SUMO-1 Overexpression Increases RbAp46 Protein Stability and Suppresses Cell Growth

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Abstract. *The retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46) is a nuclear protein of 46 kDa and contains four repeats that end with Trp-Asp (WD) residues. In this study, we reveal that the RbAp46 protein level upon SUMO-1 expression was increased. The increasing level of RbAp46 protein by SUMO-1 was not regulated at the transcriptional level. SUMO-1 does not affect the degradation of RbAp46. Co-localization of RbAp46 and SUMO-1 in the nuclei of stable NIH/3T3 cells harboring the inducible Ha-ras^{Val12} oncogene (pSVlacOras) designated as 7-4, and protein-protein interaction between RbAp46 and SUMO-1 was also detected by co-immunoprecipitation in these cells. However, SUMO-1-related sumoylation was not involved in the modification of RbAp46. It is possibly that SUMO-1 acts through formation of complex with RbAp46 to stabilize RbAp46 protein. Overexpression of RbAp46 protein suppressed the NIH/3T3 cell growth induced by Ha-Ras^{V12}. SUMO-1 further enhances the suppression of cell growth through stabilization of RbAp46 protein. This is the first report to demonstrate that SUMO-1 can suppress Ras-related cell proliferation through stabilization of RbAp46 protein.*

The retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46) is a nuclear protein of 46 kDa which contains four WD repeats that end with Trp-Asp residues (1-3). Human RbAp46 shares strong homology with MSI1, a protein characterized as a negative regulator of the Ras signal transduction pathway in *Saccharomyces cerevisiae* (2, 4). Overexpression of human RbAp46 or MSI1 suppresses heat-shock sensitivity and decreases cellular cAMP levels in a constitutively active RAS2Val-19 mutant yeast strain (2). Recently, RbAp46 has been shown to be a component of the histone deacetylase (HDAC) complex mSin3 (5), which is involved in the transcriptional repression mediated by growth-related transcription factors, such as Mad-Max, members of the oncogene Myc family (6), the acute promyelocytic leukemia-I associated PLZF protein (7), LAZ-3 (Bcl-6) oncogene (8), and acute myeloid leukemia-1 (AML-1) (9). RbAp46 is also found in NuRD (nucleosome remodeling histone deacetylase complex), a multi-subunit complex with chromosome-remodeling activity (10). Thus, RbAp46 may play an important role in modification and remodeling of chromatin during cell growth and differentiation. The exact cellular and molecular functions of RbAp46 in the above processes are as yet unknown.

A family of proteins intimately interrelated to ubiquitin has been reported. Small ubiquitin-like modifier (SUMO) is the best exemplified member of this group of ubiquitin-related proteins. In vertebrates, four diverse subtypes of SUMO1-4 have been identified and they are ~18% identical in sequence to ubiquitin (11-13). SUMO-1 is 50% indistinguishable in sequence to SUMO-2 and -3, and the conjugated forms of SUMO-2 and SUMO-3 only differ from one another by three amino terminal residues (12, 14). The targets and the *in vivo* functions of SUMO-2 and -3 modifications are still not well understood but appear to be distinct from that of SUMO-1 (14). In disparity to ubiquitination, SUMO targets proteins for proteosome-mediated degradation. SUMO-1 modification of target proteins is known to result in a number of substrate-specific functions. SUMO-1 modification of RanGAP1 is required for its localization to the nuclear pore complex (NPC) (15, 16), while SUMO-1 modification of promyelocytic leukemia protein (PML) is required for the correct assembly of nuclear bodies (17, 18). Alteration of the transcriptional inhibitor IκB-alpha by SUMO-1 takes place at the same lysine residue as ubiquitin conjugation and thus inhibits ubiquitination and stabilizes IκB-alpha (19). Most of the SUMO-1-modified substrates are nuclear proteins and function as mediators of transcription, chromatin modification and genomic stability (20).

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Sumoylation is a post translational modification by conjugation of a small peptide on a target protein (12). Conjugation occurs in 4 steps: (i) processing; (ii) activation by an E1-type enzyme; (iii) conjugation by an E2-type enzyme; (iv) ligation to the substrate by an E3-type enzyme. Ubiquitin-conjugating enzyme 9 (Ubc9) is the single E2-type enzyme for SUMO conjugation. Ubc9 directly interacts with the target proteins and sumoylates the protein with specificity. Ubc9 has been ascribed to the detection of substrate function.

