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Stabilization of P/CAF, as a ubiquitin ligase toward MDM2, suppresses mitotic cell death through p53-p21 activation in HCT116 cells with SIRT2 suppression

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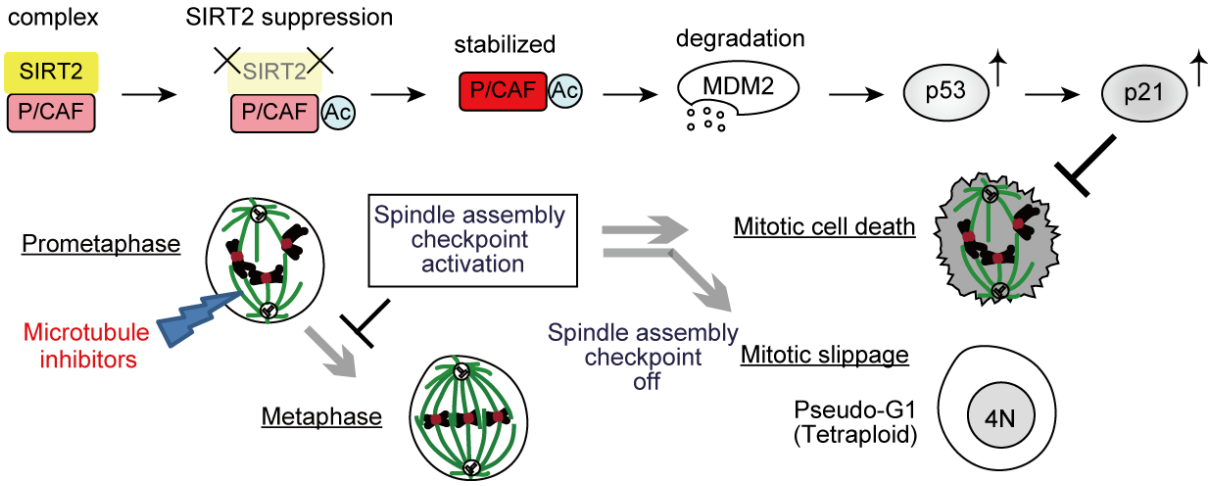
Graphical abstract



Abstract

We previously reported that the suppression of SIRT2, an NAD+-dependent protein deacetylases, induces p53 accumulation via degradation of p300 and the subsequent MDM2 degradation, eventually leading to apoptosis in HeLa cells. The present study identified a novel pathway of p53 accumulation by SIRT2 suppression in HCT116(p53+/+) cells in which SIRT2 suppression led to escape from mitotic cell death caused by spindle assembly checkpoint activation induced by microtubule inhibitors such as nocodazole but not apoptosis or G1 or G2 arrest. We found that SIRT2 interacts with P/CAF, a histone acetyltransferase, which also acts as a ubiquitin ligase against MDM2. SIRT2 suppression led to an increase of P/CAF acetylation and its stabilization followed by a decrease in MDM2 and activation of the p53-p21 pathway. Depression of mitotic cell death in HCT116(p53+/+) cells with SIRT2 suppression was released by suppression of P/CAF or p21. Thus, the P/CAF-MDM2-p53-p21 axis enables the escape from mitotic cell death and confers resistance to nocodazole in HCT116(p53+/+) cells with SIRT2 suppression. As SIRT2 has attracted attention as a potential target for cancer therapeutics for p53 regulation, the present study provides a molecular basis for the efficacy of SIRT2 for future cancer therapy based on p53 regulation. These findings also suggest an undesirable function of the SIRT2 suppression associated with activation of the p53-p21 pathway in the suppression of mitotic cell death caused by spindle assembly checkpoint activation.

Keywords: SIRT2, P/CAF, MDM2, p53, p21, mitotic cell death.

Abbreviations: MTI, microtubule inhibitor; SAC, spindle assembly checkpoint

1. Introduction

Mammalians express seven sirtuins (SIRT1–7), a family of NAD+-dependent protein deacetylases that differ widely in their specificity towards substrates, localization, and biological functions including cancer, and aging [1] .

Recently, SIRT2 has attracted attention as a potential target for cancer therapeutics, as it has been reported that SIRT2 functions as a tumor suppressor by regulating mitosis and genome integrity [2]. Furthermore, SIRT2-deficient mice exhibit tumorigenesis in multiple organs [2].

As a different mechanism of how SIRT2 suppresses tumorigenesis, our group showed that SIRT2 modulates the spindle assembly mitotic checkpoint (SAC) in the colorectal cancer cell line HCT116 expressing wild-type p53 [hereafter referred as to HCT116(p53+/+)], a mitotic checkpoint proficient near-diploid cell line [3]. It is known that suppression of BubR1 and Mad2, key molecules in the mitotic checkpoint, leads to resistance to microtubule inhibitors (MTIs) such as nocodazole by abolishing SAC function [4]. As is the case for the suppression of BubR1 and Mad2, SIRT2 suppression confers resistance to MTIs in HCT116(p53+/+) cells [3]. However, SIRT2 suppression prolongs SAC activation, suggesting that SIRT2 regulates mitotic cell death caused by SAC activation via a different mechanism from BubR1 and Mad2. Prolonged SAC activation in cells with SIRT2 suppression is thought to reflect escape from mitotic cell death, which is also observed for the case of Myc suppression leading to the inhibition of mitotic cell death [5].

In contrast to the function of SIRT2 as a tumor suppressor, SIRT2 inactivation was shown to have potential in cancer therapy. We previously reported that SIRT2 downregulation induces apoptosis in cancer cell lines such as HeLa cells. The apoptosis

was caused by p53 accumulation, which is mediated by degradation of p300, which acts as a dual regulator of p53 via acetylase and polyubiquitin ligase (E4) activities [6].

In our previous study of SIRT2 function in mitotic cell death [3], we observed that SIRT2 suppression does not result in apparent apoptotic cell death or cell cycle arrest in HCT116(p53+/+) cells in the absence of MTIs, unlike in HeLa cells. We also observed that SIRT2 suppression confers resistance to nocodazole in HCT116(p53-/-) cells in which the p53 gene was disrupted by homologous recombination with HCT116(p53+/+) cells, although the extent of resistance is much lower than in HCT116(p53+/+) cells [3]. Therefore, we initially hypothesized that p53 accumulation does not occur in HCT116(p53+/+) cells with SIRT2 suppression or is not involved in mitotic cell death. Based on this hypothesis, we evaluated the mechanism of how p53 accumulation by SIRT2 suppression is inhibited in HCT116(p53+/+) cells to improve the efficacy of inhibitors of sirtuins on tumor toxicity.

