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SUMOylation regulates Rb hyperphosphorylation and inactivation in uveal melanoma

Fengxi Meng[1,2,3] | Yiqun Yuan[1,2,3] | Hui Ren[1,2,3] | Han Yue[1,2,3] | Binbin Xu[1,2,3] | Jiang Qian[1,2,3]

[1]Department of Ophthalmology, Eye & ENT Hospital of Fudan University, Shanghai, China

[2]Laboratory of Myopia, Chinese Academy of Medical Sciences, Shanghai, China

[3]NHC Key Laboratory of Myopia, Fudan University, Shanghai, China

Correspondence
Jiang Qian, Department of Ophthalmology, Eye & ENT Hospital of Fudan University, Shanghai 200031, China.
Email: qianjiang@fudan.edu.cn

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Abstract

Small ubiquitin-like modifier (SUMO)ylation is one of the posttranslational modifications and is implicated in many tumor types. Modulation of SUMOylation can affect tumor progression, but the underlying mechanisms remain unclear. Here, we show that, for the first time, in uveal melanoma (UM), the most common intraocular malignancy in adults, global SUMOylation is upregulated and participates in tumor growth. Inhibition of SUMOylation in UM is sufficient to reduce tumor growth both in vitro and in vivo. Furthermore, we found that retinoblastoma protein (Rb) is a target protein and a critical downstream effector of the upregulated SUMOylation activity in UM. Increased SUMOylation of the Rb protein leads to its hyperphosphorylation and inactivation in UM cells, promoting UM cell proliferation. In summary, our results provide novel insight into the mechanism underlying SUMOylation-regulated tumor growth in UM.

KEYWORDS
ginkgolic acid, phosphorylation, Rb, SUMOylation, uveal melanoma

1 | INTRODUCTION

Uveal melanoma (UM) is the most common primary malignant intraocular tumor in adults, accounting for 85% of all melanomas originating from ocular and adnexal structures.[1-3] The major treatment modality for UM is either enucleation or brachytherapy/proton beam irradiation for eye salvage purposes.[4,5] Up to 50% of patients develop metastasis, especially liver metastasis, within 10 years.[6,7] The treatment options for metastatic uveal melanoma (MUM) patients are very limited due to the lack of effective adjuvant systemic therapy, and MUM is always associated with a very poor prognosis.[8]

Uveal melanoma differs greatly from cutaneous melanoma in its genetic mutation profile. Eighty-three percent of uveal melanomas harbor point mutations in GNAQ or GNA11, which codes for the G-protein α-subunit, in a mutually exclusive pattern.[9,10] GNAQ/GNA11 mutation affects diverse downstream targets in uveal melanoma, including MEK, PI3-kinase/Akt, protein kinase C, and YAP.[11] However, inhibitors of the MEK1/2 pathway were found to neither completely abolish tumor cell proliferation in vitro nor improve the overall survival rates of patients deemed at high risk.[12,13] Other genetic mutations have been reported in uveal melanoma, such as mutations in breast cancer 1 (BRCA1)-associated protein 1 (BAP1), splicing factor 3B subunit 1 (SF3B1), X-linked eukaryotic translation

Fengxi Meng and Yiqun Yuan co-first authors.

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initiation factor 1A (EIFAX1), and telomerase reverse transcriptase (TERT).[14] Therefore, the pathogenic process of uveal melanoma may involve multiple pathways, and new therapeutic targets have yet to be explored.

Small ubiquitin-like modifier (SUMO)ylation is one of the post-translational modifications and is analogous to ubiquitylation. Facilitated by the E1 activating enzymes SAE1 and SAE2, the sole E2 conjugating enzyme Ubc9 and various E3 ligases, SUMO proteins, namely, SUMO1-4, are conjugated to the target protein. SUMOylation is implicated in regulating many critical cellular processes. Accumulating evidence has shown that the SUMOylation pathway is highly upregulated in various tumors.[15] Activation of the SUMOylation pathway is closely associated with the progression of different cancers.[16-18] Downregulation of key components such as SAE1 and Ubc9 can inhibit tumor growth and metastasis, and increase the sensitivity to chemotherapy.[19-21] However, the specific target proteins of SUMOylation in the tumor biology context have not been fully investigated. Retinoblastoma protein (Rb) is a prototypical tumor suppressor vital to the negative regulation of the cell cycle and tumor progression, and its inactivation by cyclin-dependent kinase (CDK)-mediated hyperphosphorylation directly causes retinoblastoma and is closely associated with the development of several other major cancers.[22,23] Interestingly, our previous study indicated that SUMOylation of Rb facilitates cell cycle progression specifically at the early G1 phase via phosphorylation, and subsequent inactivation of this protein promotes cell growth.[24] However, it is still not clear whether SUMO modification of the Rb protein is involved in tumor growth and progression. Here, we show that SUMOylation of the Rb protein is dysregulated in UM and that Rb is a target in SUMOylation-dependent cancer cell growth.

2 | MATERIALS AND METHODS

2.1 | Plasmids and chemicals

Plasmids for His-tagged wild-type or K720R mutant Rb were previously described.[24] Lentiviral-based short hairpin RNAs (shRNAs) against Rb and SUMO1 were cloned. The sequences of the shRNAs were as follows: 5′-CCTTCATATTACCCTCTCCTT-3′ (SUMO1 shRNA1); 5′-CGACCAATGCAAGTGTTCATA-3′ (SUMO1 shRNA1); 5′-GTGCGCTCTTGAGGTTGTAAT-3′ (Rb shRNA1); 5′-CAGAGATCGTGTATTGAGATT-3′ (Rb shRNA2). Ginkgolic acid (GA) (15:1) was purchased from Sigma-Aldrich (345887). ML-792 was purchased from Selleckchem (S8697). The chemicals were initially dissolved in ethanol to produce the stock solutions, which were then diluted with culture medium to the final concentrations. For oral gavage in the mouse xenograft tumor model, GA was purchased from Chengdu Biopurify Phytochemicals Ltd (22910-60-7) and diluted with 0.9% saline to 10 mg/mL.

2.2 | Antibodies

Total Rb (9303) and phospho-Rb (9308) antibodies were purchased from Cell Signaling Technology. SUMO1 (33-2400), S100 (MA5-12969), and vimentin (MA5-11883) antibodies were purchased from Thermo Fisher Scientific. Tubulin (M30109) and His-tag (M30111) antibodies were purchased from Abmart. Ki67 (ab16667) antibody was purchased from Abcam. Cytokeratin antibody was purchased from Santa Cruz Biotechnology.

2.3 | Cell culture

All UM cell lines were kindly provided by WuXi AppTec. Immortalized human uveal melanocytes were established from a 28-year-old Chinese female healthy donor by transfection with a plasmid carrying the human telomerase reverse transcriptase (hTERT) sequence. The choroid was dissected and trypsinized, passed in a 40 μm nylon filter, and resuspended in the culture medium. Once the melanocytes showed unlimited proliferation and were cultured for more than 40 passages, the cell line was considered established. All UM cell lines and uveal melanocytes were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (P/S; Gibco) and were maintained at 37°C in a humidified incubator with 5% CO_2.