Despite the reports of targets of sumoylation accumulating, very little is known about the function and regulation of sumoylation. Whereas ubiquitin primarily mediates protein degradation, sumoylation emerges as being implicated in the regulation of the function of various proteins by altering protein-protein interactions, subcellular localization and protein stabilization by antagonizing ubiquitinylation (21).

The role of SUMO-1 in RbAp46 protein stabilization is thus worthy of exploration. The functional role of SUMO-1 in RbAp46 was also investigated.

Materials and Methods

Putative sumoylation sites were identified in RbAp46 (NCBI Accession no: BC114501) by using Sumo Plot (Abgent Inc., CA, USA).

Antibodies. RbAp46 antibody (rabbit polyclonal) was purchased from AbCam, Cambridge, UK; anti-FLAG M2 monoclonal and anti-beta-actin were obtained from Sigma, Saint Louis, MO, USA; anti-GFP was purchased from Clontech, CA, USA; Mouse anti-SUMO-1 was obtained from Zymed Laboratories Inc, South San Fransisco USA; pan-ras was from Oncogene Research Products, San Diego, CA, USA. Appropriate anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Pharmacia Biotech (Sweden). p53-Ubc9-pcDNA3.0 was a kind gift from Dr. R. Niedenthal, Institut für Physiologische Chemie, Germany.

Plasmid construction. The plasmid pCMV-FLAG-RbAp46 encoding wild-type full length RbAp46 tagged with FLAG epitope was constructed as follows: The RbAp46 open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using the sense primer 5'- A TAG ATC TTG ATG GCG AGT AAA GAG ATG TTT GAA- 3'and the antisense primer 5'-AT AAG CTT AGA TCC TTG TCC CTC CAG TTC GGA T-3' for RbAp46. The PCR conditions were 94˚C for 3 min, 94˚C for 45 s, 55˚C for 45 s, 72˚C for 2 min, and final extension at 72˚C for 10 min. The PCR product was digested with *Bgl*II and *Bam*HI and cloned in the corresponding pCMV-FLAG plasmid (STRATAGENE, LA JOLLA, CA, USA). The plasmids of pRbAp46-GFP and pRbAp46-DsRed2 were constructed by releasing with the same enzyme sites from pCMV-FLAG-RbAp46 and cloned into pEGFPN1 (CLONTECH, CA, USA) and pDsRed2N1 (CLONTECH), respectively. The plasmid SUMO-1-GG was kindly provided by Dr. G.G. Hung (NCKU, Taiwan, R.O.C). The plasmid EGFP SUMO-1-GG was kindly provided by Dr. H.M. Shih (Academia Sinica, Taiwan, R.O.C). The plasmid pRL-TK was used to drive Renilla luciferase (Promega, Madison, WI, USA). RbAp46 promoter was amplified from genomic DNA extract of E7 immortalized uroepithelial cells with the following conditions: denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, extension 1 min at 72˚C and final extension at 72˚C for 10 min for 35 cycles. The primers were used sense 5'-CCTAGCTAG CACCCTTCCAACTCTCTCTTCCCTCTC-3', and antisense 5'-CCCCAAGCTTGCGCTCTTCTCTCTCTCTCC AAAC-3'. The *NheI* and *HindIII* sites were used to clone the DNA fragment into the pGL3–basic vector (Promega) to create pGL3-RbAp46 promoter plasmid. RbAp46-Ubc9-pcDNA3.0 was constructed by using *Bam*HI and *Eco*RI. RbAp46-EGFP plasmid was amplified and digested with the above restriction enzymes and the pcDNA3.0-p53-Ubc9 plasmid was also digested with the same enzymes which resulted in the excision of p53 from the plasmid insertion of RbAp46 into the Ubc9-pcDNA3.0.

Cell culture. A mouse fibroblast NIH3T3 cell line harboring the inducible *Ha-ras*^{Val12} oncogene (pSVlacO*ras*) designated as 7-4 (22) and human embryonic kidney 293T (HEK 293T) cell line were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum at 37˚C in a 5% CO_2 incubator. All tissue culture media were supplemented with 100 μg/ml of penicillin and 100 μg/ml of streptomycin.