Unexpectedly, we observed that the p53-p21 pathway is induced in HCT116(p53+/+) cells with SIRT2 suppression in the present study. Here, we describe a novel pathway for SIRT2 in p53 regulation, in which SIRT2 controls p53 levels by regulating the stability of P/CAF, a histone acetyltransferase, which also acts as a ubiquitin ligase toward MDM2, a ubiquitin ligase that targets p53 [7]. Furthermore, we found that P/CAF accumulation followed by activation of the p53-p21 pathway mediates at least partially the escape from mitotic cell death in HCT116(p53+/+) cells with SIRT2 suppression. The present study revealed a novel pathway for p53 regulation by SIRT2 and also indicates a negative aspect of SIRT2 suppression with activation of p53-p21 pathway in the suppression of mitotic cell death by SAC activation,

2. Materials and methods

2.1 Cell culture and siRNA transfection

Cell culture anf siRNA transfection were performed as previously reported [8].

siRNAs corresponding to SIRT2 and p21 mRNAs were obtained from Qiagen (HP-validated siRNAs) (SIRT2, SI02655471; p21, SI00604905). siRNA corresponding to P/CAF mRNA was obtained from Thermo Fisher Scientific (s16895). . siRNA corresponding to p300 mRNA was designed as previously reported [9]. Negative control siRNA was obtained from Qiagen (1027281).

Nocodazole (Millipore) was resuspended in dimethyl sulfoxide (Sigma) and used at a final concentration of 200 nM. Cycloheximide (Wako) was resuspended in ethanol and used at a final concentration of 75 μg/ml. AGK2 (Cayman Chemical) was resuspended in dimethyl sulfoxide and used at a final concentration of 10 μM.

2.2 Plasmids

pcDNA3-Myc-SIRT2(wt) and pcDNA3-Myc-SIRT2(ΔNDAC) constructs were described in our previous report [8]. pCI flag PCAF was obtained from Addgene.

Transfection to 293T cells were performed using PEI Max (Polysciences).

2.3 Immunoblotting

Antibodies are listed in Supplementary Material 1. Signals were visualized with a chemiluminescence detection system (Thermo Scientific).

2.3 Immunoprecipitation

Cellular extracts were prepared from cell pellet by adding 8 volumes of Triton X-100 lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.2 % Triton X-100, 2 mM EGTA, 1.5 mM $MgCl_2$, and 10 % glycerol] containing a protease inhibitor mixture (Roche), phosphatase inhibitor mixture (Roche), and 5 mM nicotinamide (Wako).

Immunoprecipitations were carried out with 500 μL of cell extract in the presence of 2.5 μg of antibody and 10 μL Protein G Sepharose (GE Healthcare) for 12 h at 4°C. Anti-P/CAF antibody (Cell signaling), anti-SIRT2 antibody (Abcam) and anti-FLAG M2 antibody (Sigma) were used. To examine the interaction between SIRT2 and P/CAF *in vivo*, 500 μL of cell extract was precleared in the presence of 2.5 μg of normal rabbit IgG (Cell signaling) and 10 μL Protein G Sepharose for 8 h at 4°C, and then used for immunoprecipitation. Immune complexes were washed 4 times with Triton X-100 lysis buffer, and then eluted with 50 μL of SDS-PAGE sample buffer.

2.4 Real-time RT-PCR

Total RNA was prepared using NucleoSpin RNA (Macherey-Nagel) and 500 ng of RNA was used to synthesize cDNA using a ReverTra Ace kit (Toyobo). Real-time PCR was carried out in triplicate on a StepOne Plus Real Time PCR System (Applied Biosystems) using Thunder-bird SYBR qPCR Mix (Toyobo) and the following primers: P/CAF primers: forward: GCCACAGTTCTGCGACAGTCT, reverse: CCGAGCGAAGCAATGTTCTC; GAPDH primers: forward: TGTCCGTCGTGGATCTGAC, reverse: CCTGCTTCACCACCTTCTTG.

2.5 Flow cytometry analysis

Flow cytometry analyses were performed as previously reported [3].

Results

3.1 SIRT2 suppression leads to activation of the p53-p21 pathway through upregulation of P/CAF

In our previous study, we observed that siRNA-mediated SIRT2 knockdown does not result in apparent apoptotic cell death or cell cycle arrest in HCT116(p53+/+) cells, unlike in HeLa [3,6]. Thus, we originally hypothesized that p53 is not upregulated in HCT116(p53+/+) cells with SIRT2 suppression and aimed to determine the difference in p53 regulation by SIRT2 between HCT116(p53+/+) and HeLa cells. However, unexpectedly, immunoblotting analysis showed that SIRT2 suppression in HCT116(p53+/+) cells led to increased expression of p53 (Fig. 1A). Additionally, the expression of p21, a transcription target of p53, was induced in HCT116(p53+/+) cells with SIRT2 suppression (Fig. 1A).

As the molecular basis for p53 accumulation in cells with SIRT2 suppression, we reported that in HeLa cells, SIRT2 suppression causes p53 accumulation by decreasing p300 [6], which acts as a dual regulator of p53 [10]. Thus, we examined the level of p300 in HCT116(p53+/+) and HCT116(p53-/-) cells with SIRT2 suppression. As shown in Figure 1B, a minor decrease in p300 was observed in HCT116(p53+/+) and HCT116(p53-/-) with SIRT2 suppression, whereas a significant decrease in p300 was observed in HeLa cells as we previously reported [6]. Importantly, siRNA-mediated p300 suppression in HCT116(p53+/+) cells did not cause p53 accumulation or p21 accumulation unlike the case of HeLa cells in our previous study [6] (Fig. 1B). These results indicates that a minor decrease in p300 does not mediate p53 accumulation in HCT116(p53+/+) cells with SIRT2 suppression.

As a second possibility, we focused on P/CAF, a histone acetyltransferase, which

also acts as a ubiquitin ligase toward MDM2, a ubiquitin ligase that targets p53 [7]. Furthermore, SIRT1 was reported to interact with P/CAF through the conserved catalytic core domain of sirtuin proteins and deacetylate P/CAF [11]. Interestingly, immunoblotting analysis showed that SIRT2 suppression led to increased P/CAF levels and decreased MDM2 levels in HCT116(p53+/+) cells (Fig. 1A).

P/CAF expression has been reported to be induced by p53 in breast tumor cells [12]. To examine whether the increase in P/CAF was a cause or consequence of p53 accumulation in HCT116(p53+/+) cells with SIRT2 suppression, we performed siRNA-mediated P/CAF knockdown in HCT116(p53+/+) cells with SIRT2 suppression. As shown in Figure 1A, we observed that MDM2, p53, and p21 levels in HCT116(p53+/+) cells with SIRT2 suppression were restored to levels similar to those in control cells by knockdown of P/CAF. We obtained the same results in HCT116(p53-/-) cells (Fig. 1A). These results demonstrate that the increase in P/CAF in HCT116(p53+/+) cells with SIRT2 suppression caused a decrease in MDM2 followed by p53 accumulation and that P/CAF accumulation was p53-independent.