2.4 | qPCR assay

Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. One microgram of RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen), followed by PCR amplification with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Gene expression data were calculated by the $\Delta\Delta C_t$ method. Actin was used as the reference control. The following primers were used: SUMO1 (fwd, 5′-TGACCAGGAGGCAAAACCTTC-3′; rev, 5′-AATTCATTGG AACACCCTGTCTT-3′) and ACTIN (fwd, 5′-TTGCCGACAGGAT GCAGAAG-3′; rev, 5′-ACATCTGCTGGAAGGTGGAC-3′).[25]

2.5 | Proliferation assay

Cell proliferation rates were determined by using a CCK-8 Cell Counting Kit (Dojindo) according to the manufacturer's instructions. Cell number was calculated by a preset standard curve.

2.6 | Colony formation assay

Cells were digested in 0.25% trypsin (Gibco) into single cells, seeded in 12-well plates at 500 per well, and incubated at 37°C in a

humidified incubator with 5% CO_2. After 10 days of culture, colonies were gently fixed with 100% methanol and stained with crystal violet. Visible colonies were manually counted.

2.7 | Cell cycle analysis

Cultured cells were collected and fixed with 70% ethanol at −20°C overnight. Cells were washed with PBS and stained with 100 μg/mL propidium iodide (P4170, Sigma) for 30 min. Cell cycle flow cytometry was conducted on a BD FACSCalibur (BD Bioscience) and analyzed using FlowJo software.

2.8 | Apoptosis analysis

Cells were trypsinized and stained with an Annexin V apoptosis staining kit (Abcam, ab14085) and placed on a coverslip for fluorescence microscopy observation. Annexin V-positive cells were counted in three randomly selected fields of view using a 20× objective.

2.9 | SUMOylation analysis, pulldown assay, and immunoprecipitation

Rb SUMOylation analysis was conducted as previously described.[24] Briefly, cells were lysed in denaturing Radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 20 mmol/L *N*-ethylmaleimide (E3876, Sigma) as well as protease and phosphatase inhibitors (Roche). One milligram of the cell lysates was incubated with anti-Rb antibodies for 16 hours at 4°C with gentle inversion mixing. After incubation, protein A/G Sepharose (A10001, Abmart) was added and incubated for 3 hours. The beads were collected and washed and eluted with 1× SDS sample buffer. For Rb pulldown, the lysates were incubated with Ni-NTA agarose beads (30230, Qiagen) for 3 hours at 4°C. The beads were washed five times with a washing buffer containing 20 mmol/L imidazole, and eluted with 40 μL of an elution buffer containing 250 mmol/L imidazole.

2.10 | HE, immunohistochemistry, and immunofluorescence staining

The UM or xenograft specimens were fixed with neutral formalin, embedded in paraffin, and sectioned. For hematoxylin-eosin (HE) staining, the sections were processed with the conventional HE staining method for visualization under microscopic inspection. For immunohistochemistry and immunofluorescence, sections were incubated with indicated primary antibodies. Detection was performed using either the Vectastatin Elite ABC-HRP kit (Vector Laboratories) with DAB Peroxidase Substrate kit (Vector Laboratories) or a fluorescent secondary antibody (Thermo Fisher Scientific).

2.11 | Patient tissue samples

The tissue samples were obtained from nine patients with uveal melanoma treated at the Eye and ENT Hospital of Fudan University. All UM and the surrounding normal choroid samples were collected at the time of enucleation, and informed consent was obtained from each patient. This study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University.

2.12 | Animal experiments

Six-week-old male BALB/c nude mice were supplied and housed in the Animal Center of the Eye and ENT Hospital of Fudan University. OCM3 cells (5×10^6) were resuspended in 100 μL of HBSS and injected subcutaneously into the right flank of the mice. The tumor volumes were monitored with a slide caliper every 2 days with the calculation formula $0.5 \times \text{length} \times (\text{width})^2$. Twelve days later, the tumors grew into palpable sizes of 100 mm^3. GA was administered by oral gavage at 50 mg/kg, and 0.1 mL saline was administered to the control group. The tumor sizes and body weights of the mice were monitored every 2 days. Thirty days after injection, all the mice were sacrificed. The tumor tissues were carefully excised, photographed and weighed, and subsequently fixed in formalin for further analysis. All animal procedures were conducted following the guidelines of Fudan University on the Ethical Use of Animals.

2.13 | Statistical analysis

Each experiment was performed in at least triplicate. Band intensities on western blots were determined using BIO-RAD Quantity One software. Graphical presentations were performed with Prism software. All quantitative data are presented as the mean ± SEM values. Statistical significance in all experiments was analyzed using Student's *t*-test (****, $P < .0001$; ***, $P < .001$; **, $P < .01$; *, $P < .05$).

3 | RESULTS

3.1 | Global SUMOylation is increased in UM cell lines

We first established an immortalized human uveal melanocyte cell line from a healthy donate (see Materials and Methods). The cultured melanocytes showed spindle-like morphology and melanin-producing ability (Figure S1A). The proliferation rates of cultured melanocytes were increased with passage and became stable after passage 40 (Figure S1B). Cell cycle analysis showed prolonged S-phase arrest in passage 40 cells compared with passage 5 cells (Figure S1C). The uveal melanocytes were positive for both vimentin

and S100 and negative for anti-cytokeratin, ruling out the possibility of either fibroblast or retinal pigmented cell contamination (Figure S1D-F).[26]

To investigate the role of SUMOylation in human uveal melanoma, we first detected the global SUMOylation status in 10 human UM cell lines, together with two primarily cultured human skin fibroblasts and the melanocyte cell line as control groups. Among the 10 UM cell lines, OMM1, OMM2.2, OMM2.3, and OMM2.5 were cultured from metastatic lesions,[27] while the other six cell lines were of primary origin.[28-30] Western blotting using an antibody against SUMO1, one of the most frequently studied SUMO isoforms, revealed that the levels of global SUMO modification were significantly increased in almost all UM cell lines compared with the three control groups (Figure 1A,B), suggesting that SUMO1 modification in UM cells was enhanced. Consistently, we observed a significantly upregulated SUMOylation in the patient UM tissues when compared with the distal normal uvea tissues (Figure 1C,D and Table S1).

3.2 | Enhanced SUMO1 modification promotes cell growth and inhibits apoptosis in UM cell lines

Previous studies have shown that SUMOylation is significantly increased in multiple tumors of various origins and plays an important role in tumor cell proliferation,[20] therefore we next explored the functional role of upregulated SUMOylation in UM cell lines. After lentiviral transduction of two independent SUMO1 small hairpin RNAs (shRNAs) into three tumor cell lines, OCM3, Mel 285, and OMM2.3, qPCR analysis and anti-SUMO1 immunoblotting showed reduced SUMO1 levels and conjugation, respectively, in the cell lines tested when compared with cells transduced with a nontargeting shRNA control virus, confirming that the two shRNAs efficiently diminished global SUMO1 modification (Figures 2A and Figure S2A). As shown in Figure 2B, the proliferation assay demonstrated that compared with the control constructs, SUMO1 shRNAs induced significant inhibitions of proliferation in UM cell lines (Figure 2B). Similar results were observed in the colony-formation assay in vitro (Figure 2C). These data suggested that a high level of SUMO1 modification is required for UM cell growth.

