# Critical role for non-GAP function of Gαs in RGS1-mediated promotion of melanoma progression through AKT and ERK phosphorylation

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**Abstract.** Regulator of G-protein signaling 1 (RGS1) has been found to be a critical factor in melanoma and other malignancies. However, the mechanism involved in the RGS1-mediated promotion of melanoma progression is not clear. We based our study on samples collected from pathological specimens of melanoma patients. We found by immunohistochemistry that RGS1 expression was significantly higher in melanoma than that noted in nevus tissue (P<0.05). Kaplan-Meier analysis demonstrated a significant correlation between increased RGS1 expression and reduced disease-specific survival (P<0.05). RGS1 expression was also found to be related to the proliferation and migration of melanoma cells. RGS1 was able to bind to the Gαs in immunoprecipitation, but this interaction did not accelerate GTP hydrolysis in our experiment. Furthermore, we found that RGS1 may promote melanoma progression through the downstream effects of Gαs signaling, such as the increased phosphorylation of AKT and ERK by western blotting. Our results demonstrated that RGS1 promotes melanoma progression through regulation of Gαs-mediated inactivation of AKT and ERK. Therefore, RGS1 is a novel therapeutic target for melanoma treatment.

## Introduction

GTPase-activating proteins (GAPs) function as a deactivator of G-protein signaling by accelerating GTP hydrolysis. Regulator of G-protein signaling (RGS) proteins are GAPs for Gα subunits (1). RGS1 was first identified as an immediate early gene responsive to several B-cell activation signals (2), and it has been shown to be related to the regulation of chemokine-induced signaling in B cells (3). The RGS1 gene resides at 1q31, which is involved in several malignancies by gains or amplifications in certain subtypes of melanoma (4), non-Hodgkin lymphoma (5), retinoblastoma (6), pancreatic cancer (7) and nasopharyngeal carcinoma (8). RGS1 has been shown to be upregulated by gene expression profiling in several different tumor model systems. For example, RGS1 has been shown to be overexpressed in the more aggressive (blastoid) variant of mantle cell lymphoma (9), the tumorigenic variant of adult T-cell leukemia (10), and in late-stage cervical cancer (11).

RGS1 plays an important role in melanoma progression. Researchers analyzed gene profiling from 34 melanocytic neoplasms and found that RGS1 was differentially overexpressed in primary melanomas vs. benign nevi (12). Another analysis of a tissue microarray containing 301 primary melanomas showed a close relationship between RGS1 expression and the clinical outcomes associated with melanoma (13). Furthermore, RGS1 expression was shown to be an independent predictor of recurrence-free survival (RFS) and disease-specific survival (DSS) when the six factors listed by the AJCC melanoma analysis were all included. Intriguingly, in the analysis of DSS, RGS1 emerged as the top factor predicting DSS, other than tumor thickness or ulceration (13). However, none of these studies on RGS1 and melanoma revealed any hidden mechanisms.

The Gαs pathway is one of the earliest G-protein signaling pathways to be studied, and many vital concepts including that of second messengers (14), protein phosphorylation (15), and signal transducers (16,17) have come from this pathway. Gαs is a tumor suppressor in neural and epidermal progenitor-derived malignancies such as medulloblastoma, basal cell carcinoma, neuroblastoma, and melanoma (originates from neural progenitors) (18,19). In these stem cell compartments, signaling through Gαs causes GTP hydrolysis that activates the cAMP-dependent protein kinase A (PKA) signaling pathway (20), inhibits the Sonic Hedgehog (SHH) and Hippo pathways (19), and finally suppresses cell self-renewal. The loss of Gαs leads to activation of these pathways, over-proliferation of progenitor cells,

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and tumor formation. Thus, Gαs acts as a brake on excessive self-renewal or proliferation of progenitor cells.

In the present study, we explored RGS1 expression in 40 melanoma and 18 nevus samples from 58 different patients. Then, we investigated the role of RGS1 in melanoma progression using cell viability and Matrigel-based assays. Further immunoprecipitation and rescue experiments were performed to investigate the mechanism utilized by RGS1 to regulate melanoma progression.