We examined whether the P/CAF-MDM2-p53-p21 axis is also active in cells with SIRT2 suppression during SAC activation, to investigate later whether this axis is responsible for conferring resistance to mitotic cell death. The optimal length of treatment with nocodazole was determined to be 16 h to achieve SAC activation, as monitored by cyclin B1/Cdk1 activity and the MPM-2-positive population, which reached a peak at 16 h of treatment in both cells with SIRT2 knockdown and control cells in our previous study [3]. Immunoblotting analysis revealed similar results for all proteins (Fig. 1A), suggesting that the P/CAF-MDM2-p53-p21 axis is active in cells with SIRT2 suppression during SAC activation as well as in the growing state.

3.2 SIRT2 interacts with P/CAF and controls P/CAF protein stability

To determine the mechanism of how SIRT2 suppression decreased P/CAF, we examined whether SIRT2 interacts with P/CAF and controls P/CAF stability, based on a previous study showing that SIRT1 interacts with P/CAF through the conserved catalytic core domain of sirtuins that deacetylate P/CAF [11]. 293T cells were cotransfected with FLAG-tagged P/CAF and SIRT2 expression vectors. When the whole cell extracts were immunoprecipitated with an anti-FLAG antibody, SIRT2 was detected in the FLAG immunoprecipitate (Fig. 2A), suggesting that SIRT2 interacts with P/CAF. SIRT2 (ΔNDAC), a mutant version of SIRT2 lacking deacetylase activity, also interacts with FLAG-P/CAF (Fig. 2A).

We also examined whether SIRT2 deacetylates P/CAF, which is autoacetylated and acetylated by p300[13]. We examined the levels of acetyl-lysine in the FLAG immunoprecipitates by immunoblotting analysis of anti-acetyl-lysine antibody. As shown in Figure 2A, SIRT2(wt) expression reduced acetylation of P/CAF; however, SIRT2 (ΔNDAC) expression also reduced acetylation of P/CAF to a similar extent. This suggests that the complex formation of SIRT2 and P/CAF but not deacetylase activity of SIRT2 reduced acetylation of P/CAF, unlike SIRT1 [11].

We next examined whether SIRT2 interacts with P/CAF in a physiological context. When whole cell extracts of HCT116(p53+/+) cells were precipitated with anti-SIRT2 antibody, endogenous P/CAF was detected in the SIRT2 immunoprecipitate (Fig. 2B). To further confirm that the band in the immunoblot of P/CAF was derived from endogenous P/CAF, the whole cell lysate from HCT116(p53+/+) cells with siRNA-mediated P/CAF suppression was also immunoprecipitated by an anti-SIRT2

antibody (Fig. 2B). We observed that endogenous SIRT2 was immunoprecipitated with high efficiency by the anti-SIRT2 antibody (Fig. 2B). This demonstrates that SIRT2 interacts with P/CAF in a physiological context. We attempted reciprocal detection of SIRT2 in the P/CAF immunoprecipitate; however, we did not detect SIRT2 in the P/CAF immunoprecipitate (Fig. 2B). This may be because of the low efficiency of immunoprecipitation by the anti-P/CAF antibody (Fig. 2B).

We analyzed the P/CAF acetylation level in a physiological context. When the whole cell lysates were immunoprecipitated with anti-P/CAF antibody, P/CAF acetylation was not detected in immunoblotting analysis (data not shown). However, the reciprocal approach was successful (Fig. 2C). As shown in Figure 2C, SIRT2 suppression increased the level of P/CAF acetylation. Furthermore, we observed that the level of P/CAF acetylation was not increased by treatment with AGK2, a potent and selective SIRT2 inhibitor (Fig. 2D). This result is consistent with the observation that the interaction of SIRT2 but not its deacetylase activity reduced acetylation of P/CAF (Fig. 2A). Additionally, the levels of P/CAF, MDM2, p53, and p21 were not changed by treatment with AGK2 (Fig. 2D). These results not only support that the increased level of P/CAF by SIRT2 suppression leads to a decrease in MDM2 followed by increases in p53 and p21, but also indicates that the increased level of P/CAF acetylation leads to P/CAF accumulation in HCT116(p53+/+) cells with SIRT2 suppression.

We examined whether SIRT2 reduces the protein stability of P/CAF as a mechanism to explain how SIRT2 suppression causes P/CAF accumulation, although the effect of the interaction between SIRT1 on P/CAF on the protein stability of P/CAF is unknown [11]. We analyzed the protein stability of endogenous P/CAF in cycloheximide-treated HCT116(p53+/+) cells with SIRT2 suppression. As shown in

Figure 3A, P/CAF stability was increased in HCT116(p53+/+) cells with SIRT2 suppression compared to in control cells. Real-time RT-PCR analysis did not reveal significant changes in P/CAF mRNA levels between these cells (Fig. 3B). These results suggest that SIRT2 reduces the protein stability of P/CAF. The complex formation of SIRT2 and P/CAF followed by reduced acetylation of P/CAF may be a mechanism to reduce the protein stability of P/CAF.

3.3 P/CAF-MDM2-p53-p21 axis confers resistance to mitotic cell death caused by SAC activation

Our previous results suggest that both p53-dependent and p53-independent pathways conferring resistance to nocodazole exist in cells with SIRT2 suppression [3]. The results obtained in the present study prompted us to examine whether the p53-dependent pathway is mediated by the P/CAF-MDMs-p53-p21 axis.

To test this possibility, we performed clonogenic cell survival assays after nocodazole treatment (0–60 h) for HCT116(p53+/+) and HCT116(p53-/-) cells with siRNA-mediated knockdown of P/CAF or p21 alone or in combination with SIRT2, or SIRT2 alone. We performed also cell cycle analyses after nocodazole treatment by assessing the DNA content in the cells.

Clonogenic cell survival assays showed that both in HCT116(p53+/+) and HCT116(p53-/-) cells, resistance to nocodazole was observed in cells with knockdown of SIRT2 alone, as compared to in control cells, particularly at 24 h of treatment (Fig. 4A). The patterns of cell cycle progression and accumulation from 2C to 4C were similar among all samples for up to 16 h of treatment with nocodazole (Fig. 4B). In our previous study, we confirmed that SAC activation as monitored by cyclin B1/Cdk1

activity and the MPM-2-positive population reached a peak at 16 h of treatment in both HCT116(p53+/+) cells with SIRT2 suppression and in control cells; additionally, cells with SIRT2 suppression showed prolonged mitotic arrest and SAC activation [3]. Although p21 is increased in HCT116(p53+/+) cells with SIRT2 suppression. (Fig. 1A), apparent G1 arrest or G2 arrest was not observed (Fig. 4A). Importantly, suppression of P/CAF or p21 significantly reduced the resistance to nocodazole in HCT116(p53+/+) cells with SIRT2 suppression, but not in HCT116(p53-/-) cells with SIRT2 suppression (Fig. 4A). This suggests that escape from mitotic cell death in HCT116(p53+/+) cells with SIRT2 suppression is mediated by P/CAF accumulation followed by activation of the p53-p21 pathway.

