Improper distribution of chromosomes during mitosis can contribute to malignant transformation. Higher eukaryotes have evolved a mitotic catastrophe mechanism for eliminating mitosis-incompetent cells; however, the signaling cascade and its epigenetic regulation are poorly understood. Our analyses of human cancerous tissue revealed that the NAD-dependent deacetylase SIRT2 is up-regulated in early-stage carcinomas of various organs. Mass spectrometry analysis revealed that SIRT2 interacts with and deacetylates the structural maintenance of chromosomes protein 1 (SMC1A), which then promotes SMC1A phosphorylation to properly drive mitosis. We have further demonstrated that inhibition of SIRT2 activity or continuously increasing SMC1A-K579 acetylation causes abnormal chromosome segregation, which, in turn, induces mitotic catastrophe in cancer cells and enhances their vulnerability to chemotherapeutic agents. These findings suggest that regulation of the SIRT2-SCMC1A axis through deacetylation-phosphorylation permits escape from mitotic catastrophe, thus allowing early precursor lesions to overcome oncogenic stress.

INTRODUCTION

Mitotic catastrophe is a strategy for eliminating mitosis-incompetent cells in higher eukaryotes and is driven by a complex and poorly understood signaling cascade. Functionally, mitotic catastrophe can be defined as an onco-suppressive mechanism. Accordingly, the disruption of mitotic catastrophe accelerates tumorigenesis and cancer progression. Conversely, its stimulation appears to be a promising strategy in cancer treatment (I–3). However, the molecular mechanisms underlying mitotic catastrophe remain largely unknown.

Mammalian sirtuins constitute a gene family of seven (SIRT1 to SIRT7) nicotinamide adenine dinucleotide (NAD⁺)–dependent type III histone protein deacetylases that share homology with the yeast silent information regulator 2 (Sir2). SIRT2 is involved in diverse cellular functions including transcription, cell metabolism, microtubule dynamics, mitotic progression, oxidative stress response, DNA damage repair, and genome stability that affect both physiological and pathophysiological processes (4, 5). The many biological functions are attributed to the diverse deacetylation substrates of SIRT2. In particular, during the G2-M transition phase, SIRT2 serves as a histone H4 Lys16 (H4K16Ac) deacetylase through nuclear-cytoplasmic shuttling and may regulate chromosomal condensation (6). During mitosis, SIRT2 localizes to many specific structures, such as centrosome and spindles, to assist the G2-M transition (7).

Early studies found that the abnormal expression of SIRT2 is associated with a variety of malignant tumors (8–11). However, whether SIRT2 plays an important role in tumorigenesis and cancer development is controversial, as it has both tumor-promoting and tumor-suppressing functions. A previous study showed that deletion of SIRT2 promotes tumorigenesis due to several mitotic defects. Gender-specific tumorigenesis was observed in SIRT2-deficient mice (12). The SIRT2 gene and SIRT2 protein expression are reduced in gliomas and adenocarcinomas of breast, gastric, and colon origins (12–15). These observations suggest that SIRT2 acts as a tumor suppressor. However, increasing evidence suggests that SIRT2 may also be a pro-oncogenic factor. SIRT2 expression is up-regulated in hepatocellular carcinoma (11), renal cell carcinoma (16), prostate cancer (17, 18), basal-like breast cancer (19), uveal melanoma (20), and acute myeloid leukemia (21). SIRT2 not only increases the proliferation and metastasis ability of bladder and gastric cancer cells (22, 23) but also promotes the occurrence and development of hepatocellular and breast carcinomas (11, 19). Many SIRT2-specific inhibitors have broad anticancer effects (23, 24). In particular, thioymristoyl (TM), a SIRT2-specific inhibitor, has obvious
inhibitory effects on the growth of various human cancers (24), further suggesting a tumor-promoter function of SIRT2. Thus, the essential function of SIRT2 in tumorigenesis, especially during mitosis, needs to be further elucidated.

The Structural Maintenance of Chromosome (SMC) proteins are highly conserved from yeast to human. The SMC proteins are essential for sister chromatid cohesion and separation, as well as chromosome condensation (25, 26). SMC1A is a core component of the tetrameric complex cohesin, a ring-shaped multi-subunit protein complex composed of the adenosine triphosphatases (ATPases) Smc1 and Smc3, the kleisn Sccl (Mdc1/Rad21), and the HEAT-repeat domain protein Sc3 (SA1, 2) (27). Cohesin is required for both proper chromosome segregation and the DNA damage-induced G2–M checkpoint (28–30). Evidence suggests that SMC1A provides essential surveillance of proper spindle formation during mitotic catastrophe and overcoming oncogenic stress.

In an effort to fully understand the role of SIRT2 in overcoming oncogenic stress, we transfected and purified Flag-tagged SIRT2 from human embryonic kidney (HEK) 293T cells and identified potential protein interactions using mass spectroscopy (fig. S1A, supplemental data sheet). A previous study showed that SIRT2 predominantly localizes in the cytoplasm during interphase but translocates to the centrosomes at the late G2 phase and is associated with mitotic spindles and the midbody during mitosis, suggesting that it regulates certain mitotic associated proteins to ensure normal cell division (43). Proper mitosis is essential for the survival of tumor cells and tumorigenesis. In addition to α-tubulin (44), heat shock protein (45), and Anaphase Promoting Complex (APC) (12) that are known targets of SIRT2-dependent deacetylation, we identified cell cycle regulation and mitotic-associated protein SMC1A among the high-scoring interaction proteins. Western blot analyses of nuclear extracts from mouse embryonic fibroblasts (MEFs) revealed that SMC1A localized more abundantly to the nucleus, whereas SIRT2 was mainly located in the cytoplasm (fig. S1B). Moreover, two alternatively spliced transcripts, SIRT2.1 (~43 kDa) and SIRT2.2 (~37 kDa), were detected (fig. S1C). We then investigated the interaction between endogenous SIRT2 and SMC1A in the nuclei of MEFs by coimmunoprecipitation (co-IP). SIRT2 and SMC1A coimmunoprecipitated (Fig. 1G), and silencing of SMC1A and/or SIRT2 abolished the interaction (fig. S1D). Moreover, a reciprocal co-IP performed with anti-SMC1A antibodies also confirmed their interaction (fig. S1E). The SIRT2-SMC1A interaction was further confirmed by exogenous overexpression of Flag-SMC1A (Fig. 1H).