## Materials and methods

*Immunohistochemistry*. To prepare tissue sections of 40 melanoma and 18 nevus from patients for immunohistochemistry, sections from each patient were deparaffinized with xylene (3x5 min) followed by treatment with serial dilutions of ethanol (100, 100, 95 and 95%, 10 min each) and by two changes of $ddH_2O$. Antigen unmasking was conducted by boiling the slides (95-99˚C) for 10 min. Sections were rinsed three times with $ddH_2O$, immersed in 3% $H_2O_2$ for 20 min, washed twice with $ddH_2O$ and once with TBS-T (TBS, 0.1% Tween-20) and blocked for 1 h with blocking solution (5% normal goat serum in TBS-T). Antibody of RGS1 (cat. no. PA5-29579; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was diluted according to the manufacturer instructions and the sections were incubated overnight at 4˚C. Then, the sections were washed three times, 5 min each, with TBS-T and incubated for 1 h at room temperature with Signal Stain Boost (Cell Signaling Technology, Inc., Danvers, MA, USA). The negative control used for immunohistochemistry included the use of phosphate-buffered saline instead of the primary antibody. Finally, tissues were dehydrated. Images were captured with an Olympus microscope (Olympus DP80; Olympus Corp., Tokyo, Japan). All images were captured and processed using identical settings. During evaluation, for each sample, five horizons were randomly chosen to calculate the average positive ratio.

The immunostaining scores were calculated using an approved standard (13). The regions of most uniform staining were scored for each tissue array core, which included the entire midportion of the core, to exclude any 'edge effect' of increased staining. Expression of RGS1 protein was graded combining two factors. One factor was the staining intensity: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The other factor included the proportion of positive-staining cells. In all target cells of one region, the proportion of 'no staining' cells was considered 'A', and 'weak staining' was 'B', and by this analogy, the final score of this region was equal to: (0 x A) + (1 x B) + (2 x C) + (3 x D). This score was categorized into 3 grades: ≤1.0 (+); >1.0 but ≤1.5 (++); >1.5 (+++). The arrays were scored by a pathologist blinded to the identity of the patients, and each score was replicated by a separate, independent scoring trial by the study pathologist. For the melanoma patients, we divided them into high and low expression groups, and were followed up to determine their disease-related survival, and analyzed it using the Kaplan-Meier curve.

*Cell culture*. The A375 human melanoma cell line (Cell Bank in Shanghai, Chinese Academy of Sciences), RGS1-knockdown (KD) A375 cells and RGS1-overexpression cells were incubated at 37˚C in a humidified 5% $CO_2$ enriched atmosphere. These cells were cultured with Dulbecco's modified Eagle's medium with high glucose (DMEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% fungi zone (Invitrogen; Thermo Fisher Scientific, Inc.), and 1% penicillin, twice weekly, at every change in media, for normal growth by phase contrast microscopy. The cultures were grown to confluence and passaged by treatment with 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and washed in 7 ml DMEM media before being centrifuged at 120 x g for 10 min to form a pellet. The lentivirus base RGS1 overexpression system and RGS1 knockdown system (sequence of shRGS1, 5'-GATCCGCCCTGTAAAGCAGAAGAGATTTCAAGAGAATCTCTTCTGCTTTACAGGGCTTTTTTG-3') were purchased from Hanyin Biotechology (Shanghai, China) and used to infect cells as described in a previous study (21).

*Cell proliferation assay*. Cells were seeded into 96-well plates (Corning Inc., Corning, NY, USA) at a density of $2x10^3$ cells/well. Cell viability was assessed using Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). The absorbance of each well was read on a spectrophotometer (Thermo Fisher Scientific, Inc.) at 450 nm (OD450). Three independent experiments were performed in quintuplicate.

*Cell invasion assays*. For the determination of cell invasion, Transwell chambers were coated with 30 $\mu$l Matrigel (Merck KGaA, Darmstadt, Germany), and incubated at 37˚C for 40 min. In the Transwell assays with and without Matrigel, the cells were trypsinized and then seeded in chambers at a density of $1x10^4$ cells/well at 48 h after transfection. The cells were then cultured in DMEM with 2% serum. Meanwhile 600 $\mu$l of medium supplemented with 10% FBS was injected into the lower chambers. After cell harvest, the inserts were fixed and stained in a dye solution containing 1% crystal violet and 20% methanol. Cells adhering to the lower membrane of the inserts were imaged with a microscope (Olympus DP80; Olympus Corp.). Six views are randomly picked for each well.