Ginkgolic acid is a potent SUMOylation inhibitor that blocks the formation of E1-SUMO thioester complexes.[31] We next examined the effect of GA on SUMOylation and cell growth in UM cell lines. OCM3, Mel202, and OMM2.3 cells were incubated with increasing concentrations of GA for 24 hours. We confirmed that the SUMOylation levels in UM cell lines were significantly decreased by GA treatment in a dose-dependent manner (Figure 2D). In addition, 50 μmol/L of GA reduced global SUMO1 conjugation in these cells by ~60%-80% compared with untreated cells. Accordingly, cell growth rates were significantly decreased in the GA-treated groups (Figure 2E,F). Similar results were also observed in UM cell lines treated with another SUMO E1 inhibitor, ML-792, which forms an adduct with SUMO,[32] further confirming the anti-growth effects of SUMOylation inhibition in UM cells (Figure S2B). Moreover, cell cycle analysis revealed

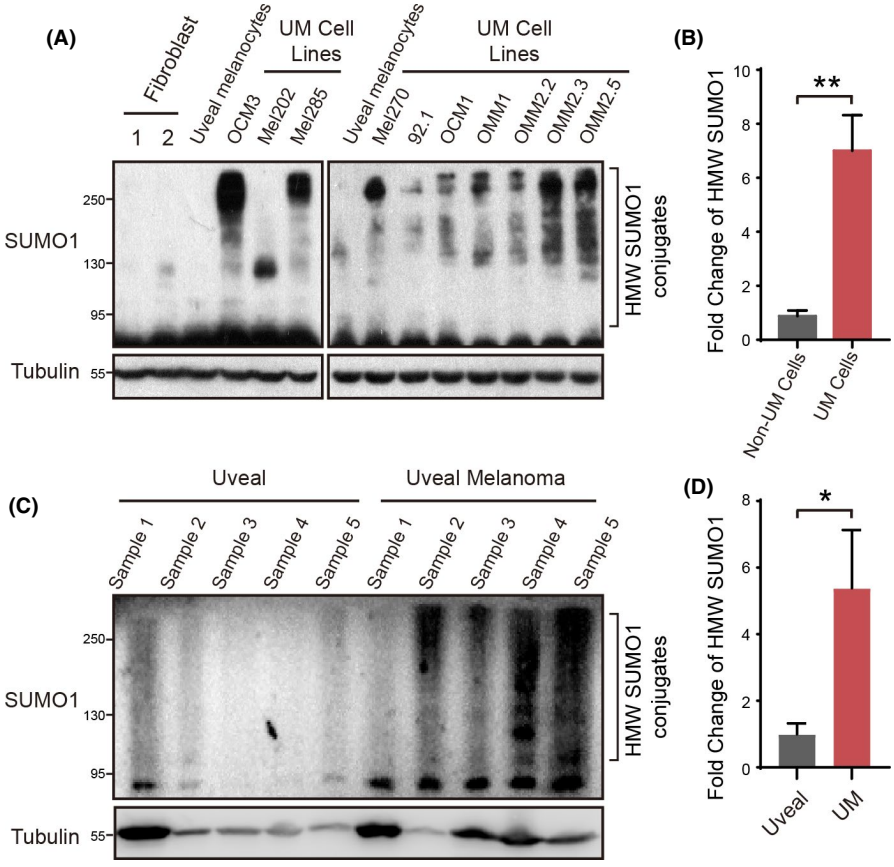

FIGURE 1 Increased global small ubiquitin-like modifier (SUMO)ylation in uveal melanoma (UM) cell lines. A, The high molecular weight (HMW) SUMO conjugates levels in 10 UM cell lines, two primarily culture human fibroblasts, and an immortalized human uveal melanocyte cell line were determined by immunoblotting for SUMO1. B, Quantification of the data shown in (A). C, The HMW SUMOylation levels in UM and the distal normal choroid tissues were determined as in (A). D, Quantification of the data shown in (C)