Cells with >4C content began to appear after 36 h and increased in number up to 48 h among control cells; however, the population with >4C DNA content was significantly lower in HCT116(p53+/+) cells with SIRT2 suppression than in control cells (Fig. 4A). These results are the same as our previously reported results [3]. Chromosome number analysis study showed that nearly all cell colonies in clonogenic cell survival assays after nocodazole treatment were derived from mitotic arrested cells that escaped from mitotic cell death and underwent a subsequent shift to the diploid G1 phase after removing nocodazole [3]. Thus, the population with >4C DNA in the presence of nocodazole is thought to reflect the population that underwent mitotic slippage followed by polyploidization at an early stage of mitotic arrest. Its delayed appearance is thought to reflect the increase in the population that escaped from mitotic cell death and finally underwent to mitotic slippage at the late stage in the presence of nocodazole, which is also observed for the case of Myc suppression leading to the inhibition of mitotic cell death [5].

Cell cycle analysis showed that suppression of P/CAF or p21 increased cell population with the >4C content of HCT116(p53+/+) cells with SIRT2 suppression (Fig. 4B). The degree of the increase was much lower in HCT116(p53-/-) cells than HCT116(p53+/+) cells (Fig. 4B). Thus, the results obtained by clonogenic cell survival assays and cell cycle analyses suggest that the P/CAF-MDM2-p53-p21 axis is responsible for escape from mitotic cell death in HCT116(p53+/+) cells with SIRT2 suppression.

Discussion

In the present study, we identified a novel pathway of p53 accumulation by SIRT2 suppression in HCT116(p53+/+) cells, in which SIRT2 suppression did not cause a significant down-regulation of p300, unlike HeLa cells (Fig. 1B). We found that SIRT2 interacts with P/CAF, a histone acetyltransferase that also acts as a ubiquitin ligase toward MDM2 (Fig. 2), and that SIRT2 suppression leads to P/CAF stabilization followed by degradation of MDM2, a ubiquitin ligase towards p53 (Fig. 1A and 3). This pathway may account for the previous observation by Matsushita et al. that the transcriptional activity of p53 is up-regulated in SIRT2-deficient chicken DT40 cells [14].

SIRT1 deacetylates p53, resulting in inhibition of p53 activity, and SIRT1 is a therapeutic target for cancer harboring wild-type p53. SIRT1 inactivation has been the most extensively studied anti-cancer drug among the SIRT family [15]. Our study showed that SIRT2 inactivation, as well as SIRT1 inactivation, may be a novel molecular target for cancer therapy to control p53 regulation.

However, the use of SIRT2 suppression for p53 activation in cancer therapy can be complicated, as our present study also suggests that SIRT2 suppression associated with activation of the p53-p21 pathway confers resistance to anti-cancer drug such as MTIs, at least in HCT116(p53+/+) cells with SIRT2 suppression. Consistent with this observation, paclitaxel and mitotic kinase inhibitors including Plk1 or Aurora kinase inhibitors cause an increase in p21 during M phase in surviving tumor cells, and enhanced p21 promotes the survival of treated cells [16].

Our results suggest that the p53-p21 pathway functions to protect cells from mitotic death caused by SAC activation. While it was reported that p21 is not an

effective inhibitor of cyclin B1/Cdk1 in vivo, some studies indicated a direct role of p21 in regulating cyclin B1/Cdk1 activity in mitosis [17]. We previously reported that cyclin B1/Cdk1 was not inhibited and cell cycle progression from 2C to 4C was not altered in HCT116(p53+/+) cells with SIRT2 suppression, despite enhanced p21 expression (Fig. 1A) [3]. This suggests that the induction of p21 protects cells with SIRT2 suppression from mitotic cell death by cyclinB1/Cdk1-independent mechanisms.

The divergent function of p21 including tumor suppressive and oncogenic functions depend on the amount, localization, modification, and cellular context [18]. Among the diverse functions of p21, several candidates have been reported to be involved in cell death by SAC activation. For example, the repression of Myc-dependent transcription by p21 is a candidate mechanism, as Myc promotes mitotic cell death by SAC activation by upregulating a cluster of redundant pro-apoptotic BH3-only proteins and suppressing pro-survival Bcl-xL [5]. Repression of caspase-2 by p21 is also a candidate mechanism, as caspase-2 has been reported to trigger mitotic catastrophe [19]. Revealing these mechanisms is important for targeting SIRT2 as a promising molecule to regulate p53 in cancer therapy.

The present study demonstrated that SIRT2 functions as a regulator of P/CAF stabilization in addition to p53. P/CAF plays a role in diverse biological processes, such as chromatin remodeling, transcriptional regulation, DNA repair, cell cycle progression, differentiation, and cell death [20]. The present study suggests that SIRT2 also functions in diverse biological processes through P/CAF and p53 regulation.

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Figure Legends

Fig. 1 SIRT2 suppression leads to increased P/CAF levels, decreased MDM2 levels, and activation of the p53-p21 pathway.

(A) HCT116(p53+/+) and HCT116(p53-/-) cells were transfected with siRNAs to the indicated genes or negative control siRNA, followed by culture for 16 h in the presence or absence of nocodazole. Changes in p53, p21, P/CAF, MDM2, and SIRT2 were determined by immunoblotting. (B) HCT116(p53+/+) ,HCT116(p53-/-), and HeLa cells were transfected with siRNAs to the indicated genes, followed by culture for 16 h in the presence or absence of nocodazole. Changes in p300, p53, p21, and SIRT2 were determined by immunoblotting. Note that HCT116 cells express a truncated form of p300.

Fig. 2 Physical interaction between SIRT2 and P/CAF, and reduced level of P/CAF acetylation by the interaction.

(A) 293T cells were transfected with a combination of expression plasmids as indicated. Whole cell lysates were immunoprecipitated by anti-FLAG antibody and then subjected to immunoblotting analysis using anti-SIRT2 antibody. The levels of FLAG-P/CAF acetylation in immunoprecipitates were examined by immunoblotting analysis using anti-acetylated lysine antibody. (B) Interaction of SIRT2 and P/CAF *in vivo*. HCT116(p53+/+) cells were transfected with siRNA to P/CAF and the negative control siRNA. Whole cell lysates were immunoprecipitated by anti-SIRT2 antibody and anti-P/CAF antibody, and then subjected to immunoblotting analysis using anti-P/CAF antibody. Detection of SIRT2 in immunoprecipitates obtained with anti-SIRT2 antibody is shown. While P/CAF was detected in the immunoprecipitate obtained with

anti-SIRT2 antibody, SIRT2 was not detected in the immunoprecipitate obtained with anti-P/CAF antibody, possibly because of the low efficiency of the precipitation. (C) Increased level of P/CAF acetylation in HCT116(p53+/+) cells with SIRT2 suppression. Whole cell lysates were immunoprecipitated by anti-acetylated lysine and then subjected to immunoblotting analysis using anti-P/CAF antibody and anti-acetylated lysine antibody. (D) Treatment with the SIRT2 inhibitor AGK2 does not increase P/CAF acetylation to activate the P/CAF-MDM2-p53-p21 axis. HCT116(p53+/+) cells were treated with AGk2 (10 μM), a selective SIRT2 inhibitor with an IC_{50} of 3.5 μM for 36 h [21].