*Apoptosis assay*. A375 cells in the three groups were measured by FACS. Annexin V-PE/7-AAD (cat# 559763; eBioscience; Thermo Fisher Scientific, Inc.) double staining was used to identify the apoptosis rate of the A375 cells. The cells ($1x10^6$ cells/ml) were harvested, washed twice with 4 centigrade PBS, and incubated for 15 min in 1X Annexin V binding buffer containing 10 $\mu$l 7-AAD and 5 $\mu$l Annexin V-PE. Finally, apoptosis was detected by FACS and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Experiments were carried out in triplicate.

*Immunoprecipitation*. The FLAG-tag RGS1 and HA-Gαs plasmids were instantly transferred into the 293T cells in the three groups and named 293T-RGS1, 293T-Gαs-GDP, 293T-Gαs-GDP-$AlF_4^-$, which was without tetrafluoroaluminate ($AlF_4$). The cells were collected and lysed with 200 $\mu$l cold RIPA buffer (RIPA buffer:PMSF = 100:1; Beyotime

Institute of Biotechnology, Haimen, China) for 30 min, followed with centrifugation at 13,200 x g, at 4˚C for 10 min. HA-G proteins, (Gαs-GDP, Gαs-GDP-$AlF_4^-$) were added into the cell lysis supernatant liquor separately and mixed. Each blend was divided into 'total' and 'co-IP' parts. The protein A agarose was prepared and washed using Lysis buffer B (pH 7.6) 4 times, 2,000 g. This was diluted by half with Lysis buffer B (pH 7.6). Protein A agarose was added into each 'co-IP' portion and was agitated slowly at room temperature for 2 h. Then, 1 $\mu$g of the Gαs flag antibody was added into the 'co-IP' parts, and swayed slowly at 4˚C overnight. Then centrifugation was carried out instantaneously at 3,000 rpm, and the precipitate was collected and washed with cold Lysis buffer B (pH 7.6) 3 times. The samples were boiled for 5 min at 100˚C, for immunoprecipitation.

Spontaneously, a group of absolute exogenous co-IP was prepared. The FLAG-tag RGS1 and HA-Gαs proteins were expressed and purified, and the binding experiment procedure was carried out as in Watson *et al* (1). The reaction buffer consisted of a solution of 50 mM Tris-HCI, pH 8.0, 0.1 M NaCl, 1 mM $MgS0_4$, 20 mM imidazole, 0.025% polyoxyethylene 10-lauryl ether ($C_{12}E_{10}$), 10 mM β-mercaptoethanol and 10% glycerol. Protein immunoprecipitation (IP) was performed, respectively using Chromatin ChIP Kits (EMD Millipore, Billerica, MA, USA). Antibody of HA-Gαs and FLAG-tag RGS1 were used.

*GTPase activity*. The HA-Gαi and HA-Gαs were purified and extracted using the method in the binding process. Then this was proceeded according to the ATPase/GTPase Activity Assay kit (MAK113; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) instructions. First, the phosphate standards were set as indicated in the kit instructions. Second, a series of dilutions of enzyme were performed in assay buffer. The sample reactions and the control well were set up according to the scheme. The reaction was incubated for the desired period of time (in our research, 1, 3, 5 and 10 min) at room temperature. Reagent (200 ml) was added to each well and incubation was carried out for an additional 30 min at room temperature to terminate the enzyme reaction and generate the colorimetric product separately. Absorbance at 600-660 nm [maximum absorbance at 620 nm (A620)] was read. We calculated the change in absorbance values (DA620) for the samples by subtracting the A620 of the control well (A620) control from the A620 of the sample well (A620) sample. The concentration (mM) of free phosphate [$P_i$] was computed in the sample from the standard curve. The formula was: Enzyme activity (units/l) = [$P_i$] (mM) x 40 ml ÷ [10 $\mu$l x reaction time (min)]. One unit is the amount of enzyme that catalyzes the production of 1 mmol of free phosphate per minute under the assay conditions.

*Western blotting*. Protein was extracted from the cultured cells and dissolved, homogenized, and quantified using the BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Sample buffer was then added and the prepared samples were stored at -80˚C after boiling. During the western blotting, protein samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes and placed in 25 mM Tris and 192 mM glycine. The membranes were blocked with 5% non-fat dry milk in PBS, 0.05% Tween-20 and probed with P-AKT (CY6569; Abways, Shanghai, China), P-ERK (CY5277; Abways), AKT (CY5551; Abways), ERK (CY5487; Abways), Gas (ab83735; Abcam, Cambridge, UK according to the manufacturers' instructions. Blots were developed with ECL reagent (Thermo Fisher Scientific, Inc.) and exposed using the FC2 Image Station (Alpha, Bellingham, WA, USA).