Transient transfection. Cells were plated on 6×10 cm Petri-plates at a density of 5×10^6 and cultured for 24 h. Transient transfection was carried out by electroporation (Gene pulser Xcell; Bio-Rad, CA, USA). The electroporation parameters were 210 mV, 500 μF, and 4 cm path length using 25 and 50 μg of plasmid DNA/plate. Expression of the transfected gene was analyzed 48 h after transfection.

Cell lysis and Western blotting. Cells were harvested after 48 h transfection in a modified RIPA buffer containing 50 mM Tris (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton®-x-100, 0.5% Nonident P40 and 0.1% sodium deoxycholate with complete protease inhibitors. Fifty μg of protein were loaded (per lane) for Western blotting experiments. Proteins were separated by sodium dodecyl (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidine fluoride (PVDF) membrane. Blots were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dried milk for 2 h at room temperature. Primary antibodies were incubated overnight at 4˚C and washed three times each in TBST. Horseradish peroxidase-conjugated IgG secondary antibodies (Chemicon International, Temecula, CA, USA) were added to TBST and membranes were incubated for 1 h in secondary antibody followed by three washes in TBST (10 min each). Western blots were incubated with ECL Western blotting reagent (Millipore, Billerica, MA, USA), and exposed to X-ray film.

Co-immunoprecipitation. Cells were harvested in lysis buffer and cellular proteins (500 μg) were incubated with anti-RbAp46 antibodies at 4˚C for 16 h. Immunocomplexes were collected by adding 50 μl of protein A agarose beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Immunocomplex was separated by adding SDS-PAGE sample buffer and boiling for 10 minutes. Samples were electrophoresed on a 10% polyacrylamide gel and transferred to a PVDF membrane; the blots were probed with anti-SUMO-1 antibody. Western blots were incubated with ECL Western blotting reagent (Millipore) and exposed to X-ray film.

