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SIRT2 mediated downregulation of FOXM1 in response to TGFβ through the RAF-MEK-ERK signaling pathway in colon cancer

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Abstract: The transcription factor forkhead box M1 (FOXM1) is frequently upregulated in many solid tumors, including those in the colon. As a master regulator, the sirtuin (SIRT) protein family is comprised of seven nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases/adenosine diphosphate (ADP) ribosyl transferases whose activities are associated with aging and cancer. In this study, we determined whether a cytoplasmic member of SIRTs, SIRT2, influences the expression of oncogenic FOXM1 in colon cancer *in vitro*. The association of SIRT2 and FOXM1 were analyzed using SIRT2 knockout mouse embryonic fibroblasts and SIRT2 knocked-down and overexpressing HCT116 colon cancer cell lines. Cell lines were treated with 10 ng/mL transforming growth factor-beta (TGF®) for 24 h. SIRT2 could downregulate FOXM1 through the TGFβ mitogen-activated protein kinase (RAF-MEK-ERK) signaling pathway in genetically altered mouse embryonic fibroblasts and colon cancer cell lines. The indirect association between SIRT2 and FOXM1 through TGFβ may be important because activators or inhibitors of SIRT2 could provide a potential approach to downregulate FOXM1 in gastrointestinal cancers.

Keywords: deacetylation; colon cancer; FOXM1; SIRT2; posttranslational

INTRODUCTION

Transcription factor forkhead box protein M1 (FOXM1), one of the members of the forkhead family of proteins, plays a role in the progression of the cell cycle and therefore in the regulation of cell division rate [1,2]. FOXM1 performs this role by binding to promoters of the target genes that are involved predominantly in cell division in actively proliferating cells, including in the small intestine, colon, thymus, testis and ovarian tissues [3,4]. An abnormal increase in FOXM1 expression is present in tumors in many organs, such as the breast [5,6], ovary [7], lung [8], liver [9] stomach [10], pancreas [11] and colon [12,13]. Excessive protein expression of FOXM1 has been associated with a poor clinical prognosis and therefore it is considered a "proto-oncogenic" transcription factor and a tumor marker [14]. In addition to continuous proliferation, oncogenic FOXM1 can trigger angiogenesis, invasion, metastasis, genomic instability and resistance of cancer cells to apoptosis [7,10,15]. Due to the oncogenic roles of FOXM1 in human cancer, several inhibitors that block the activity and expression of FOXM1 are currently being investigated to reduce cancer growth [16,17].

FOXM1 transcriptional activity can be regulated through its posttranslational modifications, including phosphorylation, acetylation, SUMOylation, methylation and ubiquitination [18]. The transcriptional activity of FOXM1 is repressed by ubiquitination while phosphorylation of FOXM1 through the mitogen-activated protein kinase (RAF/MEK/MAPK) pathway enhances its nuclear translocation and thereby its transcriptional activity during G2/M phases [19].

Continuous and uncontrolled cell proliferation is one of the most important characteristics of

colon cancer and many other types of cancer [20]. Transforming growth factor-beta (TGF-β) signaling can function as both a tumor suppressor in normal cells and a tumor promoter in advanced stages of cancer, depending on the cellular context. TGFβ ligand can activate canonical SMAD arm and noncanonical signaling pathways. The TGFβ RAS/MAPK pathway regulates important cellular events including the rate of proliferation, differentiation, survival, angiogenesis, loss of apoptotic response and migration [21].

Sirtuin 2 (SIRT2), which is one of seven sirtuin protein family members, has been shown to regulate the MEK-ERK signaling cascade, and loss of SIRT2 could result in drug resistance in colon cancer chemotherapy [22]. SIRT2 resides predominantly in the cytoplasm and unlike traditional class I and III (histone deacetylases) (HDACs), its substrates are not restricted to the histones [23]. Removal of the SIRT2 gene in mice had been associated with the development of tumors in several organs, and the antitumor activities of SIRT2 are associated with anaphase-promoting complex APC/C activity [24,25]. On the other hand, SIRT2-specific inhibitors may exhibit anticancer activity as well, and like the TGFβ signaling pathway, oncogenic or tumor-suppressor roles of SIRT2 have been reported to be context-dependent [26,27].