Given the previous finding that cytosolic SIRT2 shuttles into the nucleus during the G2–M transition, we hypothesized that the interaction between SIRT2 and SMC1A would increase during mitosis. To test this, we first synchronized HCT116 cells in the G1-S phase using a double thymidine block. After release from the thymidine block, >80% of the cells entered G1-S phase in 0 to 4 hours and progressed into G2 and the mitosis phase after 4 hours (fig. S1, F and G). The interaction between SIRT2 and SMC1A significantly increased during the mitotic phases (Fig. 1I). Immunofluorescence analysis of SIRT2 and SMC1A in mitotic HCT116 cells further demonstrated that SIRT2 and SMC1A colocalize on the mitotic spindles and spindle pole from prophase until telophase (fig. S2A).

Given the association of SIRT2 with SMC1A, we next asked whether SIRT2 might modulate SMC1A acetylation. We examined the acetylation level of SMC1A using a pan-specific anti–acetylated lysine antibody. Endogenous acetylation experiments of SMC1A were carried out by co-IP of nuclear proteins. SMC1A acetylation was enhanced after treatment with two commonly used deacetylase inhibitors. Treatment with nicotinamide (NAM) alone, an inhibitor of class III histone deacetylases (HDACs) (SIRT family deacetylases) (46), or the combination of NAM and trichostatin A (TSA), class I and II HDAC blockers (47), enhanced acetylation (fig. S2B). These results suggest that SMC1A is acetylated and is deacetylated by SIRT family deacetylases. Consistent with SIRT2-mediated deacetylation,
we found that overexpression of SIRT2 decreased SMC1A acetylation in HEK293T cells overexpressing SMC1A (Fig. 1I). In keeping with these observations, immunopurified endogenous SMC1A was effectively deacetylated by overexpression of wild-type (WT) SIRT2, whereas transfection of a catalytically inactive mutant of SIRT2 (H187Q167A) had no effect (Fig. S2C). Furthermore, comparison of the level of endogenous SMC1A acetylation in SIRT2+/+ and SIRT2−/− MEFs showed that SIRT2 deficiency significantly increased SMC1A acetylation (Fig. 1K and S2D). These findings indicate that SMC1A may be a target for SIRT2-dependent deacetylation, a speculation further confirmed by in vitro deacetylation experiments (Fig. 1L). Together, these data support that SIRT2 interacts with and deacetylates SMC1A during mitosis.

**SMC1A is acetylated by CREB-binding protein acetyltransferase at Lys 579**, which, in turn, is a target for SIRT2-dependent deacetylation

To identify the acetyltransferase responsible for SMC1A acetylation, four acetyltransferases were individually transfected into HEK293T cells, including p300 (E1A binding protein, 300 kDa), CBP [cyclic adenosine 3′,5′-monophosphate response element–binding protein (CREB)–binding protein], PCAF (p300/CBP-associated factor), and GCN5 (KAT2A). We found that ectopic expression of CBP, but not other acetyltransferases, significantly increased SMC1A acetylation (Fig. 2A). CBP bound to both endogenous and ectopically expressed Flag-SMC1A (Fig. 2, B and C). These findings confirmed CBP as an acetyltransferase that targets SMC1A.

To determine the specific site(s) on SMC1A that is deacetylated by SIRT2, we analyzed 10 acetylation sites identified from the mass spectrum available in the PhosphoSitePlus PTM database [Cell Signaling Technology (CST)]. We mutated each of the 10 putative acetylation sites to arginine (R, non-acetylatable mutant) using site-directed mutagenesis and then examined acetylation levels. Mutation of each lysine (K) to R variably decreased SMC1A acetylation, whereas only the arginine substitution of K579 caused minimal alteration of SMC1A acetylation with and without SIRT2 overexpression (Fig. S3A). Thus, K579 may be a major acetylation site in SMC1A regulated by SIRT2. K579 is evolutionarily conserved from Drosophila melanogaster to mammals (Fig. 2D). Replacement of K579 with either non-acetylatable arginine (K579R) or acetylimetic glutamine (K579Q) markedly reduced the overall acetylation of SMC1A when compared with the WT protein, suggesting that K579 is a major acetylation site in SMC1A (Fig. 2E).

To further investigate K579 acetylation, we generated an antibody that specifically recognizes ectopically expressed WT, but not the K579R mutant, SMC1A (Fig. S3B). Ectopic expression of CBP, but not other acetyltransferases, increased the level of SMC1A acetylation at K579 (Fig. 2F). AGK2, a specific inhibitor of SIRT2, significantly rescued the K579-acetylated SMC1A level in cells overexpressing SIRT2 (Fig. 2G). In keeping with this finding, overexpression of SIRT2 decreased endogenous SMC1A acetylation of K579 in HCT116 cells (Fig. 2H). Similar results were also observed in HT29 cells, another early colon cancer cell line (Fig. S3C). These findings indicate that CBP is the acetyltransferase that targets SMC1A at K579, which is also a target for SIRT2-dependent deacetylation.

**Acetylation at K579 inhibits SMC1A phosphorylation in mitosis**

Phosphorylation of Ser957 and/or Ser966 of SMC1A (SMC1A-p) plays an important role in DNA damage–induced cell cycle checkpoint regulation (28, 37–39). However, how SMC1A phosphorylation is regulated during mitosis remains unclear. Given our data showing colocalization of SIRT2 and SMC1A during mitosis (Fig. S2A),
To explore this, we first synchronized HCT116 cells to G1-S phase using a double thymidine block. After release from the thymidine block, the expression levels of SIRT2 and SMC1A-p gradually decreased of SMC1A-p in G2 and mitotic phases (fig. S4C). SIRT2 knockdown in HCT116 cells showed substantial dose-dependent rescue upon expression of WT SIRT2 that is resistant to the SIRT2 shRNA. Overexpression of 2 and 5 μg, but not 1 μg, of WT SIRT2 significantly increased the SMC1A-p level (Fig. 3F). Thus, these results further suggest that SIRT2-dependent deacetylation is required for SMC1A phosphorylation.