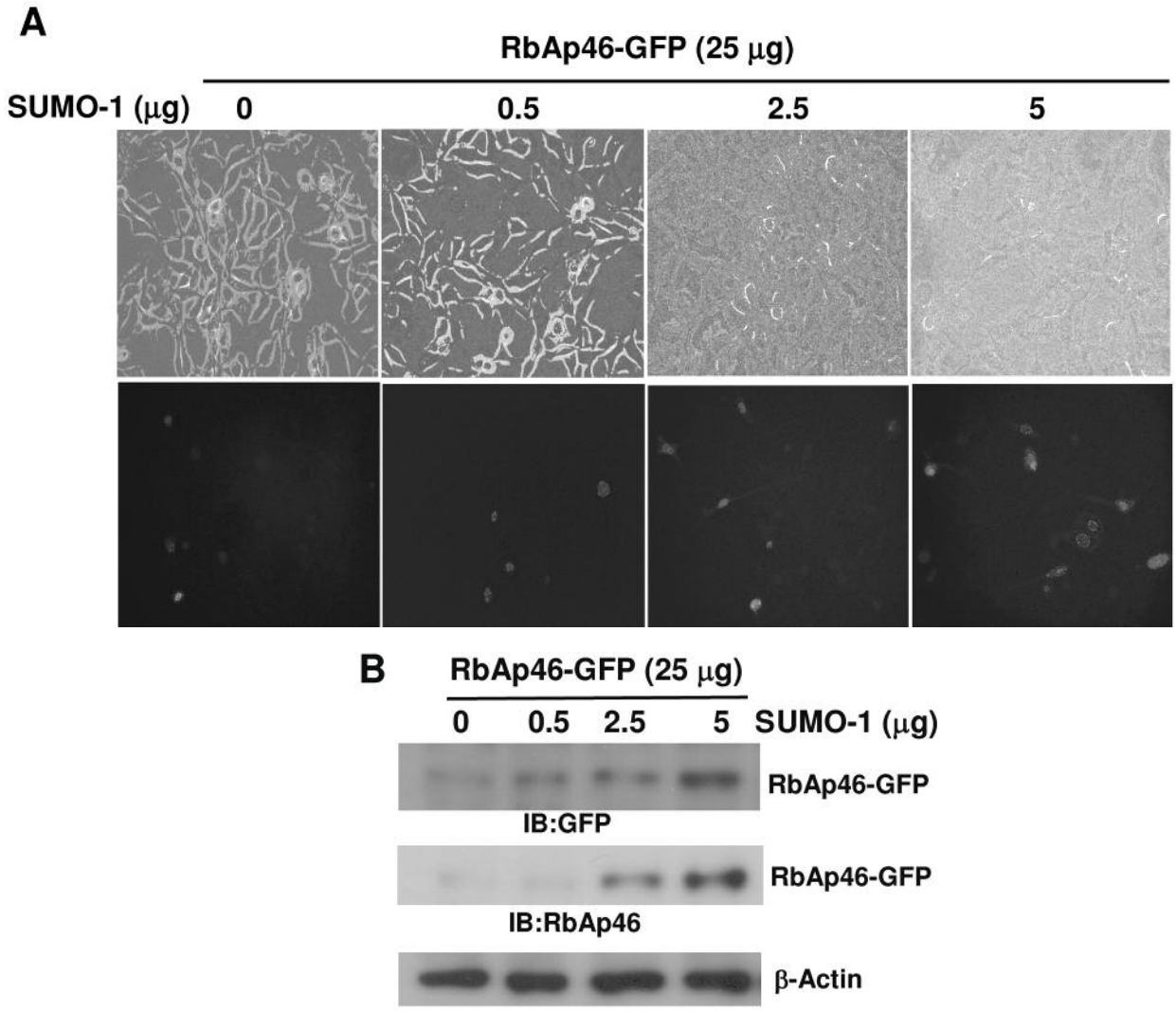

Figure 1. *SUMO-1 increases the fluorescence of EGFP-RbAp46 protein. A, Different concentrations of pCMV-Sumo-1 (0.5-5 μg) were co-transfected with pEGFP- RbAp46 (25 μg) into 7-4 cells which were observed under a fluorescence microscope at 48 h post transfection. B, The cell lysates were prepared and blotted with the antibodies against GFP and RbAp46 proteins, respectively.*

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using a single-step method with TRIzol reagent (Invitrogen Corp, Carlsbad, CA, USA). For reverse transcription-polymerase chain reaction, first strand cDNA was synthesized from 0.2 to 1 μg of total RNA with an oligo-dT primer and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Carlsbad, CA, USA).The sequences of PCR primers were as follows: RbAp46 sense primer, 5'- TG ATG GCG AGT AAA GAG ATG TTT GAA-3'; RbAp46 antisense primer, 5'-AGA TCC TTG TCC CTC CAG TTC GGA T-3'; β-actin sense primer, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; and β-actin antisense primer, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. The PCR (Perkin Elmer, OH, USA) protocol was conducted with the RbAp46 and β-actin primers at 94˚C for 3 min followed by 30 cycles of 45 s at 94˚C, 45 s at 55˚C, 2 min at 72˚C, and finally 10 min at 72˚C. After the reaction, PCR products were separated on a 0.5 x TAE-containing 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Dual luciferase assay. Cells were seeded in 12-well plates and transfected with a constant amount of DNA consisting of 2.5 μg of RbAp46 promoter luciferase reporter construct (pRbAp46-Luc) and 2.5 μg of pSUMO-1 expression construct (pCMV-SUMO-1) and 0.5 μg of pRL-TK plasmid. After 48 h transfection, cell extracts were prepared and analyzed for RbAp46 promoter luciferase activity. Relative luciferase activity was normalized by Renilla luciferase.

Degradation assay. 7-4-RbAp46-GFP cells were transfected with and without SUMO-1. Forty-eight hours after transfection, cells were treated with 50 μg/ml of cycloheximide (CHX) for 0, 3, 6, 9 h and cell lysates were prepared and analyzed by Western blotting with RbAp46 antibodies.