*Statistical analysis*. All of the statistical analyses were performed by SPSS (version 19.0) software (IBM Corp., Armonk, NY, USA) and all of the data are represented as the mean ± standard deviations (SD). Student's t-tests and analysis of variance (ANOVA) were performed to compare the differences between groups. $P<0.05$ was considered to be indicative of statistical significance. Three and more independent experiments were performed in each group.

## Results

*RGS1 is highly expressed in melanoma and is inversely associated with disease-specific survival (DSS)*. RGS1 expression has been detected throughout the cell (22). In the present study, we analyzed RGS1 expression by immunohistochemical staining in nevus and melanoma tissues. As shown in Fig. 1A, the RGS1 antibody staining intensity was darker and stained more target cells in the melanoma samples when compared to the nevus samples (Fig. 1A). Compared with the nevus tissue, RGS1 expression was significantly upregulated in the melanoma tissues (Fig. 1B, $P=0.0023$). Furthermore, we collected the DSS data and performed the Kaplan-Meier estimation. The results demonstrated that high RGS1 expression was inversely correlated with overall survival (Fig. 1C, $P<0.0001$). Collectively, RGS1 is highly expressed in melanoma and is inversely associated with DSS.

*RGS1 promotes melanoma cell proliferation and invasion*. To study the function of RGS1 in the melanoma cell line A375, the plasmid-based RGS1 overexpression and knockdown systems (shRGS1) were used for transfection. A western blot assay showed that RGS1 expression was efficiently downregulated in the shRGS1-transfected A375 cells (Fig. 2A), while expression was significantly upregulated in the RGS1-transfected overexpressing A375 cells (Fig. 2B). Next, cell viability in the shRGS1-transfected, RGS1-transfected, and negative control (NC)-transfected A375 cells were determined using the CCK-8 assay. We found that knockdown of RGS1 significantly inhibited A375 cell proliferation (Fig. 2C), and overexpression of RGS1 significantly promoted A375 cell proliferation (Fig. 2D). Furthermore, a Matrigel-based invasion assay indicated that knockdown of RGS1 significantly inhibited A375 cell invasion (Fig. 2E) and overexpression of RGS1 significantly promoted A375 cell invasion (Fig. 2F). These results demonstrated the stimulatory role of RGS1 in melanoma proliferation and invasion.

*RGS1 binds to Gαs in an endogenous environment and regulates AKT and ERK activation*. Previous research has demonstrated that Gαs is a tumor suppressor in neural and epidermal progenitor-derived malignancies (18,19). In the present study, co-immunoprecipitation (Co-IP) was performed