To further establish the relationship between SMC1A acetylation at K579 and SMC1A phosphorylation in response to oxidative stress, we examined the effect of an acetylmimetic mutation of SMC1A K579 on phosphorylation under 500 or 200 μM H2O2 conditions. SMC1A K579Q mutant significantly impaired SMC1A-p when compared to WT and SMC1A K579R proteins in SMC1A knockdown HCT116 cells (Fig. 3G and fig. S4, G and H). Furthermore, the negative correlation between K579 acetylation and phosphorylation of SMC1A in response to oxidative stress was concentration-dependent (fig. S4I). To further directly test the negative correlation, we analyzed the localizations of SMC1A K579 acetylation and phosphorylation during mitosis by immunofluorescence. Mitotic cells were labeled with anti-α-tubulin antibody to visualize the spindle, and chromosomes were stained with 4',6-diamidino-2-phenylindole (DAPI). Our results show that SMC1A K579 acetylation level decreased in cells in the mitotic phase compared to the surrounding interphase cells under normal conditions. Conversely, SMC1 phosphorylation increased in cells in the mitotic phase compared to the surrounding interphase cells (Fig. 3H). Together, these findings suggest that a negative cross-talk exists between acetylation at K579 and phosphorylation of SMC1A.

Previous studies have shown that Ataxia-Telangiectasia-Mutated (ATM) directly phosphorylates SMC1A at S957 and S966 in mammalian cells after exposure to ionizing irradiation (37). To further explore the mechanism by which acetylation of SMC1A at K579 inhibits its phosphorylation, we asked if acetylation affects the interaction between the main kinase ATM and SMC1A. AGK2 impaired both the ATM-SMC1A interaction and the SMC1A-p level induced by H2O2 stimulation (Fig. 3I). Moreover, CBP, a molecule that enhances SMC1A K579 acetylation, blocked the ATM-SMC1A interaction and decreased SMC1A-p expression in response to H2O2 stimulation (fig. S4J). Re-expression of the acetylmimetic SMC1A K579Q into HCT116-shSMC1A cells diminished the ATM-SMC1A interaction and the SMC1A-p level in response to oxidative stress (Fig. 3I). The data thus far support that SIRT2-dependent SMC1A acetylation at K579 regulates SMC1A phosphorylation by modulating the ATM-SMC1A interaction.

**SMC1A acetylation induces mitotic catastrophe**

As a growing body of data suggests that SMC1A is involved in colorectal carcinogenesis (33, 35, 36), we focused on colon cancer cells...
as our main study target. Given the colocalization of SIRT2 and SMC1A on the mitotic spindles and spindle pole during mitosis (fig. S2A), we analyzed whether SMC1A phosphorylation regulated by SIRT2 affects the mitotic process. SMC1A WT, S957AS966A (AA, a nonphosphorylatable mutant with S957 and S966 replaced by alanine), or S957DS966D (DD, a phosphomimetic mutant created by replacing S957 and S966 with aspartate) mutant was stably reexpressed in HCT116 cell lines with knockdown of SMC1A (fig. S5A). Mitotic cells were labeled with anti–α-tubulin antibody to visualize the spindle, and chromosomes were stained with DAPI. We found that cells expressing the SMC1A AA mutant, but not WT or the DD mutant, had increased spindle multipolarity from mitotic prophase until telophase under normal culture conditions (fig. S5, B and C).

To determine whether SIRT2 regulates mitosis through a mechanism that involves SMC1A Ser957/Ser966 phosphorylation, we generated SMC1A-depleted HCT116 cells in the absence of SIRT2 (fig. S5D). Reexpression of SMC1A WT resulted in released mitotic arrest in response to oxidative stress but had no effect in SIRT2 knockdown cells (fig. S5, E and F). The SMC1A DD mutant markedly decreased the levels of cyclin B1 and H3-p in response to oxidative stress, whereas SMC1A AA mutant had no such effect, even in cells lacking SIRT2 (fig. S5, E and F). These data strongly suggest that defects in SMC1A phosphorylation, which is regulated by SIRT2, result in aberrant chromosome segregation and mitotic defects.

To further understand the effect of acetylation on SMC1A, we first analyzed whether SMC1A acetylation affects SMC1A location in mitosis. We found that both SMC1A K579R and K579Q mutants colocalized on the mitotic spindles similar to WT and showed no effect on localization of SMC1A (fig. S6A). Next, we analyzed whether SMC1A acetylation affects spindle formation and chromosome segregation by regulating its phosphorylation. SMC1A WT, K579Q (acetyl mimetic mutant), or K579Q-DD (K579Q-DD, phosphorylation rescue mutant) mutant was stably reexpressed in HCT116 cell lines with knockdown of SMC1A (fig. S6B). We found that cells expressing the SMC1A K579Q mutant, but not WT or K579Q-DD mutant, had increased spindle multipolarity from mitotic prophase until telophase under normal culture conditions, similar to those expressing the SMC1A AA mutant (fig. 4A and fig. S6C). Multipolar spindles were significantly rescued in cells expressing the SMC1A K579Q-DD mutant when compared to those expressing the K579Q mutant (fig. 4A and fig. S6C). We further found that stable expression of the SMC1A K579Q mutant resulted in an increase in the number of multinucleated cells and enlarged cells when compared to the WT and K579Q-DD mutant groups. However, the SMC1A K579Q-DD mutant partially rescued the multinucleation phenotype (fig. 4B). Metaphase chromosome spread analysis showed that stable expression of SMC1A K579Q or K579Q-DD mutant did not cause significant changes in chromosome structure when compared to the WT cells. However, ~15% of SMC1A K579Q cells were aneuploid (chromosome numbers ranging from 48 to 90 per cell), whereas <4% of WT and K579Q-DD cells were aneuploid (Fig. 4C). These data strongly suggest that enforced K579 acetylation of SMC1A results in abnormal mitotic features and chromosome instability by inhibiting SMC1A phosphorylation.