Fig. 3 SIRT2 suppression stabilizes P/CAF

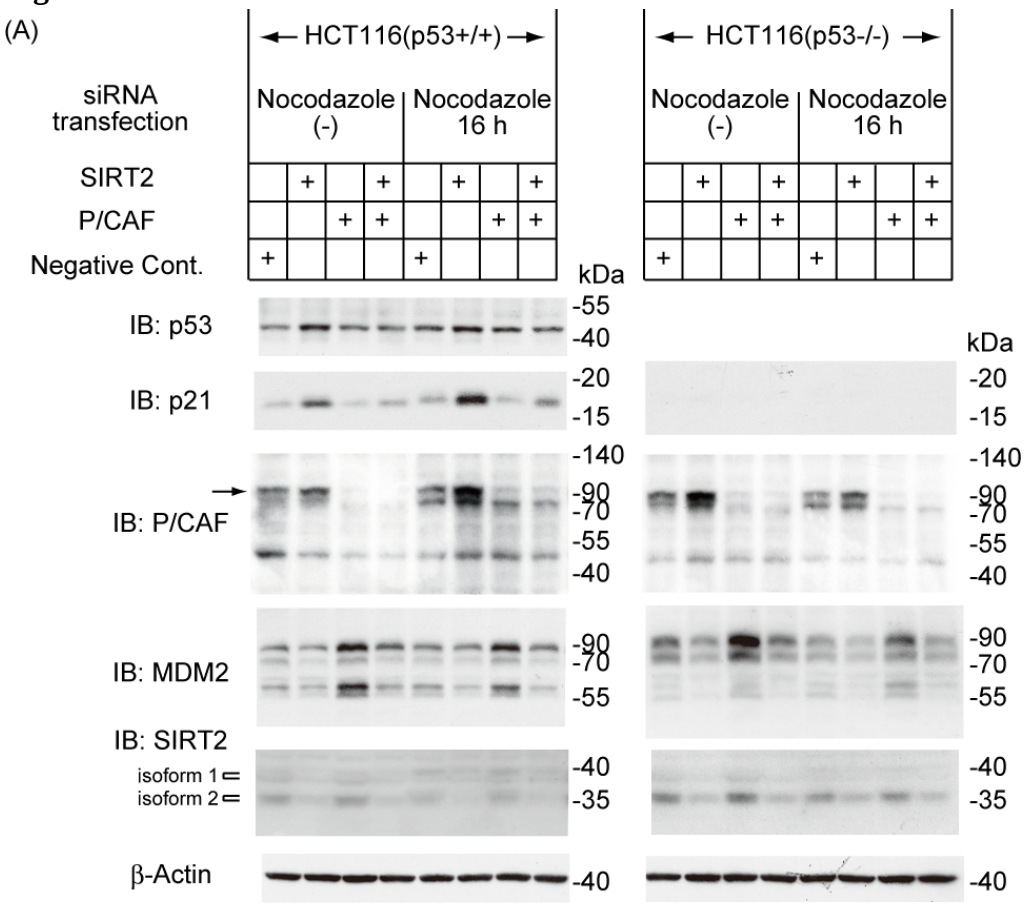
(A) Time course of P/CAF protein stability in HCT116(p53+/+) cells with SIRT2 suppression and control cells after treatment with 75 μg/mL cycloheximide. β-Actin was used as an internal control. Quantification of the immunoblotting was performed with Image J software (NIH). A similar result was obtained in an independent experiment (supplementary figure 1). (B) Real-time RT-PCR analysis of P/CAF mRNA in HCT116(p53+/+) cells with SIRT2 suppression and control cells. The expression of GAPDH was used as an internal control.

Fig. 4 Resistance to nocodazole and prolonged mitotic arrest in the presence of nocodazole in HCT116(p53+/+) cells with SIRT2 downregulation is abolished by knockdown of P/CAF or p21.

(A) Clonogenic cell survival assays for HCT116(p53+/+) and HCT116(p53-/-) cells were performed. Cells were transfected with siRNAs to the indicated genes. Cells were

incubated in the presence of nocodazole for the indicated times, incubated in drug-free medium for 8–10 days. Assays were performed in triplicate and error bars denote standard deviation. (B) Cell cycle analyses for cells transfected with siRNAs to the indicated genes. DNA content and the ratio of >4C population, which reflect mitotic slippage, were evaluated by flow cytometry analyses.

Figure 1


(A)
HCT116(p53+/+)
HCT116(p53-/-)
siRNA transfection
Nocodazole (-)
Nocodazole 16 h
SIRT2
P/CAF
Negative Cont.
kDa
IB: p53
IB: p21
IB: P/CAF
IB: MDM2
IB: SIRT2
isoform 1
isoform 2
β-Actin
-55
-40
-20
-15
-140
-90
-70
-55
-40
-90
-70
-55
-40
-35
-40