In this study, SIRT2 overexpressing and SIRT2 knockdown colon cancer cells and mouse embryonic fibroblasts were used to investigate the influence of SIRT2 on the expression and intracellular localization of FOXM1.

MATERIALS AND METHODS

Cancer cell culture and generation of genetically altered cell lines

The HCT116 colorectal carcinoma (CRC) cell line was maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic agent in a 37°C incubator. Mouse embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 15% FBS, 1% antibiotic-antimycotic agent and 1% non-essential amino acid solution in a 37°C incubator as described [27]. Genetically altered stable cell lines were constructed by infecting cells with lentivirus expressing SIRT2 for overexpressing SIRT2 or shSIRT2 (short hairpin SIRT2) for knocking down SIRT2 as described previously [27].

Immunoblotting

Cells were lysed and quantified by the bicinchoninic acid (BCA) assay. Forty micrograms (μg) of each homogenate were loaded into each well and proteins were resolved by 10% tris-glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (TG SDS-PAGE). Immunoblotting on polyvinylidene (PVDF) membranes was performed using the Transblot Turbo transfer system (BioRAD, CA, USA) as described [28,29]. Membranes were incubated with anti-FOXM1 (Santa Cruz Biotechnology, USA), anti-SIRT2 and anti-Flag antibody (Sigma, USA), anti-actin, anti-GAPDH, anti-tubulin and anti-P-ERK1/2 (all fom Cell Signaling, MA, USA) primary antibodies for 16 h at 4°C. Membranes were washed three times with PBS-T for 15 min each, followed by horseradish peroxidase (HRP)-conjugated secondary antibody treatment for 30 min.

Colony formation assay

Stable inactive non-silencing (shNS) and SIRT2 overexpressing HCT116 cells were plated at low cell densities (100 cells per well onto a 6-well plate). After two weeks, each well was stained with crystal violet stain, and the number of colonies for each group was determined as described [29].

Immunofluorescence

ShNS control and SIRT2 knockdown HCT116 cells (1×10^4 cells/slide) were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. Cells were incubated at 4°C with antibodies to mouse anti-FOXM1 (Santa Cruz Biotechnology, USA). After washing the cells three times in PBS, the sections were incubated with the Alexa Fluor® 594 goat anti-mouse IgG (H+L) secondary antibody (Thermo Fisher Scientific, MA, USA) for 1 h, and mounted in Vectashield mounting medium