To further study the molecular mechanism by which K579 acetylation of SMC1A regulates spindle formation, we examined whether ectopically overexpressed SIRT2 in cells under oxidative stress affects the formation of the cohesin complex with ectopically overexpressed SMC1A. We found that the interaction of the cohesion subunit RAD21 with Flag-SMC1 was not affected by SIRT2 overexpression under oxidative stress (fig. S6D). Consistently, the RAD21-SMC1 interaction was further confirmed by co-IP of endogenous SMC1 and RAD21 (fig. S6E). Therefore, SIRT2-dependent SMC1 deacetylation does not cause abnormal cohesin complex formation.
We next asked if the spindle multipolarity induced by SMC1A during mitosis, which is required for bipolar spindle formation, results from impeding the binding of SMC1 and Rae1. We found that reexpression of acetylmimetic SMC1 A K579Q into HCT116-shSMC1A cells significantly reduced SMC1A-Rae1 interaction (Fig. 4D). These results support that SIRT2-dependent SMC1A deacetylation at K579 induces mitotic defects by inhibiting SMC1A phosphorylation and thus blocking SMC1A-Rae1 interaction.

Mitic machinery has been shown to ensure correct chromosome segregation. On the other hand, aberrant mitotic machinery may generate mitotic catastrophe and lead to cell death. We therefore tested whether SMC1A acetylation affects survival of tumor cells. In this regard, we found that transient reexpression of the SMC1A K579Q mutant, but not SMC1A WT and the K579Q-DD mutants, in SMC1A-depleted HCT116 cells markedly increased the percentage of apoptotic cells formed in response to oxidative stress, indicating that K579 acetylation of SMC1A may induce cell apoptosis (Fig. 4, E and F). Furthermore, the cleaved poly(adenosine 5′-diphosphate–ribose) polymerase (C-PARP) and cleaved caspase-3 (C-Cas3) were enhanced in the SMC1A K579Q mutant, but not in WT or the K579Q-DD mutant cells under oxidative stress (fig. S7, A and B). SMC1A K579Q mutant also inhibited cell survival at 24 hours under oxidative stress (fig. S7C). Similar results were obtained in HT29 cells when we probed for C-PARP and C-Cas3 (fig. S7D).

To further clarify the role of SIRT2 regulation of SMC1A in mitosis, we examined whether mutations in SMC1A K579 could rescue SIRT2-deficient phenotypes in response to oxidative stress. As expected, reexpression of SMC1A WT resulted in released mitotic arrest in response to oxidative stress (Fig. 4G and fig. S7E) but had no effect in SIRT2 knockdown cells (Fig. 4H and fig. S7F). Reexpression of the SMC1A K579Q mutant significantly increased the levels of cyclin B1 and H3-p in response to oxidative stress, even in cells lacking SIRT2, suggesting that hyperacetylation of SMC1A at K579 results in mitotic arrest (Fig. 4, G and H, and fig. S7, E and F). Together, the role of SIRT2 in the mitotic stress response, at least in part, can be attributed to SMC1A phosphorylation at Ser957 and Ser966 regulated by SMC1A acetylation. The Nomenclature Committee on Cell Death (NCCD) has defined the characteristics of mitotic catastrophe as one of the main forms of cell death (30). Our data suggest that K579 acetylation of SMC1A induces mitotic catastrophe characterized by mitotic defects and arrest, and promotes cell death.

**SMC1A acetylation inhibits cell proliferation and tumor growth**

A previous study has shown that a SIRT2-specific inhibitor (TM) has a broad anticancer effect in various human cancer cells and in breast cancer mouse models (24). To further clarify whether SIRT2 inhibition exerts anticancer effects by modulating SMC1A at K579, we examined the effect of AGK2, a selective SIRT2 inhibitor, in mouse tumor xenografts. SMC1A-deficient HCT116 cells with stable reexpression of either WT or the K579R mutant SMC1A were injected subcutaneously into immunodeficient mice. The tumor volume and weight were significantly higher in the SMC1A-K579R-expressing HCT116 xenografts when compared to those derived from HCT116-expressing WT SMC1A (Fig. 5A and fig. S8A). The growth of AGK2-treated tumors expressing WT SMC1A was significantly inhibited, whereas tumors expressing the K579R mutant SMC1A...
showed no such effect (Fig. 5A and fig. S8A). These data confirm that the anticancer effect of SIRT2 inhibition is achieved by regulating SMC1A at K579, and further indicate that Lys579 may be an essential determinant of cell mitotic catastrophe and a potential anticancer target.

Our previous studies have shown that phosphorylation of SMC1A promotes hepatocellular carcinoma cell proliferation and migration (51). To explore the biologic significance of SMC1A K579 acetylation in cell proliferation, we examined the proliferation of HCT116 cells stably expressing SMC1A WT, K579Q, or K579Q-DD. We found that the SMC1A K579Q mutant, but not WT or the K579Q-DD mutant, significantly inhibited the growth of HCT116 cells (fig. S8B). To investigate whether the K579 acetylation of SMC1A also reduces tumor growth in vivo, we performed xenograft experiments and monitored tumor growth over a period of 26 days. In line with the in vitro observations, the tumor volume and weight were significantly lower in the SMC1A-K579Q–expressing HCT116 xenografts when compared to those derived from HCT116-expressing WT or the SMC1A K579Q-DD mutant, with the latter developing the largest tumors (Fig. 5, B and C). In support of these findings, immunohistochemical analyses demonstrated that the tumor cells in the SMC1A-K579Q–expressing HCT116 xenografts displayed the highest Ki-67 proliferation index, whereas the cells in SMC1A K579Q xenografts yielded the lowest Ki-67 score (fig. S8, C and D). Moreover, HCT116 SMC1A-K579Q xenografts displayed significantly lower SMC1A-p expression than SMC1A WT xenografts (fig. S8, E and F), resulting from the inhibition of SMC1A phosphorylation by K579 acetylation. This result suggests that the tumor suppressor function of K579 acetylation of SMC1A might be achieved through its phosphorylation.