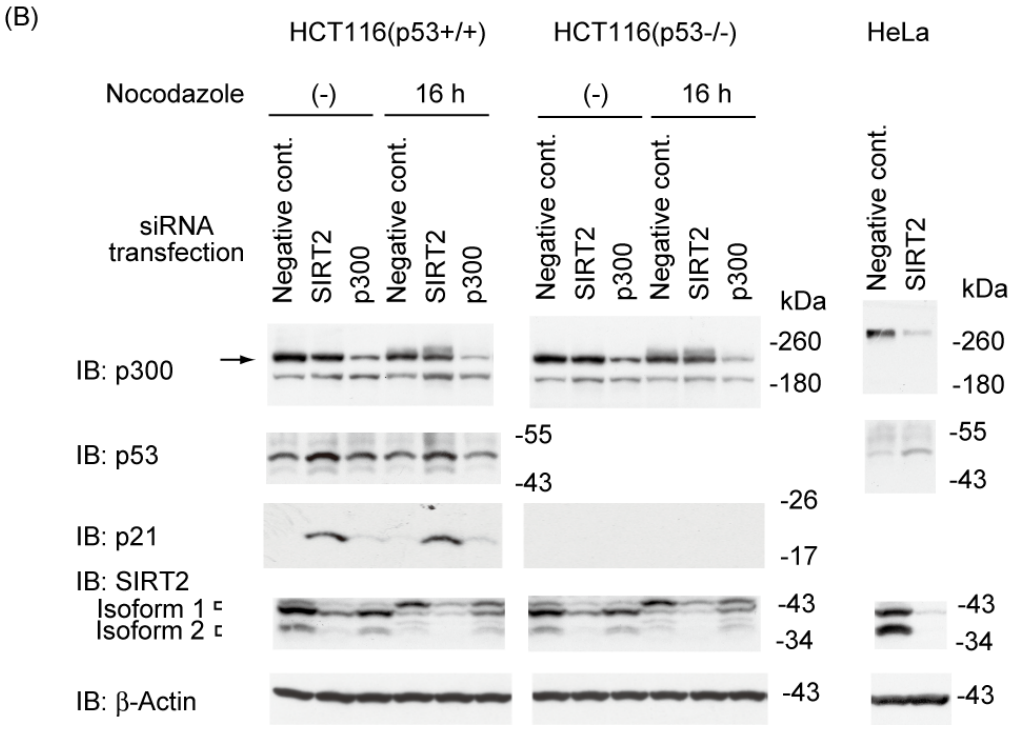

(B)
HCT116(p53+/+)
HCT116(p53-/-)
HeLa
Nocodazole
(-)
16 h
siRNA transfection
Negative cont.
SIRT2
p300
kDa
IB: p300
IB: p53
IB: p21
IB: SIRT2
Isoform 1
Isoform 2
IB: β-Actin
-260
-180
-55
-43
-26
-17
-43
-34
-43

Figure 2

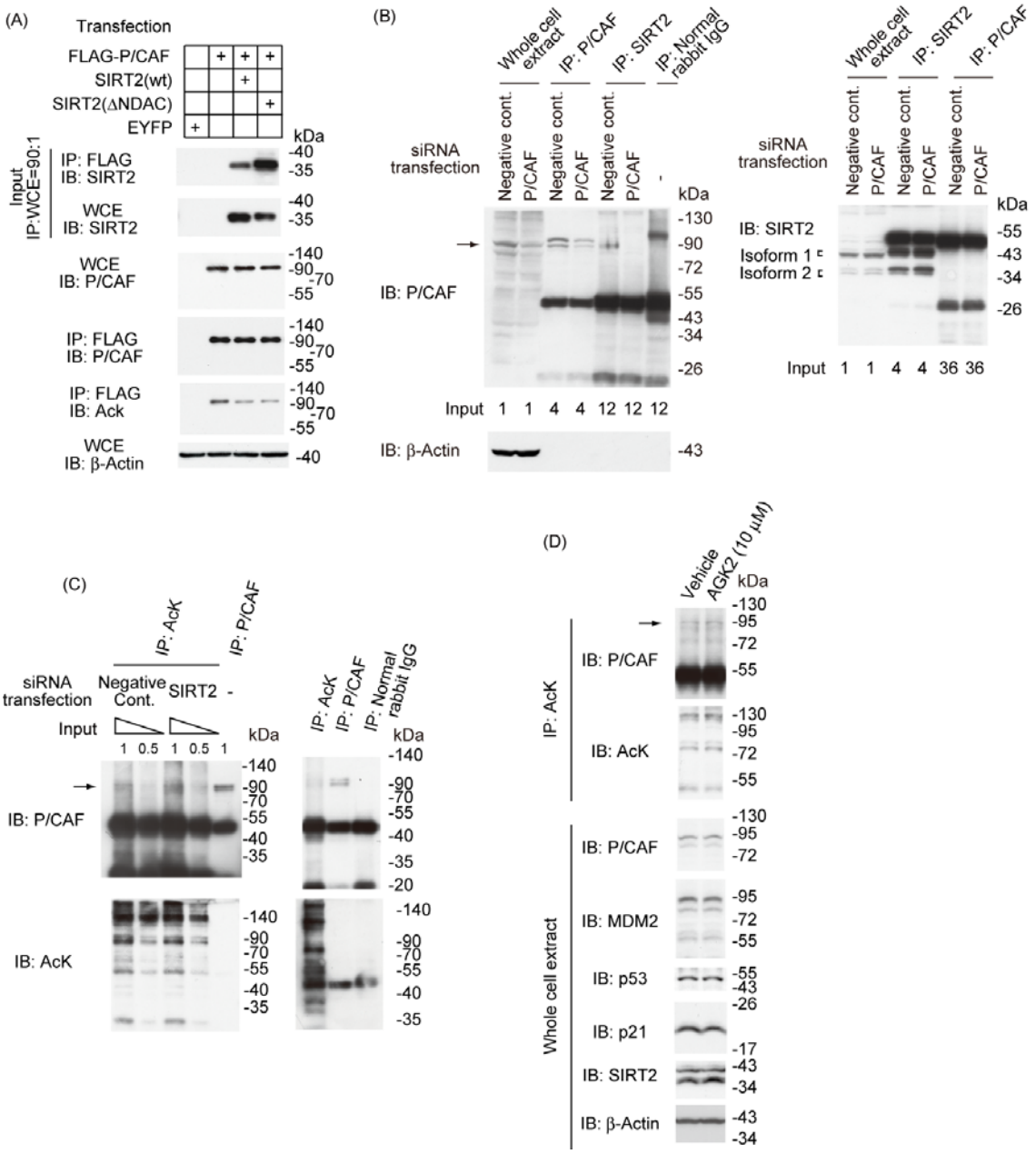
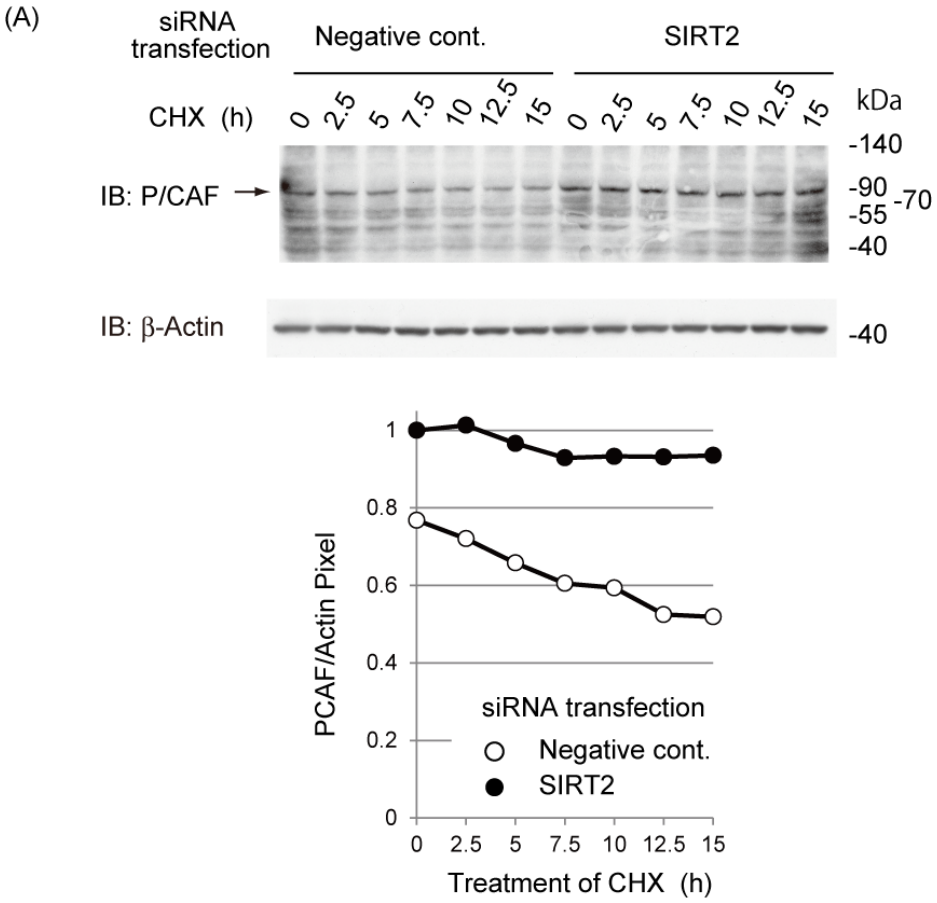