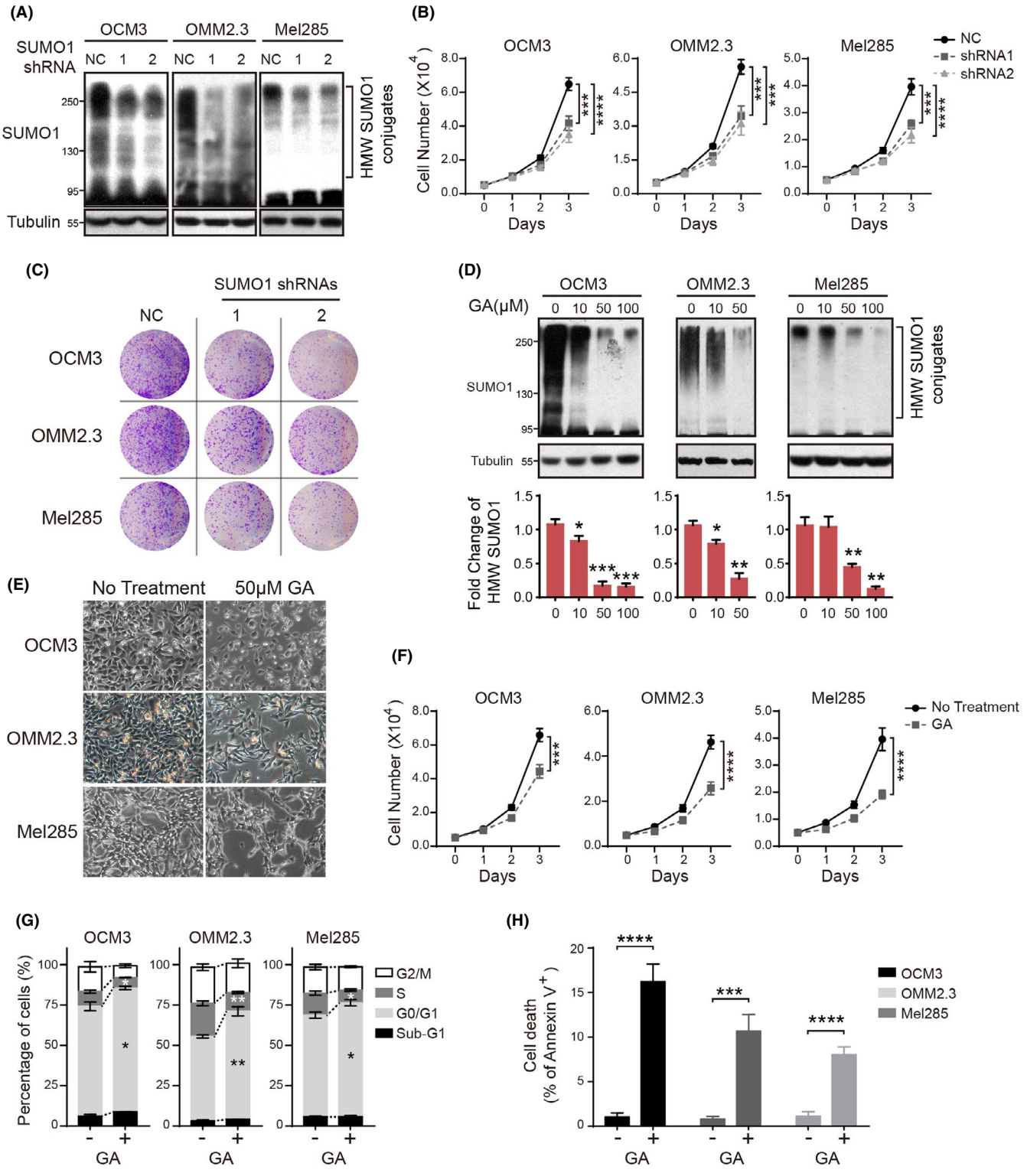

FIGURE 2 Enhanced small ubiquitin-like modifier (SUMO)ylation promotes uveal melanoma (UM) cell growth and inhibits apoptosis. A, Three indicated UM cell lines were transduced with two short hairpin RNAs (shRNAs) against SUMO1 or a nontargeting control (NC) shRNA for 72 h. SUMO1 levels were determined by immunoblotting. B, C, UM cells transduced with the shRNAs were subjected to either cellular growth determination (B) or colony formation evaluation (C). D, UM cells were treated with the indicated concentration of ginkgolic acid (GA) for 24 h and subjected to immunoblotting analysis of SUMO1. E, F, Representative images (E) and quantification (F) of cell growth for 72 h with or without GA treatment. G, UM cells were treated with 50 μmol/L GA for 24 h. Cell cycle progression was assessed using Propidium iodide staining and flow cytometry. H, Apoptosis rates of UM cells treated with 50 μmol/L GA were measured by annexin V staining

that G1-phase cells were increased while the percentage of S-phase cells was decreased in GA-treated cells compared with the control group (Figure 2G). We also examined the effect of SUMOylation on the survival of UM cells. UM cells were treated with GA, and apoptosis was determined by staining for annexin V, a marker for cell death, followed by microscopic examination. Inhibition of SUMOylation by GA treatment increased the proportion of apoptotic cells (Figure 2H).

Taken together, our data indicate that inhibition of protein SUMOylation by both genetic and pharmacological approaches decreases cell proliferation and induces cell death in UM.

3.3 | Pharmacological inhibition of SUMOylation suppresses the growth of uveal melanoma tumor xenografts in vivo

To further confirm the suppressive function of GA against UM tumor growth in vivo, a subcutaneous xenografted UM mouse model was employed. OCM3 cells were injected subcutaneously (s.c.) into 6-week-old male nude mice. After implantation, the tumor volumes and body weights of the mice were measured every 2 days. After 12 days, when the tumors were palpable, the mice were randomly divided into two groups, and GA was administered via oral gavage daily as described in the Materials and Methods section. Treatment with GA induced marked tumor growth inhibition (Figure 3A). The overall body weights of the xenograft mice in the GA group were not significantly different from those in the control group (Figure 3B). After the mice were sacrificed at day 30, the tumor volumes were measured, and the GA group showed significantly smaller tumor volumes than the corresponding vehicle-treated mice (Figure 3C). Ki-67 levels in xenograft tumor samples were analyzed to assess the proliferative status of different tumor samples. As shown in Figure 3D, the percentages of Ki-67-positive cells in the GA-treated groups (8.3%) were significantly smaller than those in the control group (38.8%). In addition, when the histology of these tumors was evaluated by HE staining, larger necrotic areas were identified in the tumors from the GA-treated group (Figure 3E). Finally, the SUMOylation inhibiting effects of GA on the xenograft tumor tissues were confirmed by immunoblotting (Figure S3A). These results supported the notion that enhanced SUMO conjugation participated in UM growth and demonstrated the antineoplastic efficacy of GA in UM in vivo.

3.4 | Rb is near completely hyperphosphorylated and inactivated in UM

Next, we aimed to understand how SUMO modification regulates UM cell growth. Rb protein is a well-established tumor suppressor whose inactivation results from hyperphosphorylation and is crucial for cancer cell growth, apoptosis inhibition, and tumor progression. Rb can be SUMOylated on K720 in the B domain of the pocket region, and this modification controls its phosphorylation and activity, which is crucial for cell growth.[33,34] Therefore, we hypothesized that SUMO modification of Rb might be the key step that connects the overall SUMO conjugation capacity with cell growth control in UM cells.

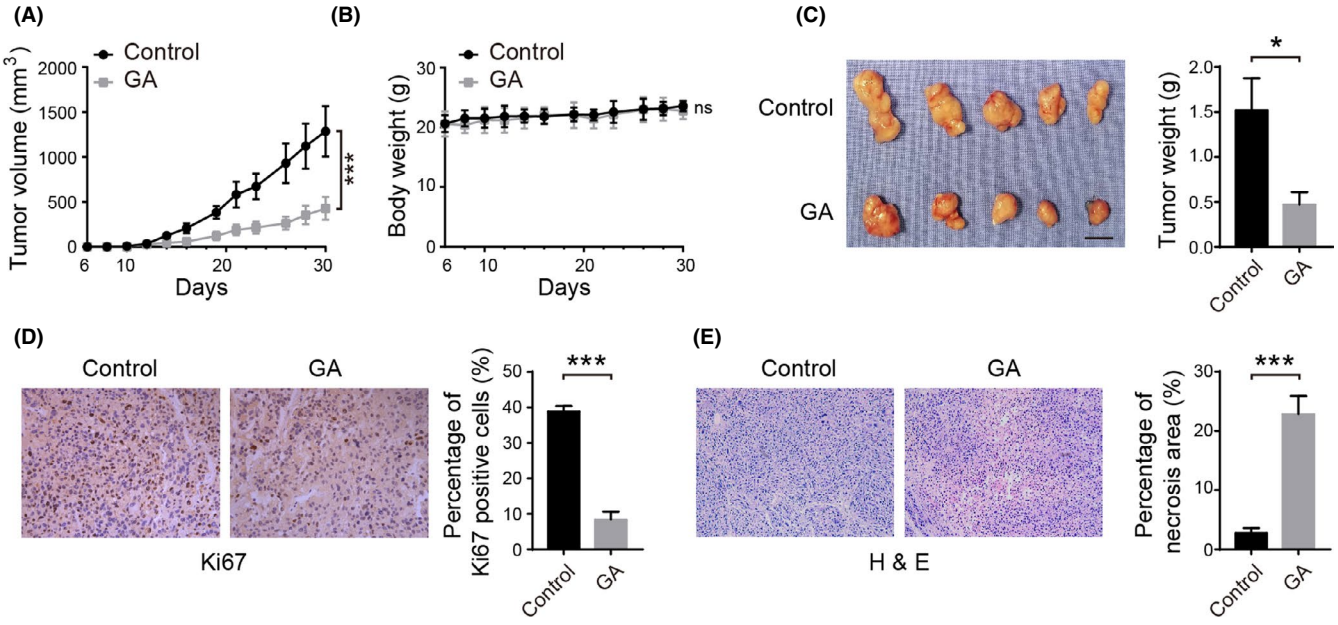

FIGURE 3 Small ubiquitin-like modifier (SUMO)ylation inhibition by ginkgolic acid (GA) inhibits tumor growth in vivo. A, Volumes of xenografted tumors derived from uveal melanoma (UM) OCM3 cells in nude mice treated with GA or saline for 24 d. B, The body weight of mice was recorded. C, Representative images and weights of xenograft tumors from saline or GA-treated groups at the end time point. Scale bar, 1 cm. D, Representative images of Immunohistochemistry staining of tumor xenografts for Ki67. The percentages of Ki67 positive cells are shown. E, Representative images of hematoxylin-eosin staining of tumor xenografts. The percentages of necrosis areas are shown

To test this idea, we first assessed the Rb function in UM. Although the implications of Rb inactivation in several major cancers have been demonstrated, the functional role of the Rb in uveal melanoma is not fully understood. Since the *Rb* gene is not mutated in UM, we analyzed the protein levels and phosphorylation status of the Rb protein in UM tissue samples and cell lines. Immunofluorescence staining analyses of the enucleated eyes of UM patients revealed significantly elevated levels of Rb in the tumors compared with the normal uvea tissue distal to the tumors (Figure 4A,B and Table S1). We further confirmed the upregulation of Rb in tumor tissues by western blotting (Figure 4C). Furthermore, by immunoblotting for two major phosphorylation sites of Rb (807/811, ppRb), we confirmed that the phosphorylation of Rb was significantly upregulated in UM tissues (Figure 4C).