To further clarify the clinical significance of K579 acetylation of SMC1A in inhibiting tumor growth, we treated cells with 5-fluorouracil (5-FU) or oxaliplatin, which are both first-line chemotherapeutic agents for colon cancer (52). We found that treatment with 5-FU or oxaliplatin did not affect the K579 acetylation level of SMC1A (fig. S8G). However, 5-FU or oxaliplatin significantly inhibited cell survival at 96 hours (Fig. 5, D and G) and resulted in a lower Proliferating cell nuclear antigen (PCNA) proliferation index (Fig. 5, E and H) and higher C-PARP and C-Cas3 apoptosis index (Fig. 5, F and I). The decrease in cell viability and increase in apoptosis observed with 5-FU or oxaliplatin treatment were rescued by overexpression of SIRT2 (Fig. 5, D to I). These data strongly suggest that SIRT2 acts as an oncogene to protect tumor cell survival. We further examined the effects of different concentrations of 5-FU or oxaliplatin on the survival of cells expressing SMC1A K579 mutants. Our data showed that K579 acetylation of SMC1A significantly inhibited cell survival at lower doses of oxaliplatin (Fig. 5I) or 5-FU (Fig. S5L) and promoted cell apoptosis (Fig. 5, K and M) when compared to the SMC1A-WT group, suggesting that K579 acetylation of SMC1A enhances the chemosensitivity of these anticancer drugs by promoting cell apoptosis. Together, these results demonstrate that the acetyliminic substitution at Lys579 impairs the ability of SMC1A to promote colon cancer cell survival and tumor growth, suggesting that Lys579 may be a potential anticancer target.

**Down-regulation of SMC1A acetylation and up-regulation of SMC1A phosphorylation in early-stage human cancers**

Our results indicate that SMC1A acetylation at K579 results in mitotic catastrophe and inhibits tumor cell growth by regulating its phosphorylation. To further address the role of SIRT2-SMC1A axis in tumorigenesis, we asked whether an association between SIRT2 and SMC1A is present in colon cancer. Analysis of the TCGA public datasets contained in the GEPIA database revealed a positive correlation between SIRT2 and SMC1A mRNA levels in colon cancer tissues (fig. S9A).

We then examined the alteration of SMC1A acetylation and SMC1A phosphorylation in tissue samples of early-stage (stage I and II) colon
cancer, invasive ductal carcinoma of the breast, as well as squamous cell carcinoma of the esophagus. We optimized an anti–acetyl-SMC1A (K579) antibody in paraffin-embedded normal and colon cancer tissues. The signals were blocked by the acetyl-K579 antigen peptide, confirming its specificity (fig. S9B). We used this antibody to analyze colorectal, breast, and esophageal carcinomas and their adjacent normal tissues. The K579-acetylated SMC1A signal was significantly reduced, and the SMC1A-p expression was significantly elevated in cancerous tissues when compared to the adjacent normal tissues (Fig. 6, A to I). Specifically, a higher SIRT2 protein level was found in 39 colon cancer samples when compared to the adjacent normal epithelia. These tumors also exhibited decreased K579-acetylated SMC1A and increased SMC1A-p (fig. S9, C and D). Furthermore, the K579-acetylated SMC1A levels were negatively correlated with SIRT2 (r = −0.2716; P = 0.0274; fig. S9, C and E) and SMC1A-p levels (r = −0.2862; P = 0.0180; fig. S9, D and F). Similar observations were obtained when analyzing breast (fig. S9, G and H) and esophageal carcinoma samples (fig. S9, I and J). These data indicate that SIRT2 and K579-acetylated SMC1A are potential universal biomarkers for early-stage human cancers.

DISCUSSION

In this study, we uncovered a novel mitotic phase–associated non-histone protein, SMC1A, as a target of SIRT2 regulation. We also found the molecular mechanism by which SIRT2 affects early tumorigenesis by regulating SMC1A acetylation. SIRT2 directly interacts with and deacetylates SMC1A at Lys579, which, in turn, regulates its phosphorylation under oxidative stress. Hyperacetylation of SMC1A at K579 induces mitotic catastrophe and inhibits tumor growth, thus ultimately enhancing the effect of anticancer agents. Reduced K579-acetylated SMC1A expression and elevated SMC1A-p and SIRT2 levels were found in early-stage colorectal, breast, and esophageal carcinomas (Fig. 6J). These observations highlight the importance of the SIRT2-SMC1A axis in overcoming oncogenic stress and suggest that SMC1A K579 is a potential therapeutic target for human early-stage cancers.

In addition to the known α-tubulin, heat shock protein, and APC factors, we also found several interesting potential interactors based on our mass spectra results (RAD50 and SMC3). Although SMC3 is also a mitotic-associated protein and was found among the high-scoring interaction proteins, SIRT2 does not affect its acetylation level and is not an SMC3 deacetyltransferase (S3). Rad50, which is not found among the high-scoring interaction proteins, is a key component of the Mre11-Rad50-Nbs1 (MRN) complex involved in DNA double-strand break repair. Although RAD50 is reported to regulate mitotic progression independent of DNA repair functions, it is preferentially colocalized with chromosomes rather than centrosomes, spindle poles, or spindles, which more directly affect the assembly of cell division (S4). Considering that SIRT2 is localized on the mitotic spindles and spindle pole from prophase until telophase, SIRT2 is colocalized with SMC1A but not RAD50 in mitosis.