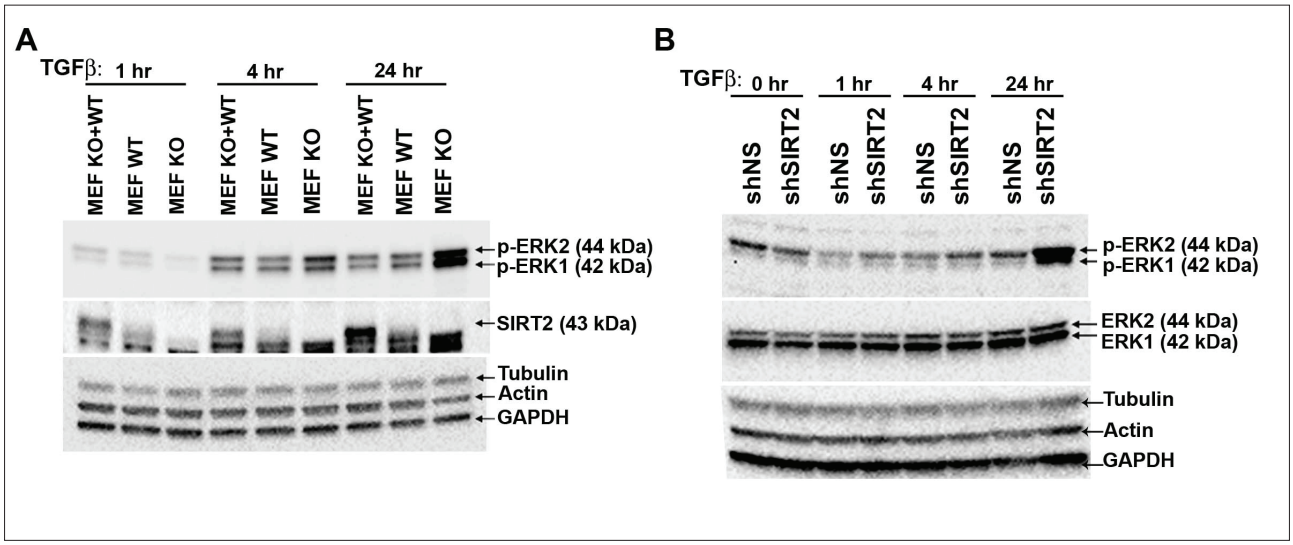

Fig. 1. Removal of SIRT2 increased the phosphorylation of ERK1/2 in response to TGFβ. SIRT2$^{+/+}$, SIRT2$^{-/-}$ and SIRT2 overexpressing SIRT2$^{-/-}$ MEFs (**A**) and non-specific knockdown control (shNS) and SIRT2 knockdown (shSIRT2) HCT116 colorectal carcinoma cell lines (**B**) were exposed to 10 ng/mL TGFβ for 1 h, 4 h and 24 h. Cells were separated and immunoblotted with anti-ERK1/2, p-ERK1/2, anti-SIRT2, including anti-actin/tubulin/GAPDH as a load control. All experiments were performed in triplicate.

with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA). Images were obtained with a fluorescence microscope (Olympus, FV1000).

Statistical analysis

For the comparison of three groups, one-way ANOVA with Tukey post hoc analyses were performed via GraphPad Prism software (CA, USA).

RESULTS

Removal of SIRT2 increased the phosphorylation of ERK1/2 in response to TGFβ

It was previously reported that SIRT2 influences the TGFβ-MEK-ERK signaling pathway [22]. We tested if TGFβ exposure changes the activity of mitogen-activated protein kinase (MEK) enzyme, and accordingly alters the phosphorylation of ERK1/2 among SIRT2$^{+/+}$, SIRT2$^{-/-}$ MEFs, and SIRT2 overexpressing MEFs. Removal of SIRT2 increased the phosphorylation of ERK1/2 in response to 10 ng/mL TGFβ for 4 h and strongly for 24 h (Fig. 1A). Then, TGFβ treatment using a stably SIRT2 knocked-down and overexpressing CRC cell line, HCT116 was performed. As with MEFs, SIRT2 knockdown in HCT116 colon cancer cells increased the phosphorylation of ERK1/2 in response to 10 ng/mL TGFβ for 4 h and strongly for 24 h (Fig. 1B).

Removal of SIRT2 increased the expression of FOXM1 protein

Next, the role of SIRT2 on the expression of FOXM1 in response to TGFβ using SIRT2$^{+/+}$ and SIRT2$^{-/-}$ MEFs was investigated. Exposure to TGFβ enhanced the expression of FOXM1, especially at 4 h in SIRT2$^{+/+}$ MEFs. An increase in FOXM1 expression in SIRT2$^{-/-}$ was apparent as early as 1 h after TGFβ exposure and peaked at 4 h and was stronger than in SIRT2$^{+/+}$ MEF at the same time point (Fig. 2A). SIRT2 knocked-down and overexpressing HCT116 cells were treated with TGFβ. SIRT2 overexpressing cells displayed reduced expression of FOXM1 when TGFβ was absent or applied for 1 h. When these cells were treated with TGFβ for 4 h, FOXM1 expression increased and there was no difference among the knockdown control, SIRT2 knockdown and SIRT2 overexpressing cells (Fig. 2B).

SIRT2 overexpression decreased the number of colony formations and proliferations

To test if SIRT2 overexpression has some antitumorigenic activities, stable HCT116 cells overexpressing