Similar upregulated levels of Rb protein were found in all UM cell lines compared with fibroblasts and uveal melanocytes (Figure 4D). Inactivation of the Rb by hyperphosphorylation results in a band shift to a more slowly migrating band when examined by immunoblotting against the Rb protein. We found that only 25% or ~35% of the total Rb content in normal uveal melanocytes or fibroblasts, respectively, was hyperphosphorylated (Figure 4D). Interestingly, we found that 80% of the total Rb content in the OMM2.2, MEL270, and Mel285 cells and more than 90% of the total Rb content in the remaining seven UM cell lines were in the hyperphosphorylated form, as evidenced by the higher molecular weight of the Rb bands in UM cells (Figure 4D, Rb panel). In contrast, the faster migrated, hypophosphorylated forms of Rb were significantly diminished in UM cells compared with normal uveal melanocytes. Consistently, the phosphorylation levels of Rb were significantly increased in UM cells (Figure 4D, ppRb panel).

Next, to examine the roles of the Rb in UM cells, we compared the growth rates of OCM3, OMM2.3, and Mel 285 cells transduced with a control or two independent Rb-specific shRNAs. Compared with control shRNA, transduction of Rb shRNAs significantly reduced Rb protein levels and increased the proliferation rates in the tested cells (Figure 5A,B). These data suggest that, although most Rb protein was in the hyperphosphorylated form in all tested UM cells, the remaining active, hypophosphorylated Rb still functions to inhibit cancer cell growth. To further investigate the functional role of the Rb in UM cell growth regulation, we overexpressed the Rb in normal uveal melanocytes and UM cell lines. Overexpression of the Rb significantly reduced the growth rate of normal uveal melanocytes (Figure 5C). However, the growth rates of UM cells were not affected by Rb overexpression, suggesting that UM cells overcame the antiproliferative effects resulting from the ectopically overexpressed Rb (Figure 5C). Indeed, we confirmed that the overexpressed Rb was functionally inactivated, as evidenced by immunoblotting showing that they almost exclusively existed as the slow-migration, hyperphosphorylation form and exhibited strong immune signals for the anti-phospho-Rb antibody (Figure 5D).

Together, our data suggest that while Rb protein expression is upregulated in UM cells, they are functionally inactivated by hyperphosphorylation.

3.5 | Enhanced SUMO1 conjugation of Rb causes its inactivation and promotes cell growth in UM cell lines

SUMO conjugation of the Rb stimulates its hyperphosphorylation and inactivation, which facilitates cell cycle progression and proliferation.[24] Therefore, we hypothesized that due to the upregulated global SUMOylation activity in UM cells, the SUMO conjugation level of the Rb could be increased in UM cells and subsequently promoted its phosphorylation. To test this idea, we first analyzed the SUMOylation status of Rb in UM cells. After immunoprecipitation (IP) of endogenous Rb in normal uveal melanocytes and three UM cell lines, we detected strong SUMO1 signals in the tumor cells, suggesting that, in accordance with the upregulated global SUMO1 conjugation activity in these cells, SUMOylation of Rb protein was enhanced in UM cells (Figure 6A). To further test whether increased SUMO1-Rb promoted its phosphorylation, we treated UM cells with GA to inhibit SUMO1 conjugation activity. SUMOylation of Rb was significantly decreased in UM cells treated with GA (Figure 6B). Immunoblotting showed that GA treatment significantly reduced Rb protein phosphorylation (ppRb panel) in a dose-dependent manner (Figure 6C). Similar reductions in Rb phosphorylation were observed in the GA-treated xenograft tumor tissues (Figure S3B). Moreover, a switch from slow-migrating hyperphosphorylated to fast-migrating hypophosphorylated Rb protein was observed in each UM cell lines when treated with GA (Figure 6C). Similar results were obtained in UM cells after SUMOylation inhibition by a SUMO1 shRNA (Figure 6D). Thus, the data suggest that the hyperphosphorylation of Rb in UM is likely caused by its enhanced SUMO modification due to the upregulated global SUMO-conjugation activities in these cells. To further confirm this idea, we took advantage of the fact that replacing the SUMO-accepting lysine residue of Rb with an arginine (K720R) is sufficient to abolish the SUMO modification of this protein.[34] After overexpressing wild-type and SUMO-deficient Rb in UM cell lines, immunoblotting demonstrated that approximately half of the total mutant Rb content still existed as faster-migrating, functional forms, in contrast to the entirely hyperphosphorylated wild-type Rb proteins, suggesting that SUMOylation of the Rb protein was necessary for its hyperphosphorylation in UM (Figure 7A). Consistent with this finding, due to the partially restored Rb function in UM cells, overexpression of the SUMO-deficient K720R mutant but not wild-type Rb significantly inhibited UM cell growth (Figure 7B). Such anti-growth effects of mutant Rb could be abolished by knocking down Rb protein itself (Figure 7C). Moreover, inhibition of global SUMOylation by GA administration conferred the wild-type Rb protein a similar capacity as mutant Rb to inhibit UM cell growth, further confirming that SUMOylation underpinned the differential anti-growth effects between wild-type and SUMO-deficient Rb proteins (Figure 7D). Together, the results suggested that, as a result of enhanced global SUMOylation activity, SUMO modification of the Rb protein promotes UM cell growth by stimulating its hyperphosphorylation and inactivating this protein.