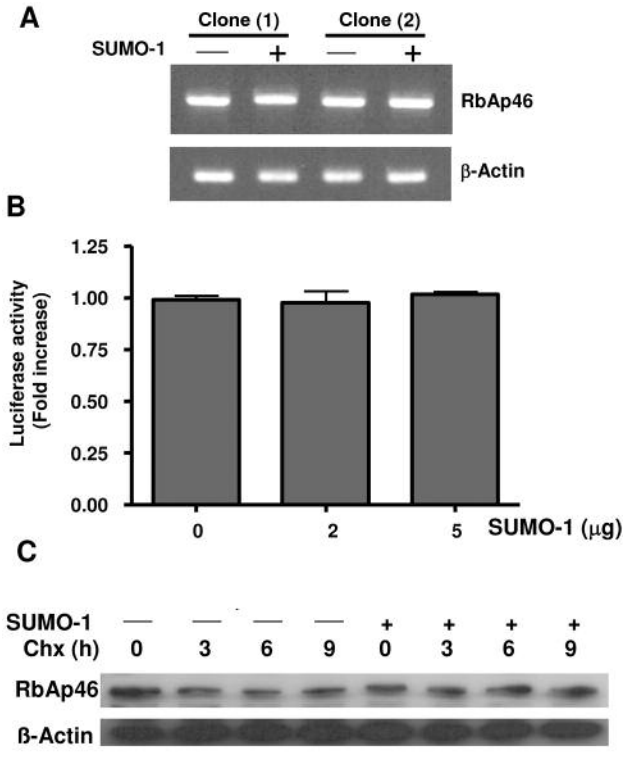

Figure 2. *SUMO-1 does not increase RbAp46 expression at a transcriptional level. A, SUMO-1 was transiently transfected into two 7-4-RbAp46GFP stable cell lines (clones 1 and 2). Total RNA of the transfected cells was prepared and analyzed by RT-PCR 48 h after transfection. B, Different concentrations of SUMO-1 plasmid DNA (pCMV-SUMO-1) (2-5 μg) were co-transfected with RbAp46 promoter reporter plasmid (pRbAp46-LUC) into 7-4 cells. After 48 h, cell lysates were prepared and luciferase activity was analyzed. C, Degradation of RbAp46 protein in a 7-4-RbAp46-GFP stable clone. The 7-4-RbAp46 stable cells were transfected with SUMO-1. After 48 h, the cells were treated with cycloheximide (CHX, 50 μg/ml) for different times. Cell lysates were then prepared and analyzed for RbAp46 protein degradation by Western blotting using RbAp46 antibody.*

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (5×10^3/well) were plated in 96-well plates. After 24 h, the cells were then pulsed with 10 μl of MTT (5 mg/ml; Sigma) and incubated for an additional 4 h at 37˚C. After lysis buffer (100 μl of 10% SDS in 0.01 M HCl) was added and reduced MTT was measured by a microplate reader (Dynatech Mr 5000; Dynatech Laboratories, USA) at 590 nm.

Statistical analysis. Statistical analysis was carried out by Student's *t*-test using the software SigmaPlot version 9.

Results

RbAp46 protein expression level is increased by SUMO-1. To determine whether the RbAp46 protein level is affected by SUMO-1, RbAp46-GFP fusion protein (pEGFP-RbAp46, 25 μg) and different concentrations of SUMO-1 plasmid DNA (pCMV-SUMO-1, 0, 0.5, 2.5, 5 μg) were co-transfected into 7-4 cells and the level of RbAp46-GFP fusion protein expression in the cell was evaluated under a fluorescent microscope. Figure 1A shows that SUMO-1 increased the fluorescence (RbAp46-GFP) in a dose-dependent manner in 7-4 cells. Figure 1B shows that RbAp46 protein levels were significantly increased with the increasing concentrations of SUMO-1 DNA evaluated by Western blotting with GFP (upper panel) and RbAp46 (middle panel) antibodies, respectively. Our data clearly show that SUMO-1 can increase RbAp46 protein expression level in 7-4 cells.

SUMO-1 up-regulates the expression of RbAp46 at a post-transcriptional level. Similarly, two stable clones (clone 1 and 2) constitutively expressing RbAp46-GFP fusion protein showed significant increase of fluorescence (RbAp46-GFP fusion protein) while SUMO-1 was transiently introduced, indicating up-regulation of RbAp46-GFP fusion protein by SUMO-1 (data not shown). The expression levels of RbAp46 mRNA in these two clones were unchanged in the presence or absence of SUMO-1 as demonstrated by RT PCR (Figure 2A). Moreover, RbAp46 promoter activity was evaluated in the presence or absence of SUMO-1 using RbAp46 luciferase reporter plasmid (pRbAp46Luc). Figure 2B shows that the RbAp46 promoter activity was not altered after SUMO-1 was introduced. Taken together, these results clearly demonstrate that the SUMO-1 up-regulation of RbAp46 is not at the transcriptional level.