(A)
Transfection
FLAG-P/CAF
SIRT2(wt)
SIRT2(ΔNDAC)
EYFP
kDa
Input IP:WCE=90:1
IP: FLAG IB: SIRT2
WCE IB: SIRT2
WCE IB: P/CAF
IP: FLAG IB: P/CAF
IP: FLAG IB: Ack
WCE IB: β-Actin
(B)
Whole cell extract
IP: P/CAF
IP: SIRT2
IP: Normal rabbit IgG
siRNA transfection
Negative cont.
P/CAF
IB: P/CAF
Input 1 1 4 4 12 12 12
IB: β-Actin
IB: SIRT2
Isoform 1
Isoform 2
Input 1 1 4 4 36 36
(C)
IP: AcK
IP: P/CAF
siRNA transfection
Negative Cont.
SIRT2
Input
1 0.5 1 0.5 1
IB: P/CAF
IB: AcK
IP: Normal rabbit IgG
(D)
Vehicle
AGK2 (10 μM)
IP: AcK
IB: P/CAF
IB: AcK
Whole cell extract
IB: MDM2
IB: p53
IB: p21
IB: SIRT2
IB: β-Actin

Figure 3


(A)
siRNA transfection
Negative cont.
SIRT2
CHX (h)
0 2.5 5 7.5 10 12.5 15 0 2.5 5 7.5 10 12.5 15
kDa
-140
-90
-70
-55
-40
IB: P/CAF
IB: β-Actin
-40
PCAF/Actin Pixel
1
0.8
0.6
0.4
0.2
0
siRNA transfection
Negative cont.
SIRT2
0 2.5 5 7.5 10 12.5 15
Treatment of CHX (h)

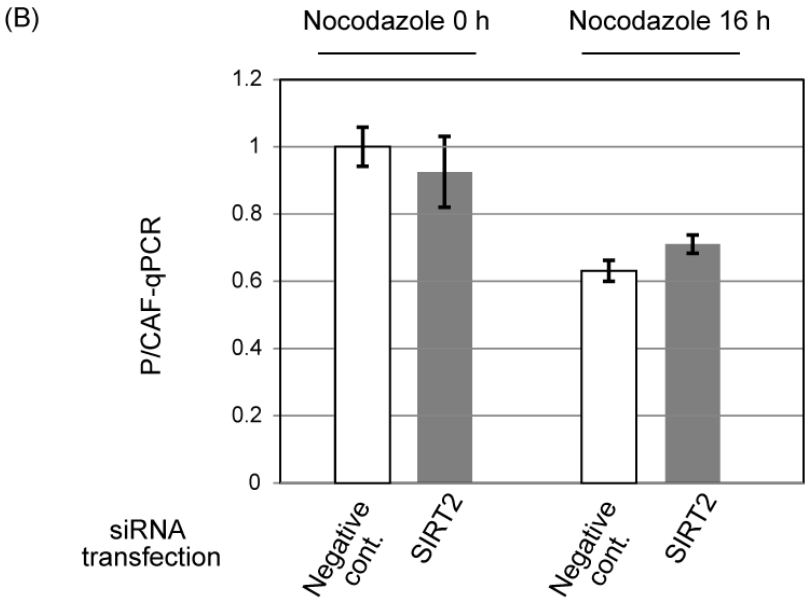
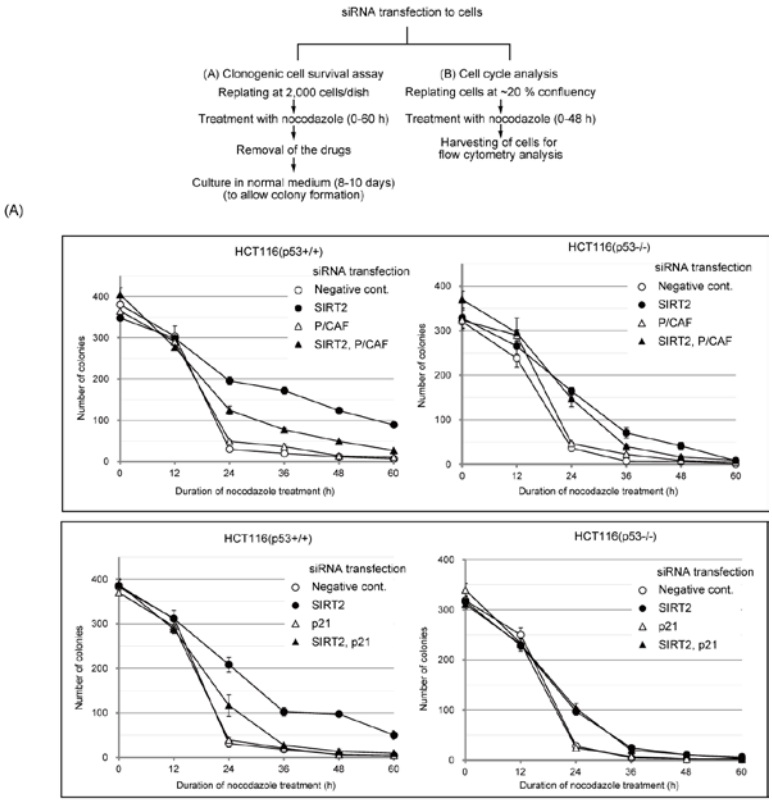
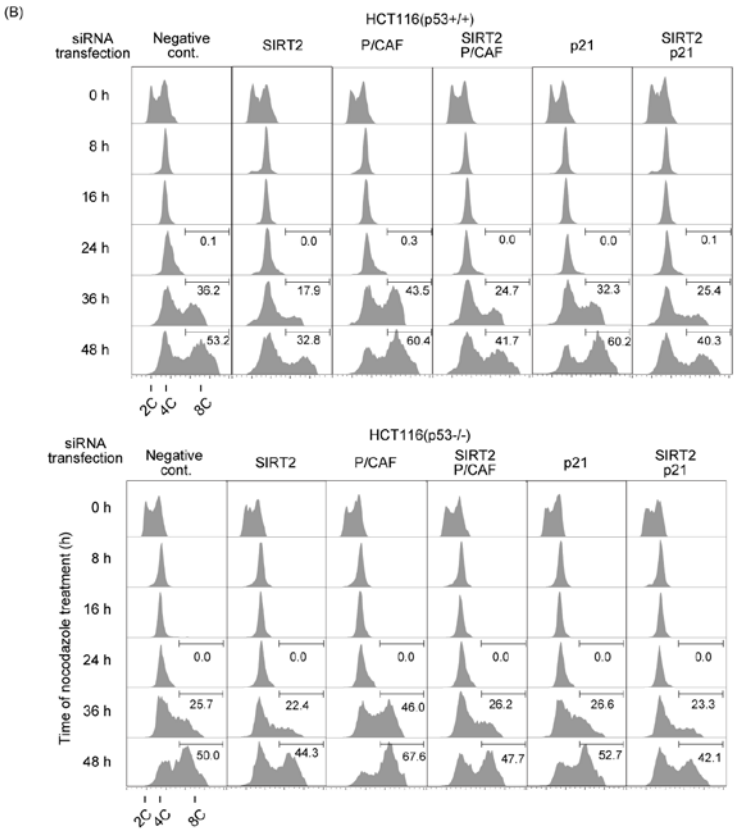