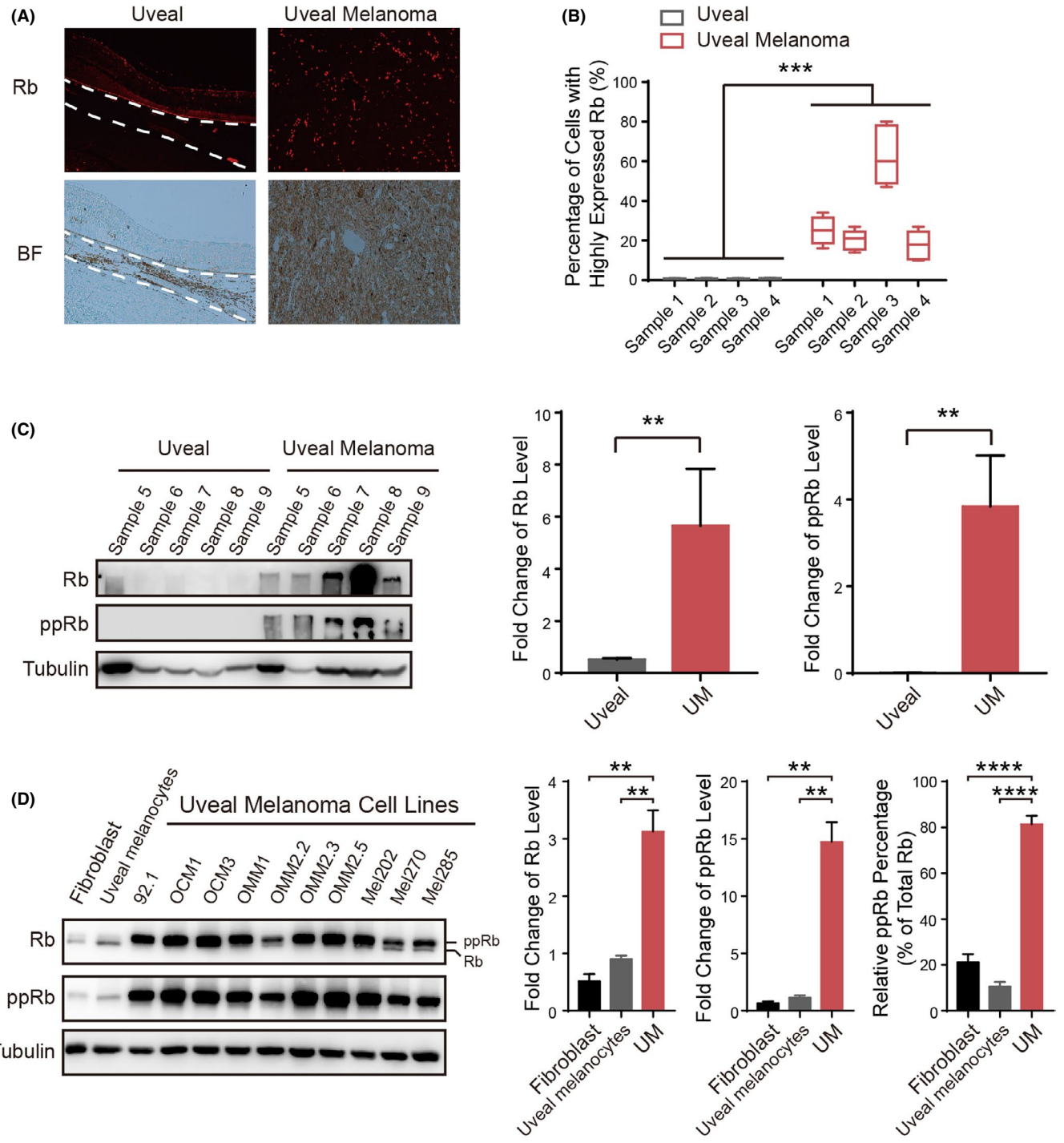

FIGURE 4 Retinoblastoma protein (Rb) is dramatically hyperphosphorylated and inactivated in uveal melanoma (UM). A, Representative images of immunofluorescence staining for Rb and bright-field images of UM and the surrounding normal choroid. B, The percentage of Rb-positive cells in samples from (A). C, D, Total Rb protein levels (Rb panel) and hyperphosphorylated Rb levels (Ser 807/811; ppRb panel) in UM and the distal normal choroid tissues (C) or indicated UM or non-UM cell lines (D) were determined by immunoblotting and quantified. Positions of hypophosphorylated Rb (Rb) and hyperphosphorylated Rb (ppRb) were indicated (D)

4 | DISCUSSION

SUMOylation is one of the most common posttranslational modifications and regulates protein activities by altering their functions. This highly dynamic modification participates in many critical cellular processes, such as transcription, replication, genome integrity, DNA repair, and cell cycle progression.[35] Accumulating evidence suggests that dysregulation of SUMOylation is involved in cancer development and progression. In an shRNA-mediated genome-wide screen, several SUMO pathway enzymes, including SAE1 and Ubc9, were identified as essential genes for cancer cell proliferation.[36] Overexpression of Ubc9, the sole SUMO E2

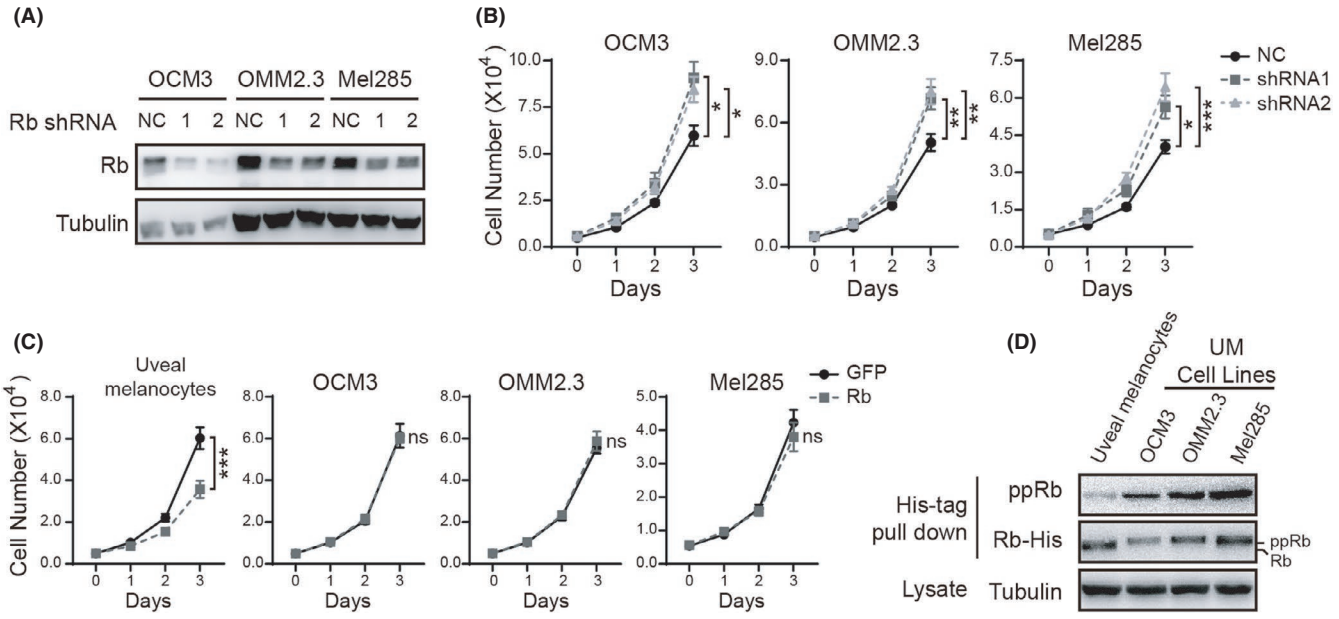

FIGURE 5 The antiproliferative potential of retinoblastoma protein (Rb) is constitutively inhibited in uveal melanoma (UM). A, The knockdown efficiencies of two Rb short hairpin RNAs (shRNAs) in three indicated UM cell lines were analyzed by western blot. NC, nontargeting control. B, C, UM cells transduced with either indicated shRNAs (B) or Rb or green fluorescent protein control (C) were subjected to cellular growth determination. D, Uveal melanocytes and indicated UM cell lines were transduced with Rb-His and subjected to pulldown of Rb-His using nickel-nitrilotriacetic acid-agarose beads. Total or hyperphosphorylated forms of Rb were determined by western blot. Positions of hypophosphorylated Rb (Rb) and hyperphosphorylated Rb (ppRb) were indicated