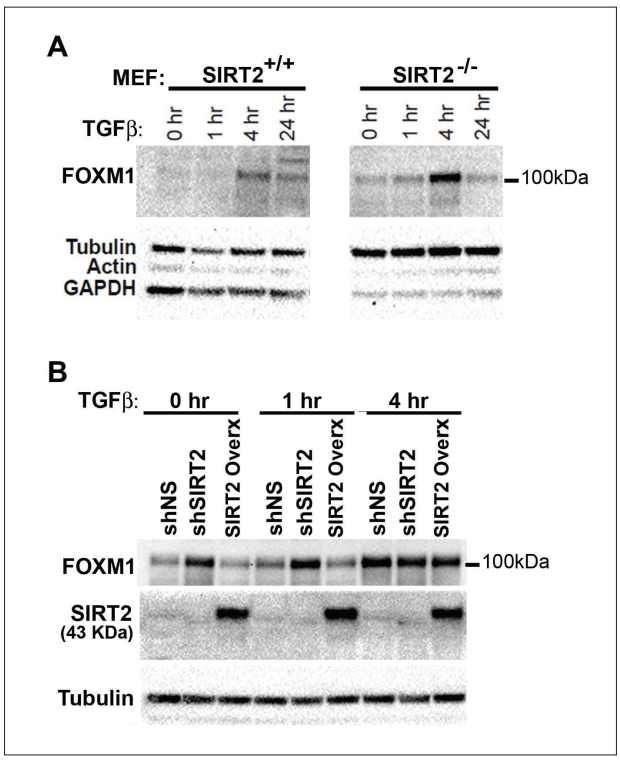

Fig. 2. SIRT2 decreases the expression of FOXM1 through the TGFβ signaling pathway. SIRT2$^{+/+}$ and SIRT2$^{-/-}$ MEFs (**A**) and non-specific knockdown control (shNS), SIRT2 knockdown (shSIRT2) and SIRT2 overexpressing HCT116 cell lines (**B**) were exposed to 10 ng/mL TGFβ for 1 h, 4 h and 24 h. Cells were separated and subsequently immunoblotted with anti-FOXM1, anti-SIRT2, and anti-actin/tubulin/GAPDH antibodies for loading controls. All experiments were performed in triplicate.

SIRT2 were used. SIRT2 overexpression significantly decreased the number of colonies relative to shNS (Fig. 3A). Additionally, overexpression of SIRT2 lengthened the doubling time to 22.37 h, which was about 4.5 h longer than the control cells.

Next, the subcellular localization of FOXM1 in control and SIRT2 knocked-down HCT116 cells was investigated. In both types of cells, FOXM1 staining was both in the cytoplasm and nucleus. FOXM1 staining was punctate in the cytoplasm, while it was diffuse in the nucleus (Fig. 3B).

DISCUSSION

FOXM1 belongs to the forkhead superfamily of transcription factors and has crucial roles in cell cycle progression and proliferation. The transcriptional activity of FOXM1 can be regulated through various post-translational modifications, including phosphorylation and acetylation [19,30]. While the transcriptional activity of FOXM1 is repressed by ubiquitination during the G1 and early S phases [31], phosphorylation of FOXM1 by cyclin-CDK complexes stimulates its transcriptional activity [32,33]. Phosphorylation of FOXM1 recruits the p300/CREB histone acetyltransferases and increases its acetylation. p300/CBP transcriptional adaptors acetylate FOXM1 at lysines K63, K422, K440, K603 and K614, and increase FOXM1 transcriptional activity by increasing its DNA binding affinity, protein stability and phosphorylation sensitivity [30]. Additionally, phosphorylation of FOXM1 on S331 and S704 via the RAF-MEK-ERK signaling pathway enhances nuclear translocation of FOXM1 and thus its transcriptional activity [19]. Mutated *KRAS* gene can activate the downstream MEK/ERK (MAPK-extracellular signal-regulated kinase) pathway. MEK inhibitors are in clinical trials for the treatment of targeted KRAS-dependent CRC. Inhibition of MEK along with CDK4/6 synergistically downregulates FOXM1 in KRAS-dependent CRC [34]. In other studies, a specific MEK1/2 inhibitor was observed to decrease the translocation of FOXM1 into the nucleus [19]. Additionally, when the dominant-negative form of MEK1 is co-expressed with FOXM1 and the cyclin B1 reporter in human fibroblasts, it decreases the transactivating activity of FOXM1 [22,35]. It was proposed that the deacetylation of MEK by SIRT1 and SIRT2 decreases its oncogenic potential *in vitro* [36]. Overexpression of SIRT1 in a breast cancer cell line decreased FOXM1 levels and cellular invasion. Moreover, knockout mouse embryonic fibroblasts (MEFs) enhanced ERK signaling activation, leading to a decrease in FOXM1 level and activity compared to control cells [6]. Herein, overexpression of SIRT2 in a gastrointestinal cancer cell line decreased the expression of FOXM1 in response to TGFβ, possibly through inhibition of the MEK/ERK pathway.

