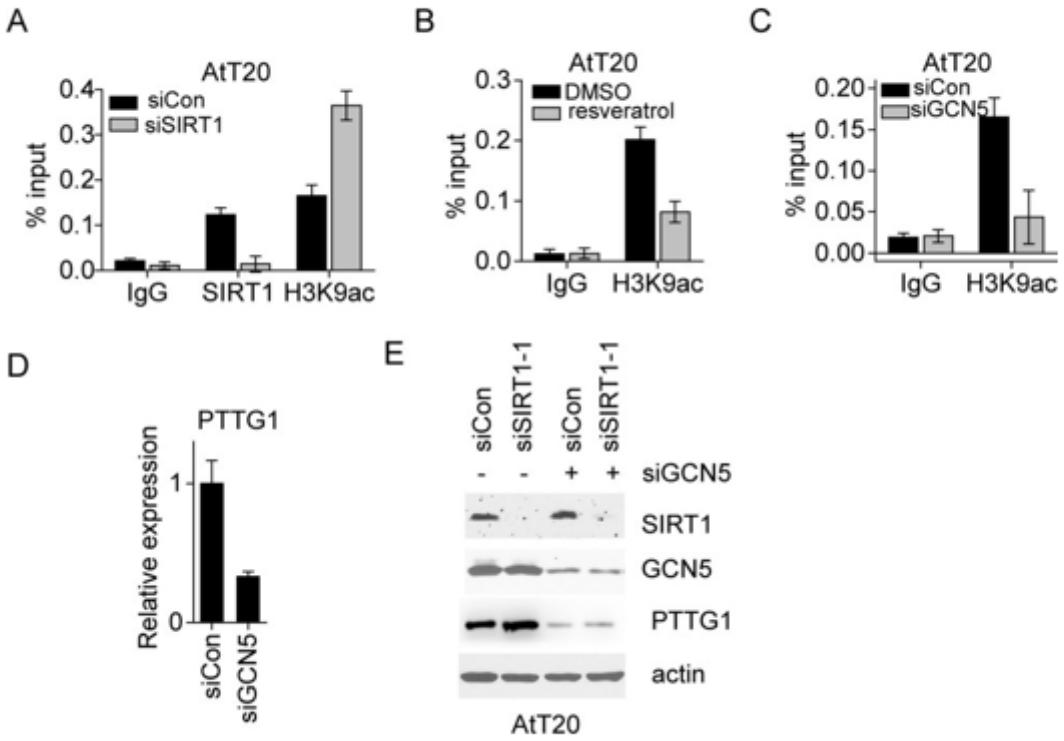

Figure 4

SIRT1 deacetylates the histone H3K9 to regulate PTTG1 expression (A-C) Bar plot showing the quantity of enriched PTTG1 promoter region. (D) Bar plot showing relative mRNA expression. (E) WB result showing protein expression levels in cells.

Figure 5

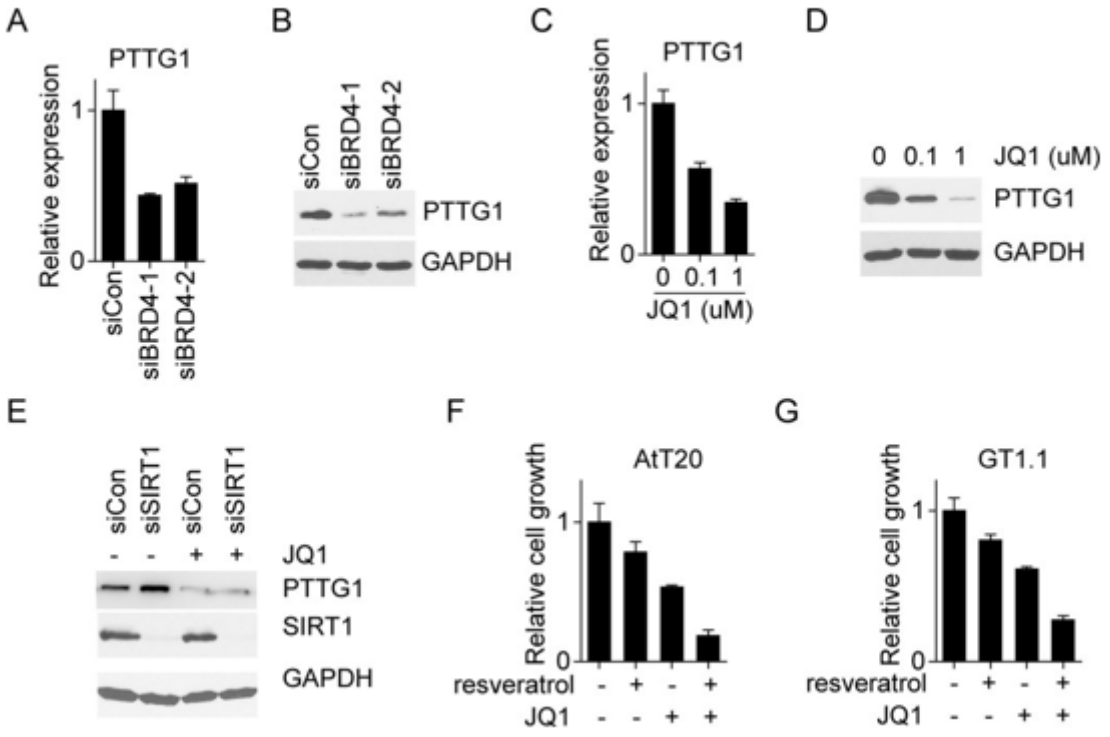

Figure 5

H3K9ac reader BRD4 is required for PTTG1 expression (A) Bar plot showing relative mRNA expression. (B) WB result showing protein expression levels in cells. (C) Bar plot showing relative mRNA expression. (D) WB result showing protein expression levels in cells. (E) WB result showing protein expression levels in cells. (F, G) Bar plot showing cell growth.