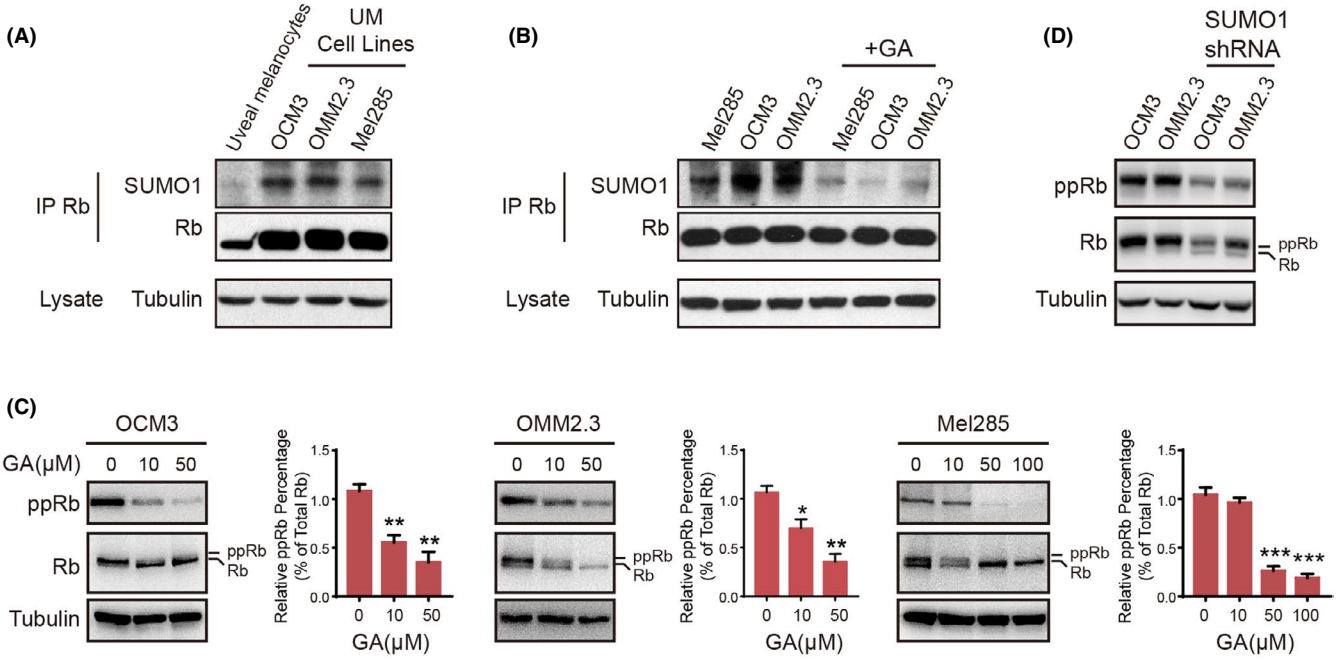

FIGURE 6 Downregulation of retinoblastoma protein (Rb) small ubiquitin-like modifier (SUMO)ylation decreases its phosphorylation level in uveal melanoma (UM) cell lines. A, Uveal melanocytes and three UM cell lines were lysed in denaturing RIPA buffer, immunoprecipitated with an Rb antibody, and examined by western blotting using SUMO1 and Rb antibody. B, Rb-SUMO1 species in indicated UM cell lines with or without ginkgolic acid (GA) treatment for 24 h were determined as described in A. C, D, Total or hyperphosphorylated forms of Rb in three UM cell lines treated with indicated concentrations of GA for 24 h (C) or two UM cell lines transduced with a SUMO1 shRNA (D) were determined by western blot

enzyme catalyzing the conjugation of SUMO to target proteins, was found in several malignancies, including lung and ovarian carcinoma.[16,18,37] Pharmacological or genetic inhibition of the activity of key enzymes in SUMOylation, including SAE1/2 and Ubc9, has been found to suppress cancer growth and induce synthetic lethal effects and regression in multiple cancers.[19-21,38]