SIRTs belong to class III histone deacetylases (HDACs) and are phylogenetically conserved from bacteria to humans. Seven sirtuins are identified in mammals and are considered master regulators. All sirtuins except SIRT4 have been reported to have deacetylase activity. SIRTs have a wide range of physiological functions in aging, longevity, regulation of metabolism and gene silencing, to name a few.

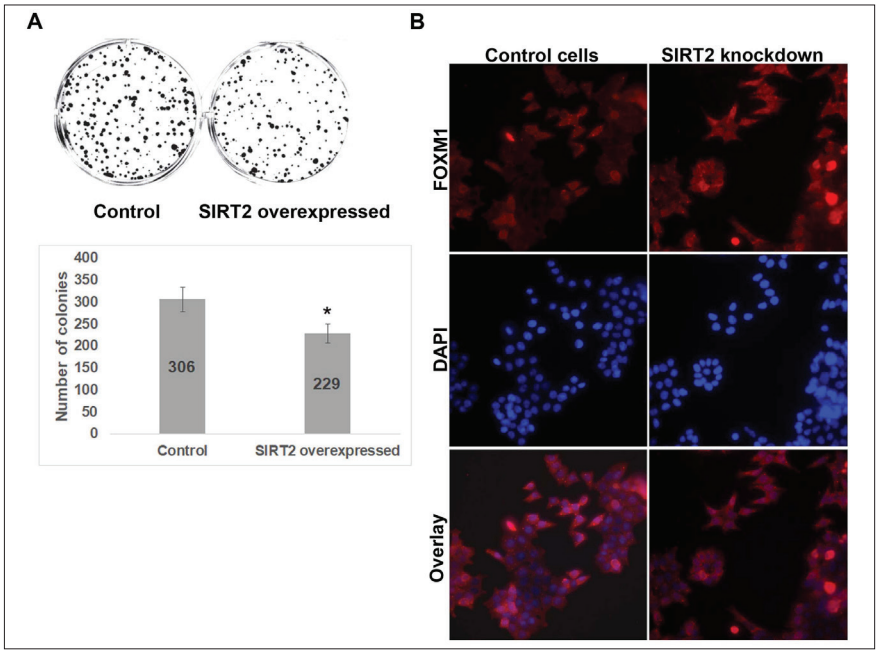

Fig. 3. SIRT2 overexpression decreases the number of colony formations. HCT116 and SIRT2 overexpressing HCT116 cells were trypsinized and 100 cells were seeded into a 6-well plate and incubated for 14 days Representative images are shown (upper panel in **A**). After incubation, colonies were stained with crystal violet and counted using ImageJ software. Experiments were repeated three times and data expressed as mean +/- standard deviation (lower panel in **A**). FOXM1 proteins were stained by anti-FOXM1 antibody in control and SIRT2 knocked-down HCT116 cells and subsequently visualized by fluorescence microscopy (**B**). All experiments were performed in triplicate. Representative images are shown.

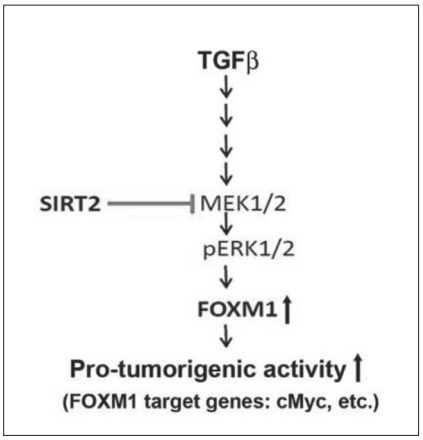

Fig. 4. Schematics of association between SIRT2 and FOXM1 proteins. SIRT2 inhibits the TGFβ MEK-ERK signaling pathway and accordingly decreases the expression of FOXM1. TGFβ triggers the MEK-ERK signaling pathway and increases FOXM1 protein levels. SIRT2 downregulates FOXM1 expression, at least partly, through the suppression of ERK1/2 phosphorylation. In agreement with this, the removal or decrease in the expression of SIRT2 liberates TGFβ MEK-ERK signaling, upregulating protein levels of FOXM1.