As a key component of cohesin, SMC1A is required for the DNA damage–induced G2-M checkpoint (S8, S55). Consistently, we found that SMC1A knockdown induced a higher mitotic index under oxidative stress. Moreover, only WT and the phosphorylation mimetic mutant S957DS966D, but not the nonphosphorylatable mutant S957AS966A, could rescue this phenomenon, at least in part, in SMC1A or SMC1A-SIRT2 knockdown cells. These results indicate that a higher mitotic index caused by deletion of SIRT2 or SMC1A is only partly due to abnormal SMC1A-p, and that other signal pathways may also be involved. SMC1A is essential for proper spindle formation (S30–S32). In this study, we found that cells expressing the SMC1A K579Q mutant, but not WT or the phosphorylation rescue K579Q-S957DS966D mutant, exhibit distinct multipolar spindles during mitosis through a mechanism of SMC1A-p inhibition, suggesting a critical role of this mutant in mitotic spindle multipolarity characteristic of cancer cells.

How might SMC1A acetylation regulate spindle formation? Previous work showed that phosphorylation of Ser597 and Ser566 of SMC1A stimulates binding to Rae1 during mitosis, which is required for bipolar spindle formation (S32). We tested whether the spindle multipolarity, which is induced by SMC1A acetylation through inhibiting its phosphorylation, occurs through impeding the binding
of SMC1 and Rae1. We found that reexpression of acetyl mimetic SMC1A K579Q in HCT116-shSMC1A cells significantly reduced SMC1A-Rae1 interaction. These results elucidate the molecular mechanism by which SMC1A acetylation affects the formation of multipolar spindles.

Mitotic catastrophe is considered a form of cell death triggered by dysregulated or failed mitosis. Cells undergoing mitotic catastrophe are usually driven to irreversible fates including apoptosis, necrosis, or senescence. Thus, activating the mitotic catastrophe is an attractive anticancer therapy. At present, microtubule-targeting agents are the most important antimitotic drugs used clinically (56, 57). Consistent with an early study on mitotic catastrophe characteristics (50), we have found that hyperacetylation of SMC1A at K579 leads to mitotic arrest with defective mitotic features and ultimately cell apoptosis. Many functions of SIRT2 have been disclosed since its discovery. A previous study showed that SIRT2 maintains genome stability by regulating cell cycle progression (48). We have also demonstrated that SIRT2 participates in the mitotic stress response as a mitotic checkpoint. Given that SIRT2 deacetylates SMC1A at K579, small-molecule inhibitors of SIRT2 that interfere with mitotic checkpoint signaling may also be of potential clinical use to disrupt tumor cell proliferation. There has been increasing evidence that potent SIRT2-specific inhibitors have broad anticancer activities (24, 58). In contrast to its potential tumor-promoting property, SIRT2 may also serve as a tumor suppressor in some cancer types. SIRT2 protein and RNA levels are down-regulated in glioma (14), gastric carcinoma (13, 59), hepatocellular carcinoma, breast cancer (12), and melanoma (60). Gender-specific tumorigenesis was observed in the SIRT2-deficient mice (12). Although the role of SIRT2 in tumorigenesis remains controversial, we believe that the essential function of SIRT2 is to maintain genome stability. Under physiological conditions (normal cell), the inhibition of SIRT2 leads to genomic instability and carcinogenesis. However, under pathological conditions (tumor cell), most tumors might be stable aneuploids. Inhibition of SIRT2 in tumors inevitably leads to gross genomic instability and ultimately tumor cell death. As shown in our study, the significant up-regulation of SIRT2 levels in multitype early-stage cancers is likely to maintain genomic stability to enable tumor cells to survive and tumor development. Consistent with these results, we also found an increase in SIRT2 expression under oxidative stress. Furthermore, the SIRT2-specific inhibitor AGK2 showed significant tumor growth inhibition function. Overexpression of SIRT2 significantly protected cell survival when treated with 5-FU or oxaliplatin. Therefore, our results provide a plausible explanation for how SIRT2 acts as an oncogene in early-stage cancers.

Mutation and/or up-regulation of SMC1A is commonly observed in human colorectal carcinomas (33, 34, 36), breast cancers (61, 62), and gliomas (63). Furthermore, overexpression of SMC1A has been found in advanced diseases and is associated with a poor prognosis in colorectal cancer (36). On the contrary, SMC1A expression is inversely correlated with the clinical outcomes in acute myeloid leukemia (64). Therefore, the data thus far suggest that SMC1A might play a dual role in tumorigenesis and progression, acting as a promoter in solid tumors and an inhibitor in hematologic malignancies. Moreover, PTM of SMC1A in tumorigenesis and progression has not been well studied. We previously reported that phosphorylation of SMC1A promotes hepatocellular carcinoma cell proliferation and migration (51), a phenomenon in line with the results observed in colorectal cancer. Here, we found that SIRT2-mediated SMC1A deacetylation at K579 promotes phosphorylation of SMC1A that, in turn, enhances cell proliferation and tumor growth. A low dose of oxaliplatin or 5-FU, both first-line therapeutic agents for colon cancer (52), significantly suppresses the survival of SMC1A-K579Q cells by promoting apoptosis. These findings suggest a potential for combined cytotoxic and SMC1A K579-targeted therapy to achieve a synergistic treatment effect. In addition, K579-acetylated SMC1A expression was significantly decreased, whereas SMC1A-p and SIRT2 levels were significantly increased in early-stage human cancers of various origins, suggesting that the deacetylation-phosphorylation regulation of SIRT2-SMC1A axis is universal in early-stage human cancers. These findings not only indicate that SIRT2 plays a vital role in early precursor lesions to overcome oncogenic stress but also suggest that K579 acetylation of SMC1A may be a potential novel anticancer target via inhibition of SMC1A phosphorylation.

In summary, we have identified for the first time an acetylation-dependent regulatory mechanism governing SMC1A function in mitosis. SMC1A acetylation causes mitotic catastrophe and inhibits tumor growth. Our findings support that SMC1A may be a key factor in SIRT2-mediated genomic stability, providing a potential mechanism by which SIRT2 acts as an oncogene in early-stage cancers. These observations further indicate the potential utility of SIRT2 inhibitors or targeting of SMC1A K579 acetylation for early-stage cancer treatment.