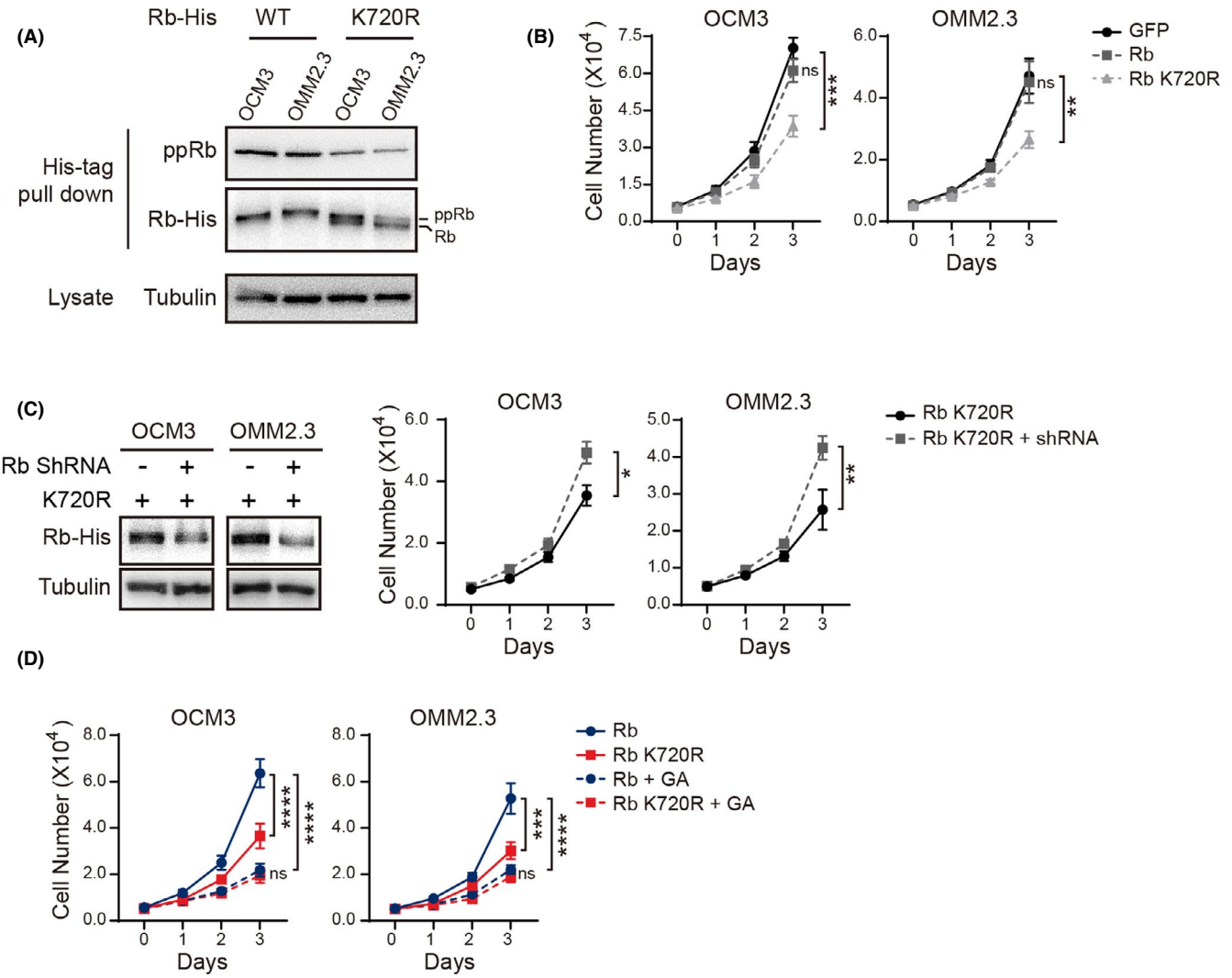

FIGURE 7 Small ubiquitin-like modifier (SUMO)ylation deficiency of retinoblastoma protein (Rb) restores its antiproliferative effect in uveal melanoma (UM) cell lines. A, Two UM cell lines were transduced with either His-tagged wild-type or a SUMO-deficient mutant (K720R) Rb and subjected to pulldown procedures. Total or hyperphosphorylated forms of ectopic His-tagged Rb proteins in the pulldowns were determined by western blot. B, Two UM cell lines transduced with either wild-type or mutant Rb-His were subjected to cellular growth determination. C, Two UM cell lines expression mutant Rb-His were transduced a shRNA targeting Rb protein and then subjected to cell growth analyses. Rb-His levels were determined by immunoblotting. D, Cell growth analyses of indicated UM cell lines expressing either wild-type or mutant Rb-His, with or without ginkgolic acid (GA) treatment

Overexpression of a dominant-negative mutant form of Ubc9 is associated with increased sensitivity to anticancer drugs.[39] However, whether SUMOylation contributes to the development and progression of UM is still unclear. Here, we showed that global SUMO1 conjugation activity was enhanced in 10 UM cell lines and UM tissues. Furthermore, we demonstrated that such upregulated SUMOylation activity is required for cancer cell growth. Therefore, our data add to the growing evidence for the functional connection between SUMO modification and cancer. Moreover, multiple chemical compounds have been reported to inhibit SUMOylation.[40] GA, a natural compound that blocks SUMOylation by inhibiting the formation of the SAE1/2 thioester complex and the subsequent conjugation of SUMO to target proteins, has been demonstrated to inhibit growth and invasion and promote apoptosis in a number of cancers both in vitro and in vivo.[41-43] Here, we further demonstrated that administration of GA blocks upregulated SUMOylation in UM cell lines and is sufficient to reduce cancer cell growth and promote apoptosis. Thus, our data suggest that blockade of global SUMOylation may inhibit UM development, making SUMOylation a potential therapeutic target.

While the enhanced activity of the global SUMOylation cascade has been implicated in developing multiple cancers, the mechanisms by which cancer cells are regulated are not fully understood. SUMO conjugation changes the activities of the modified target proteins by altering the protein-protein interaction, subcellular localization, stability, and interplay with other posttranslational modifications. Thus, to understand the SUMOylation-regulated phenotypes in cancers, the key is to identify the substrates whose SUMO statuses and functions are altered in cancer cells with enhanced global

SUMO conjugation capacity. Indeed, a previous study demonstrated that global SUMOylation in glioblastoma is enhanced and promotes cancer cell growth; consequently, CDK6 is SUMOylated and therefore stabilized and drives the cell cycle for cancer development and progression.[44] Similarly, SUMOylation of SMAD4 and Rac1 is required for breast and oral cancer growth, respectively.[41,42] Here, we revealed that the Rb protein is a key downstream effector of the upregulated global SUMOylation capacity in UM. The Rb protein is the first identified tumor suppressor whose inactivation is involved in retinoblastoma and several other major cancers. In noncycling cells, the Rb protein is present in its hypophosphorylated, active form to bind E2F transcription factors to repress the transcription of cell cycle target genes. On growth stimulation, Rb becomes hyperphosphorylated to its inactivated form by cyclins and CDKs, such as cyclin E/CDK2 and cyclin D/CDK4/6, resulting in the transcription of S phase genes.[23,45] In a previous study, we found that after cell cycle synchronization, SUMOylation of the Rb protein specifically occurs at the early G1 phase.[24] This modification promotes hyperphosphorylation and inactivation of the Rb protein and cell cycle progression by enhancing the interaction between Rb and CDK2 through a SUMO-interaction motif (SIM). Interestingly, we found that SUMOylation of the Rb protein is significantly increased in UM cells and that this modification can be readily detected without cell cycle synchronization, suggesting that SUMOylation of the Rb protein is constitutively enhanced in UM cells. GA administration significantly reduces Rb SUMOylation, suggesting that the increased Rb SUMOylation is caused by elevated global SUMO conjugation activity in UM cells. SUMO1 modification is crucial for Rb protein function, and blocking SUMOylation of the Rb protein restores its antiproliferative function in UM cells. Therefore, our detailed mechanistic study revealed a new pathway through which global SUMOylation controls cancer cell growth and apoptosis.

Although the Rb protein has never been found to be mutated in UM cells, Brantley et al reported that, while Rb proteins can be identified in most of the cells from UM or normal uveal melanocytes, the hyperphosphorylation signal can only be detected in a portion of cancer cells (1.4%-3.3%), suggesting that Rb protein is frequently dysregulated in UM cells.[46,47] Consistent with this finding, Pardo et al[48] reported that, at least in one primary cultured UM cell line, the interaction between Rb and E2F is disrupted. However, it is still unclear how the Rb protein is inactivated and to what extent its dysregulation contributes to UM cell growth. Here, we demonstrated that the Rb protein was near completely hyperphosphorylated and inactivated in UM samples and all 10 tested UM cell lines, strongly suggesting that Rb protein inactivation is closely associated with UM development. While we also observed an upregulation of Rb protein levels in UM cells, such increased Rb protein amounts fail to restore its activity, since they are almost exclusively hyperphosphorylated and inactivated, suggesting that the overall function of Rb protein is limited in UM. The upregulated Rb protein levels in UM cells could result from an unknown compensatory mechanism aimed at restoring normal cell growth. Downregulation of global SUMO-conjugation activity by both genetic and pharmacological approaches could decrease Rb protein SUMOylation and its phosphorylation level, leading to inhibition of cell growth and increased apoptosis, suggesting that global SUMOylation activities in UM cells promotes Rb hyperphosphorylation by directly stimulates its SUMO-conjugation. However, we noticed that the hyperphosphorylation levels of Rb are not proportional to the global SUMOylation levels in each of the UM cell lines, indicating that the SUMOylation-controlled hyperphosphorylation of Rb in UM involves additional regulatory mechanisms. Moreover, our study does not exclude the possibility that additional substrates may also contribute to the pro-growth effect of the enhanced global SUMOylation in UM. In summary, our study showed that Rb acts as a critical effector of the general SUMOylation upregulation during tumor development, and SUMOylation-induced hyperphosphorylation of Rb provides novel insight into the mechanism of tumorigenesis and progression.

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DISCLOSURE

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ORCID

Fengxi Meng https://orcid.org/0000-0002-1742-3283

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SUPPORTING INFORMATION

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