The protein degradation rate was then evaluated using the protein inhibitor CHX to block protein synthesis after introducing SUMO-1 into the cells. Figure 2C shows that there was no significant difference of RbAp46 protein degradation in a total of 9 h period in the presence and absence of SUMO-1. Taken together, our data indicate that the increase of RbAp46 protein expression by SUMO-1 is not at the transcriptional level and is not through reduced degradation.

Co-localization and co-immunoprecipitation of RbAp46 and SUMO-1. To understand the relationship between RbAp46 and SUMO-1 proteins, co-localization of RbAp46 and SUMO-1 was assessed by co-transfection of the plasmids pDSred2-N1-RbAp46 (red) and pEGFP-Sumo-1 (green) into 7-4 cells. Both of the proteins were detected in the nuclei and were co-localized (Figure 3A), suggesting that SUMO-1 may interact with RbAp46. A co-immuno-precipitation assay was conducted to confirm that RbAp46 could form a complex with SUMO-1 (Figure 3B). Our data indicate that SUMO-1 and RbAp46 may form a complex in the nuclei.

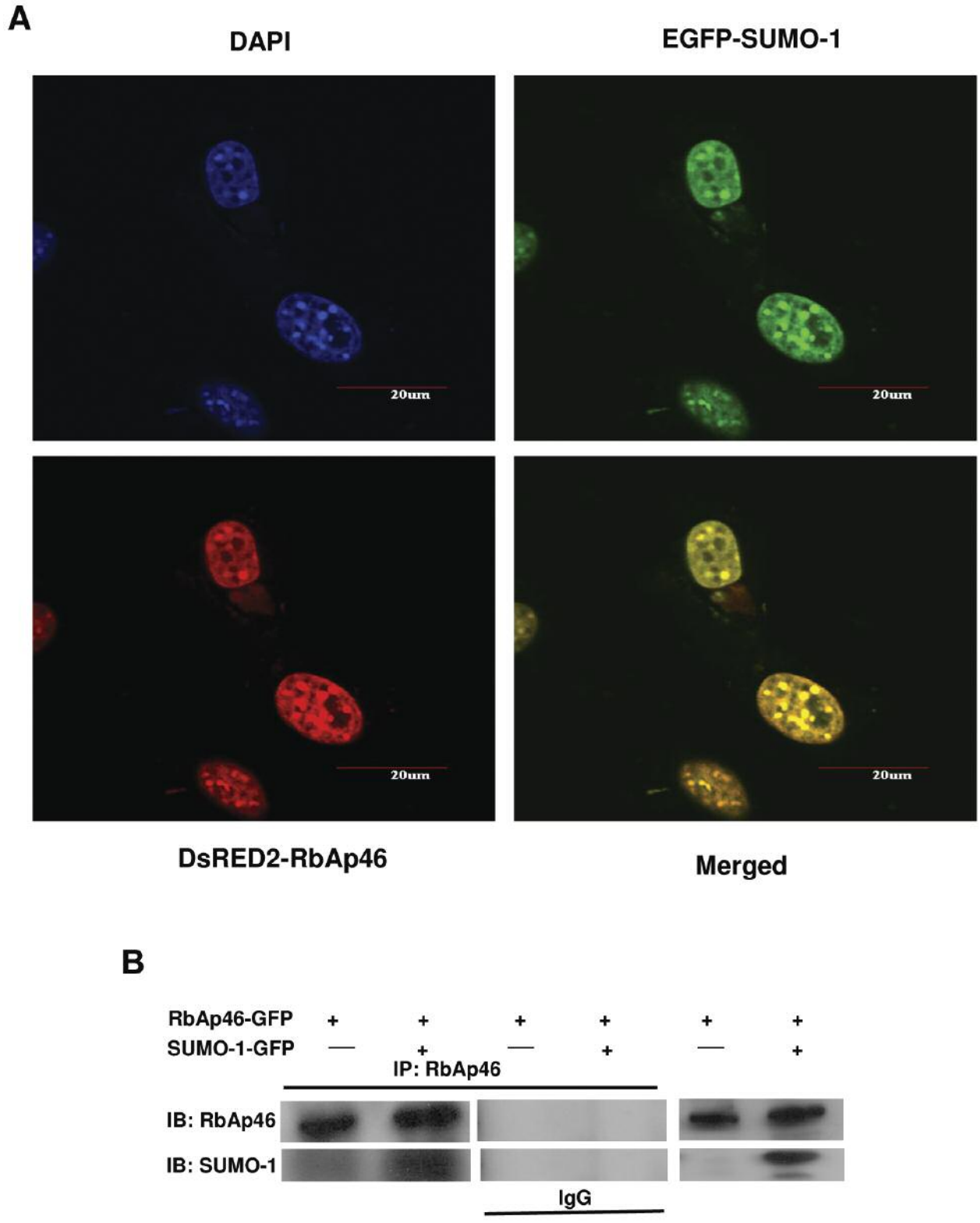