Therefore, dysregulation of the activity and/or expression of SIRTs are associated with age-related diseases, including tumorigenesis [37]. SIRT2 mainly resides in the cytoplasm but it can translocate into the nucleus during the G2/M cell cycle transition to control cell cycle progression [38]. SIRT2 has been reported to deacetylate numerous substrates, including alpha-tubulin [38], p300 [39] and FoxO1 family members [40]. It is one of the key regulators of normal homeostasis of cells, including genome integrity and mitosis regulation. The roles of SIRT2 in cancer are largely unknown, complex and context-dependent [41]. In our study, forced expression of SIRT2 significantly decreased both the number of colony formations and the doubling time in the HCT116 cell line. SIRT2 displayed an antiproliferative effect in HCT116 cells, at least partly, by decreasing the expression of FOXM1.

FOXM1 overexpression is frequently observed in numerous tumors in humans [1]. The inhibition of FOXM1 in most cancer models through small molecule inhibitors or RNA interference significantly diminishes cancer progression and is currently being investigated [16,18,42]. For example, honokiol, a natural biphenolic compound isolated from the plant *Magnolia officinalis*, has been reported to downregulate both gene and protein expression of FOXM1 [16]. Several small molecules have been identified as capable of both activating and inhibiting sirtuin expression. Honokiol has also been reported to activate SIRTs, specifically SIRT3. Herein it was found that TGFβ increased the phosphorylation of ERK1/2, and that the presence of SIRT2 decreased the ERK phosphorylation status for at least 24 h. In parallel, SIRT2 keeps the level of FOXM1 from upregulation in response to TGFβ as early as 1 h in both non-cancerous normal and HCT116 cancer cell lines (Fig. 4). When SIRT2 is removed or reduced in CRC cell lines, FOXM1 levels are markedly higher in response to at least 1 h of TGFβ exposure. TGFβ signaling is commonly reported to maintain cell homeostasis and suppress tumor progression in normal cells; however, it can be altered in advanced stages of cancer and exert

tumor-promoting effects. In our study, prolonged treatment with TGFβ for longer than 3 h in HCT116 cancer cells increased the expression of FOXM1, even when SIRT2 was overexpressed. This result suggests that the effect of SIRT2 on FOXM1 expression upon exposure of TGFβ for longer than 3 h in cancer cells was limited, and possibly other crosstalk mechanisms, for example between FOXM1 and TGF-β/SMAD signaling-dependent pathways, become more dominant.

FOXM1 displays punctate staining in the cytoplasm that could be the result of its interaction with microtubules. FOXM1 is associated with the polymerization of microtubules [24]. Immunofluorescent FOXM1 staining in the shNS control and SIRT2 knocked-down HCT116 cell lines was performed, and we observed that posttranslational modification directed by SIRT2 did not change the subcellular localization of FOXM1 or its subcellular staining pattern. It seems that the downregulation of FOXM1 in the presence of SIRT2 is linked to FOXM1 stability or mRNA expression. These results suggest that SIRT2 prevents oncogenic FOXM1 from upregulation, and this indirect regulation of SIRT2 on FOXM1 may be lost after the early phases of cancer formation because of either a decrease in SIRT2 protein levels and/or overactivation of the TGFβ ERK-MEK signaling pathway.

CONCLUSIONS

Dysregulation of FOXM1 is associated with tumorigenesis and cancer development. Understanding the regulation and interacting partners of FOXM1 is crucial because this information provides new insights into the roles of this oncogenic protein in cancer. The sirtuin family members (SIRT1-7) are crucial regulators whose activities can delay age-associated disorders, including cancer. SIRT2 has been reported to be involved in cell cycle regulation, genome stability and cell death. In the present study, an antitumorigenic role of SIRT2 through downregulation of FOXM1 and inhibition of the TGFβ MEK/ERK pathway is reported. SIRT2 activating small molecules may be used to downregulate FOXM1 to diminish the proliferative potential of cancer cells. Further in-depth *in vivo* animal and clinical studies on SIRT2 and FOXM1 association are needed to develop novel potential therapeutic strategies against cancer development.

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