(B)
Nocodazole 0 h
Nocodazole 16 h
P/CAF-qPCR
1.2
1
0.8
0.6
0.4
0.2
0
siRNA transfection
Negative cont.
SIRT2
Negative cont.
SIRT2

Figure 4

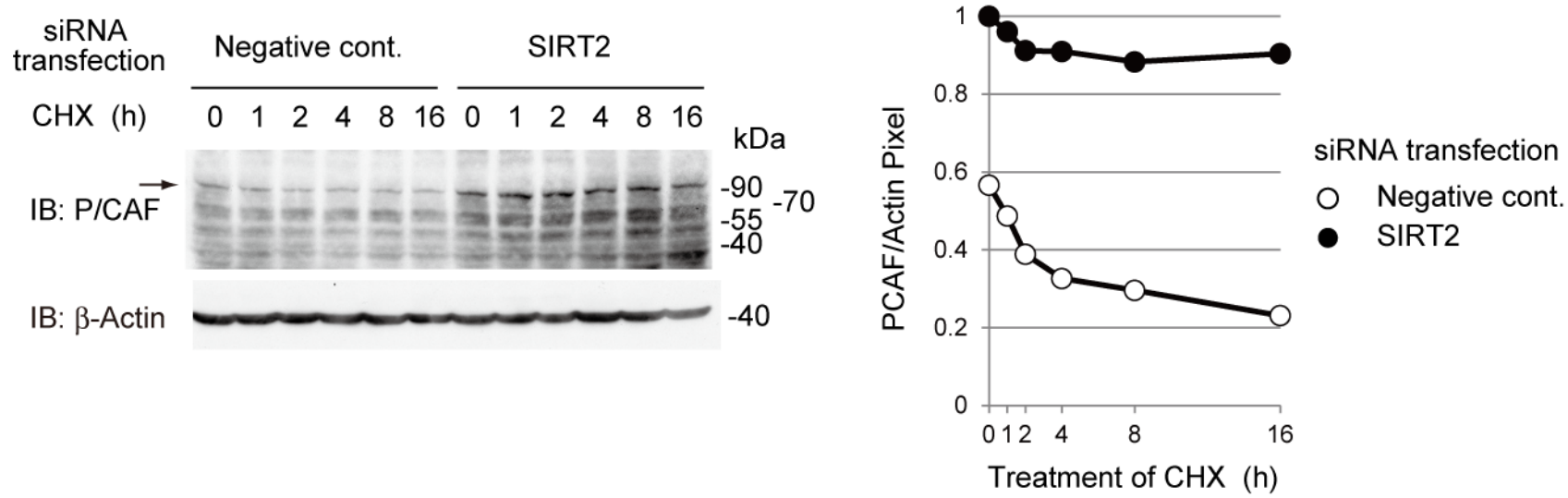

siRNA transfection to cells
(A) Clonogenic cell survival assay
Replating at 2,000 cells/dish
Treatment with nocodazole (0-60 h)
Removal of the drugs
Culture in normal medium (8-10 days)
(to allow colony formation)
(B) Cell cycle analysis
Replating cells at ~20 % confluency
Treatment with nocodazole (0-48 h)
Harvesting of cells for
flow cytometry analysis
(A)
HCT116(p53+/+)
HCT116(p53-/-)
siRNA transfection
Negative cont.
SIRT2
P/CAF
SIRT2, P/CAF
p21
SIRT2, p21
Number of colonies
Duration of nocodazole treatment (h)


(B)
HCT116(p53+/+)
HCT116(p53-/-)
siRNA transfection
Negative cont.
SIRT2
P/CAF
SIRT2 P/CAF
p21
SIRT2 p21
Time of nocodazole treatment (h)
0 h
8 h
16 h
24 h
36 h
48 h
2C
4C
8C

Supplementary table 1: List of antibodies that were used for immunoblotting analyses

Antibody	Host species	Company	Dilution
p53	rabbit	Santa Cruz Biotechnology sc-6243	1:1000, Immunoreaction Enhancer Solution (Toyobo)
MDM2	mouse	Calbiochem OP46	1:350, 3 % skim milk/TBS-T(0.05 %)
P/CAF	rabbit	Cell Signaling Technology #3378	1:2000, Immunoreaction Enhancer Solution
p21	rabbit	Cell Signaling Technology #2947	1:2000, 1 % bovine serum albumin/TBS-T(0.05 %)
p300	rabbit	Cell Signaling Technology #70088	1:2000, Immunoreaction Enhancer Solution
SIRT2	rabbit	Sigma HPA011165	1:1000, Immunoreaction Enhancer Solution
SIRT2	rabbit	Abcam EPR20411-105	1:1000, Immunoreaction Enhancer Solution
acetylated lysine	rabbit	Cell Signaling Technology #9441	1:1000, 1 % bovine serum albumin/TBS-T(0.05 %)
β-actin	mouse	Sigma A5441	1:8000, 1 % bovine serum albumin/TBS-T(0.05 %)
Rabbit IgG HRP-linked	Donkey	GE Healthcare NA934	1:4000
Mouse IgG HRP-linked	Sheep	GE Healthcare NA931	1:4000

Supplementary figure 1



SIRT2 suppression stabilizes P/CAF. Time course of P/CAF protein stability in HCT116(p53+/+) cells with SIRT2 suppression and control cells after treatment with 75 μg/mL cycloheximide. β-Actin was used as an internal control. Quantification of the immunoblotting was performed with Image J software (NIH). This experiment was performed in an independent manner from the experiment shown in figure 3A.