Figure 3. *Co-localization and co-immunoprecipation of RbAp46 and SUMO-1 in 7-4 cells. A, 7-4 Cells were co-transfected with the plasmid pRbAp46-RFP and pSUMO-1-GFP. The cells were observed under a confocal microscope at 48 h. B, Co-immunoprecipitation of RbAp46 and SUMO-1. Cells (7-4-RbAp46-GFP) were transfected with SUMO-1-GFP. After 48 h cell lysates were prepared and immunoprecipitation was conducted by RbAp46 antibody followed by Western blotting with RbAp46 and SUMO-1 antibodies, respectively. IgG treatment was a negative control.*

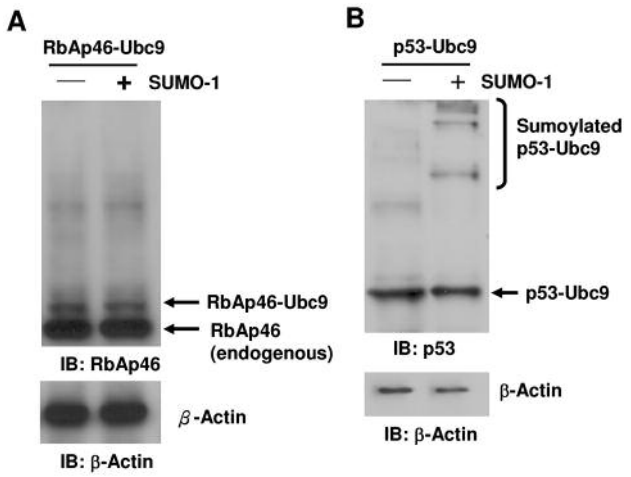

Figure 4. *SUMO-1 cannot modify RbAp46 through sumoylation. A, To detect the sumoylation of RbAp46 protein, RbAp46 gene was fused with Ubc9 (pRbAp46-Ubc9) and transfected into 7-4 cells in the presence or absence of SUMO-1-GFP. Cell lysates were prepared and RbAp46 band shift for sumoylation modification was detected by Western blotting using RbAp46 antibody. β-Actin was used as the internal control. B, Similar to the procedure in A, p53-Ubc9 was co-transfected with or without Sumo1-GFP into 7-4 cells and was used as a positive control for sumoylation.*

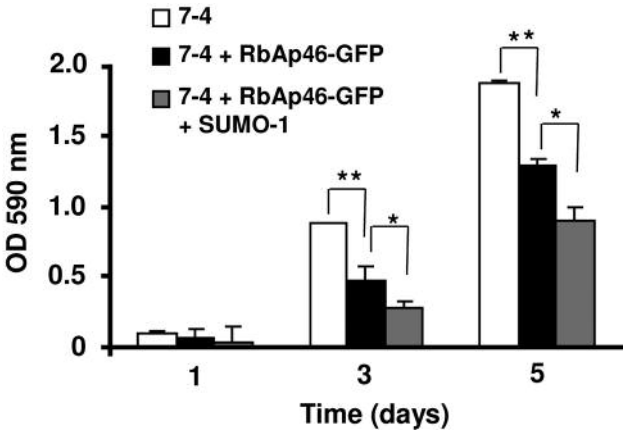

Figure 5. *RbAp46 can suppress the proliferation of 7-4 cells. The 7-4 cell stable clone (7-4-RbAp46-GFP) in the presence or absence of SUMO-1 was used to evaluate the growth of the cells at day 1, 3 and 5 by MTT assay.*