MATERIALS AND METHODS

Mice

Sirt2−/− mice with a deletion of exons 5 to 8 were a gift from C. X. Deng (12). Nude mice (BALB/C, 6-week-old male) were purchased from Beijing Vital River Laboratory Animal Technology Company. All animal experiments were approved by the Animal Care and Use Committee of China Medical University.

Cell culture

HEK293T, HCT116, and HT29 cells were purchased from Cell Bank in Chinese Academy of Sciences Shanghai. Cells were cultured in high-glucose Dulbecco’s Modified Eagle’s medium (DMEM) (for HEK293T cell line), RPMI 1640 medium (for HCT116 cell line), or McCoy’s 5A medium (for HT29 cell line), supplemented with fetal bovine serum (FBS) (10%, CLARK, Australia), penicillin (100 U), and streptomycin (100 µg/ml). MEFs were cultured in DMEM containing 15% FBS.

Antibodies and reagents

Antibodies used in this study include SIRT2 (Western blot: S8447, Sigma-Aldrich; IP staining: ab211033, Abcam), SMC1 (Western blot/IP: NB100-204, Novus; immunofluorescent staining: A300-834A, Bethyl), pSer966 SMC1 (Western blot: ab124783, Abcam), pSer312 SMC1 (200-301-397, Rockland), acetylated-lysine (#9441, CST), Flag (SG4110-16, Shanghai Genomics Technology), Myc (SG4110-18, Shanghai Genomics Technology), cyclin B1 (#4135S, CST), H3 (#4499, CST), phospho–histone H3 (Ser10) pSer10 H3-P (#3377, CST), α-tubulin (AT819, Beyotime), CBP (#3724S, CST), RAD21 (#4321, CST), Rae1 (ab124783, Abcam), cleaved PARP (#5625, CST), cleaved caspase-3 (#25546-1-AP, Proteintech), Flag M2 affinity gel (B23102, Biomage), donkey anti-mouse immunoglobulin G (IgG) secondary antibody, Alexa Fluor 488 (A21202, Thermo Fisher Scientific), donkey anti-goat IgG secondary
Stable cell lines were selected by puromycin (5 μg/ml) treatment for infection efficiency of the target cells was determined by Western blot. The mass spectrometry procedure and analysis were performed by the corresponding non-acetylated peptide coupled to AminoLink Coupling Resin (Thermo Fisher Scientific) and purified by affinity chromatography.

AGK2 (S7577), NAM (S1899), and TSA (S1045) were purchased from Selleck. Flag peptide (F3290), thymidine (T1895), isopropyl-β-d-thiogalactopyranoside (IPTG; I6758), Giemsa stain (G5500), colchicines (C3915), 5-FU (F6627), and oxaliplatin (O9512) were from Sigma-Aldrich. PI (propidium iodide, ST511) was from BeyoTime. Blasticidin S HCl was from Thermo Fisher Scientific (R21001). Cell Counting Kit-8 (CCK8, CK04) was from Dojindo.

Plasmid constructions and transfection
Human SMC1A was cloned into the indicated vectors including pcDNA3.1-flag/HA and pUB6/V5-His. Human SIRT2 was cloned into pcDNA3.1-flag/HA and pCMV-Myc-N, respectively. The plasmids containing specific point mutations of SMC1A or SIRT2 were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA). Flag-P300, Flag-CBP, and Myc-GCN5 were provided by Q. Lei (Shanghai Medical College, Shanghai, China). Flag-PCAF was a gift from W. Zhu (Shenzhen University, Shenzhen, China). GST-SIRT2 was supplied by C. Wang (Shanghai General Hospital, Shanghai, China). The plasmids were confirmed by sequencing and transfected into HEK293T, HCT116, and HT29 cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. Cells were used 24 to 72 hours after transfection.

Mass spectrometry
Flag-tagged SIRT2 expression plasmids were transfected into HEK293T cells and cultured for 36 hours. Cells were collected and lysed with lysis buffer [137 mM NaCl, 10 mM NaF, 50 mM tris-HCl (pH 7.6), 1 mM EDTA, 0.1 mM Na3VO4, 10% glycerol, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The acetylated SMC1A and recombinant GST-SIRT2 were incubated with ice-cold 4% paraformaldehyde (PFA) for 15 min, followed by washing with PBS. Cells were permeabilized with 0.5% Triton X-100 for 15 min. Cells were then blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature and incubated with appropriate primary antibodies overnight at 4°C. After washing three times with PBS, cells were incubated with the corresponding fluorescent secondary antibodies for 1 hour at room temperature. The nucleus was stained with DAPI at room temperature for 15 min. The cells were imaged using a confocal microscope (Olympus).

Immunofluorescence
HCT116 cells with and without specific point mutations were washed three times with phosphate-buffered saline (PBS) and then fixed with ice-cold 4% paraformaldehyde (PFA) for 15 min, followed by washing with PBS. Cells were permeabilized with 0.5% Triton X-100 for 15 min. Cells were then blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature and incubated with appropriate primary antibodies overnight at 4°C. After washing three times with PBS, cells were incubated with the corresponding fluorescent secondary antibodies for 1 hour at room temperature. The nucleus was stained with DAPI at room temperature for 15 min. The cells were imaged using a confocal microscope (Olympus).

In vivo acetylation assay
Cells were transfected with Flag-CBP plasmid and treated with TSA (0.5 μM, 16 hours) and NAM (5 mM, 6 hours), then harvested, and lysed. TSA (5 μM) and NAM (20 mM) were added to the cell lysis buffer for 3 hours at 4°C and subsequently with protein A/G-Sepharose beads for 3 hours or overnight at 4°C. The protein–antibody complexes were then washed three times at 4°C with cold lysis buffer and eluted with SDS loading buffer by boiling for 10 min.

GST-SIRT2 fusion protein purification
The bacterial expression constructs (pGEX-4T-1) containing the indicated genes (Sirt2) were transformed into BL21-competent cells (Takara). Cells containing the appropriate GST-fusion protein were grown until the OD600 was between 0.4 and 0.6, and then protein expression was induced by 1 mM IPTG at 30°C for 3 hours. Cells were resuspended in bacterial lysis regent (PBS containing 5 mM β-mercaptoethanol, 0.5% Triton X-100, 2 mM EDTA, and 1 mM PMSF), followed by shaking and ultrasonication. The proteins were purified using MagneGST particles according to the MagneGST Protein Purification manufacturer’s protocol (Promega Science).