SUMO-1 stabilization of RbAp46 protein is not through sumoylation. In general, SUMO-1 stabilizes target proteins through sumoylation (15). The sequential steps for sumoylation are processing, activation, Ubc9 conjugation and ligation (12). Since RbAp46 and SUMO-1 form a complex, it is interesting to know whether RbAp46 protein is stabilized through SUMO-1-mediated sumoylation. However, in the presence of SUMO-1, the RbAp46-Ubc9 fusion protein cannot form a sumoylation super-shift (Figure 4A). Figure 4B shows that in the presence of SUMO-1, the positive control p53-Ubc9 fusion protein becomes super-shifted. Our data indicate that the stabilization of RbAp46 protein by SUMO-1 is not through sumoylation.

SUMO-1 suppresses further proliferation of 7-4 cells. The biological significance of RbAp46 protein in the cell was explored by measuring the growth rate of the cells overexpressing RbAb46. Figure 5 shows that the proliferation rate was significantly (**$p<0.0001$) reduced by exogenous RbAp46 protein (constitutive expression) when compared with the parental 7-4 cells. Furthermore, a significant (*$p<0.001$) decrease in cell growth was seen in cells transfected with SUMO-1 when compared with the cells without SUMO-1 (Figure 5). Our current data support the auxiliary role of SUMO-1 in RbAp46 protein stability.

Discussion

Sumoylation affected the localization of target proteins in the cell (23). It can guide the nuclear proteins (some are transcription factors) to the so-called PML nuclear bodies (24) to sequester them away from the DNA (24-26). The protein transportation between the cytoplasm and the nucleus is also frequently coupled with sumoylation (27, 28). SUMO-1 can regulate the distribution of many proteins in the cell through posttranslational modification (23). Unmodified RanGAP1 is exclusively cytoplasmic and SUMO-1 modification targets it to the nuclear pore complex (15, 16). In this study, RbAp46 formed a complex and co-localized with SUMO-1 (Figure 3B and 3A), however sumoylation of RbAp46 was not detected (Figure 4A) and the localization of RbAp46 protein was not affected by SUMO-1 (Figure 3A).

SUMO-1 increased the expression of RbAp46 protein when it was transiently expressed in the cells. Further analysis clarified that the regulation of SUMO-1 on RbAp46 expression is not at the transcriptional level. We have shown that the RbAp46 protein degradation ratio was unchanged in the presence of CHX when SUMO-1 was introduced, indicating that the regulation is not through any decrease of degradation. The possibility that SUMO-1 can induce RbAp46 protein synthesis cannot be excluded. In addition, SUMO-1 may maintain the RbAp46 protein level at the posttranslational level. SUMO-1 can affect the stability of its target proteins through posttranslational modification (19). However, the sumoylation of RbAp46 either by endogenous or exogenous SUMO-1, or with the help of ubiquitin-conjugating enzyme 9 [Ubc9, the single

E2-type enzyme for small ubiquitin-like modifier (SUMO) conjugation] could not be detected here, while the positive control p53 clearly shows sumoylation shift (Figure 4A and 4B). Taken together, our findings indicate that SUMO-1 stabilizes RbAp46 protein in a sumoylation-independent manner. However, the possibility that the level of sumoylated RbAp46 was too low to be detected, or that other SUMO family members may cause sumoylation of RbAp46 cannot be excluded. It is also possibly that SUMO-1 acts through inhibition of ubiquitinylation as well as formation of complex with RbAp46 to stabilize RbAp46 protein.

SUMO modification appears to play a role in a variety of cellular processes, including protein-protein interaction, subcellular localization, protein stabilization and transcriptional regulation (29). In this study, SUMO-1 seems to contribute to the stabilization of RbAp46 protein.

The functional role of stabilization of RbAp46 by SUMO-1 in the cell remains elusive. Here, we showed that overexpression of RbAp46 significantly suppressed the proliferation of 7-4 cells (Figure 5). RbAp46-related suppression was further enhanced when SUMO-1 was overexpressed (Figure 5). We clearly demonstrate that RbAp46 *per se* was able to suppress cell proliferation and SUMO-1 can further suppress cell proliferation through stabilization of RbAp46 (30, 31). In conclusion, our data provide evidence that RbAp46 protein is stabilized by SUMO-1 and the function of RbAp46 was subsequently promoted.

Acknowledgements

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