In vitro deacetylation assay
Flag-tagged SMC1A and Myc-tagged CBP were transiently expressed in HEK293T cells. Cells lysates were incubated with anti-Flag M2 agarose, and the protein was purified with Flag peptide (0.2 μg/ml). The acetylated SMC1A and recombinant GST-SIRT2 were incubated in deacetylation buffer [50 mM NaCl, 50 mM tris-HCl (pH 9.0), 4 mM MgCl2, 5% glycerol, 0.5 mM dithiothreitol, 0.02% NP-40, and 1 mM PMSF], supplemented with 50 μM NAD+ at 30°C for 2 hours.
were subjected to SDS-PAGE, and the level of SMC1A acetylation was assayed with anti-acetylated lysine (Ac-K) antibody.

Fluorescence-activated cell sorting analysis
For analysis of cell cycle, cells were harvested with EDTA-free trypsin, fixed with ice-cold 70% ethanol at −20°C overnight, and then treated with 0.25% Triton X-100 for 15 min. DNA was stained by PI. Cells were analyzed with FACS Calibur (Becton Dickinson).

For analysis of apoptosis, HCT116 cells were treated with 500 μM H2O2 for 6 hours. Cells were harvested with EDTA-free trypsin, and 1 × 107 cells were stained according to the FITC Annexin V Apoptosis Detection Kit (#556547, BD Biosciences) instructions. Cells were analyzed by FACS Calibur (Becton Dickinson).

Cell synchronization with thymidine
The double-thymidine block was carried out by incubating cells in culture medium containing 2 mM thymidine at 50% confluency for 18 hours. After the first thymidine block was removed, cells were washed with PBS and then incubated in fresh medium for 8 hours. Cells were subsequently treated with 2 mM thymidine for an additional 16-hour block. Cells were released from the second thymidine block and then harvested at different time points for flow cytometry and Western blot analysis.

Chromosome spread assays
Cells were treated with colcemid (50 ng/ml) for 6 hours at 37°C, collected, and washed with PBS. Cells were incubated in 75 mM KCl for 15 min at 37°C and fixed with freshly prepared Carnoy’s solution (25% acetic acid and 75% methanol) for 30 min. Cells were dropped onto slides and stained with 5% Giemsa solution. The chromosome morphology was analyzed using an oil immersion lens (Olympus microscope).

Colon cancer samples and immunohistochemistry
The tissue microarrays of early stage of colon cancer (HCol-Ade060CS1-01, HCol-Ade150CS-01; CO601), invasive ductal carcinoma of the breast (HBre-Duc060CS-04; nine cases of customized sections), esophageal squamous cell carcinoma (OD-CT-DgEs04-002, HEsq-Squ150CS-02), and adjacent benign tissue, along with the patients’ clinical information, were purchased from Shanghai Outdo Biotech Company, China and Xi-an Alenabio Inc., China.

Immunohistochemistry was performed as previously described (51). Sections were incubated with SIRT2 (1:8000), phospho-SMC1 (Ser966) (1:500), or acetyl-SMC1A (K579Ac) (1:100) antibody, respectively. Immunohistochemical staining was semiquantitated by determining the percentage of tumor cells stained using 5% increments (0%, 5%, 10%, 15%, …, 100%) as in a previous study (65). The mean values from two evaluators were used for statistical analysis.

Cell proliferation analysis
Cells were seeded in triplicate in 96-well plates at a density of 3 × 103 cells per well. Basic RPMI 1640 (90 μl) and CCK8 staining solution (10 μl) was added to cells for 2 hours at 37°C. The absorbance at 450 nm was measured daily using an absorbance reader (TECAN, Switzerland).

Drug sensitivity analysis
Cells (3 × 103 cells per well) were plated in 96-well plates. After a 24-hour incubation in complete RPMI 1640 with 10% FBS, cells were exposed to 5-FU or oxaliplatin at different concentrations. The absorbance at 450 nm was measured by using CCK8 staining solution. The percentage of cell survival was calculated.

Tumor xenograft analysis and AGK2 treatment of tumor xenograft
Nude mice (BALB/C, 4-week-old female, N = 10) were injected subcutaneously into the right flank region with 5 × 106 stable HCT116 SMC1A knockdown cells with reexpression of WT, K579Q, or K579Q-S957DS966D (K579Q-DD) mutant SMC1A. Tumor volume was measured every 4 days for 26 days using the formula [tumor volume = π/6 (L × W2)]. The tumors were removed on day 26 for subsequent analysis.

When the volume of most tumors reached 100 mm3, after the initial injection, intratumor injection was performed with vehicle control {DPH solution [8% dimethyl sulfoxide + 30% PEG300 (polyethylene glycol, molecular weight 300) + ddH2O]} or inhibitor (100 μM AGK2 in DPH per tumor), three times per week for 20 days.

Statistical analyses
SPSS version 22.0 software (SPSS Inc., Chicago IL, USA) was used for statistical analysis. The differences between groups were evaluated using two-tailed Student’s t test for continuous variables (expressed as the mean ± SEM) and nonparametric t test for categorical data. The semiquantitative immunohistochemical analysis was carried out by dividing the scores into low (SIRT2 ≤ 30; K579 ≤ 45; SMC1A-p ≤ 50), medium (30 < SIRT2 < 70; 45 < K579 < 70; 50 < SMC1A-p < 70), and high (≥70) groups. Spearman’s rank correlation test was used to evaluate the correlation between SIRT2, SMC1A acetylation, or phosphorylation expression. P ≤ 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/9/eabe5518/DC1

View/request a protocol for this paper from Bio-protocol.

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The deacetylation-phosphorylation regulation of SIRT2-SMC1A axis as a mechanism of antimitotic catastrophe in early tumorigenesis

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