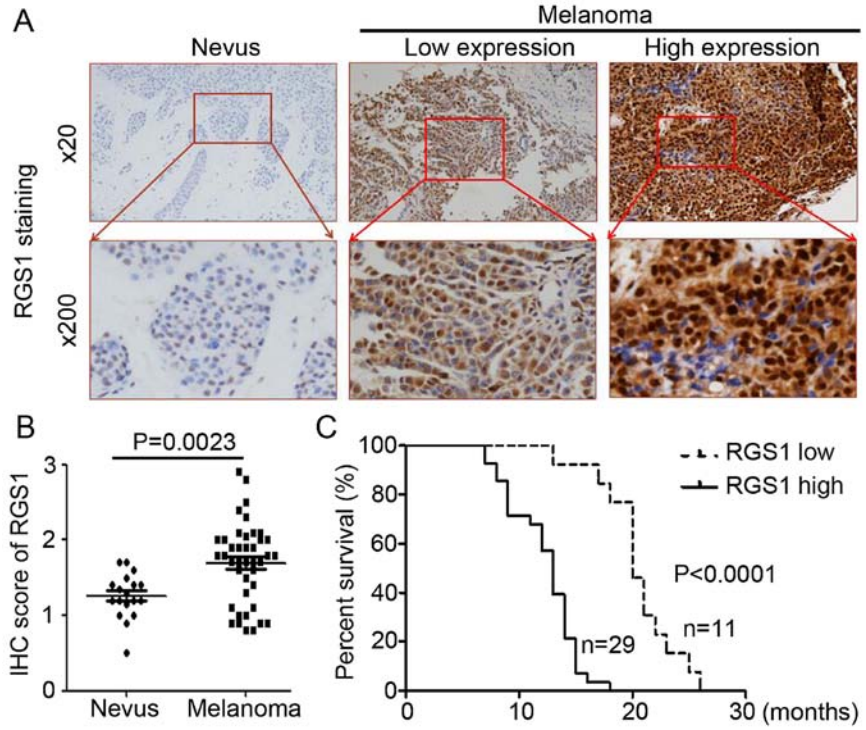


Figure 1. RGS1 is highly expressed in melanoma and is inversely associated with disease-specific survival (DSS). (A) Staining detection of RGS1 in nevus and melanoma tissues. The bottom panels are the high power magnification (x200) of the red square in the low power lens image (x20). (B) Analysis of RGS1 expression in nevus (n=18) and melanoma (n=40) tissues (P=0.0023). (C) Comparison of DSS curves of the melanoma patients according to RGS1 expression. RGS1 expression was divided according to the immunohistochemical staining score. Scores >1.5 were considered as having high expression. Scores ≤1.5 were categorized as low expression (P<0.0001).

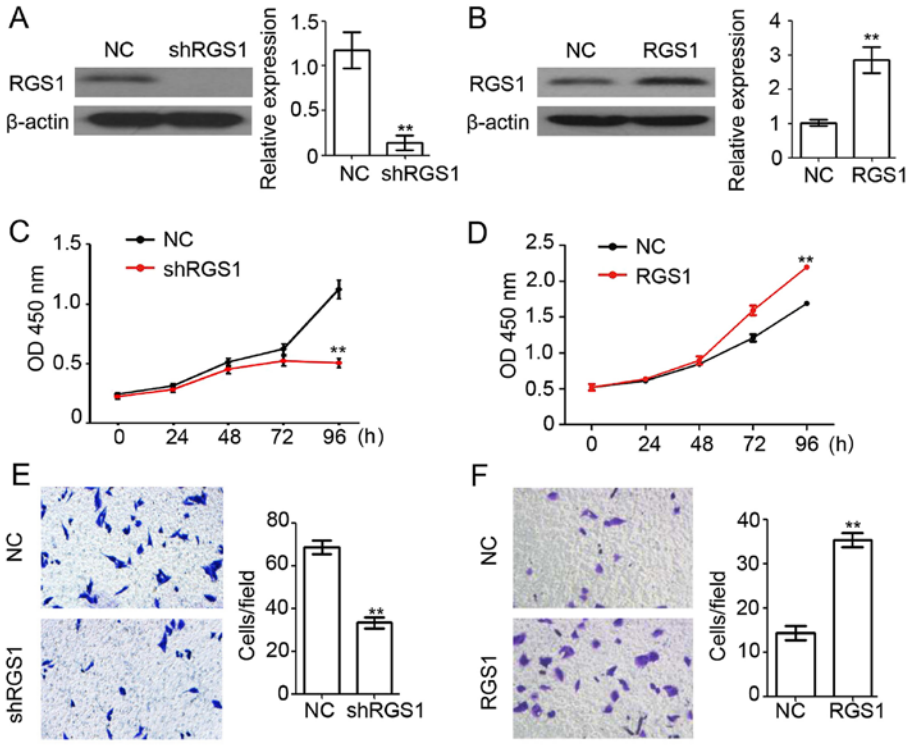


Figure 2. RGS1 promotes melanoma proliferation and invasion. (A) Western blot analysis of RGS1 expression in NC (negative control)-transfected and shRGS1-transfected A375 cells. β-actin was used as a loading control. The relative expression of RGS1 was determined by ImageJ and analyzed. $^{**}$P<0.01. (B) Western blot analysis of RGS1 expression in NC (negative control)-transfected and RGS1-transfected A375 cells. β-actin was used as a loading control. The relative expression of RGS1 was determined by ImageJ and analyzed. $^{**}$P<0.01. (C) CCK-8 assay was utilized to analyze A375 cell proliferation at 0, 24, 48, 72 and 96 h post NC (negative control) and shRGS1 transfection. $^{**}$P<0.01. (D) CCK-8 assay was utilized to analyze A375 cell proliferation at 0, 24, 48, 72 and 96 h post NC (negative control) and RGS1 transfection. $^{**}$P<0.01. (E) Matrigel-based invasion assay was performed to determine A375 cell invasion ability post NC (negative control) and shRGS1 transfection. $^{**}$P<0.01. (F) Matrigel-based invasion assay was performed to determine A375 cell invasion ability post NC (negative control) and RGS1 transfection. $^{**}$P<0.01.

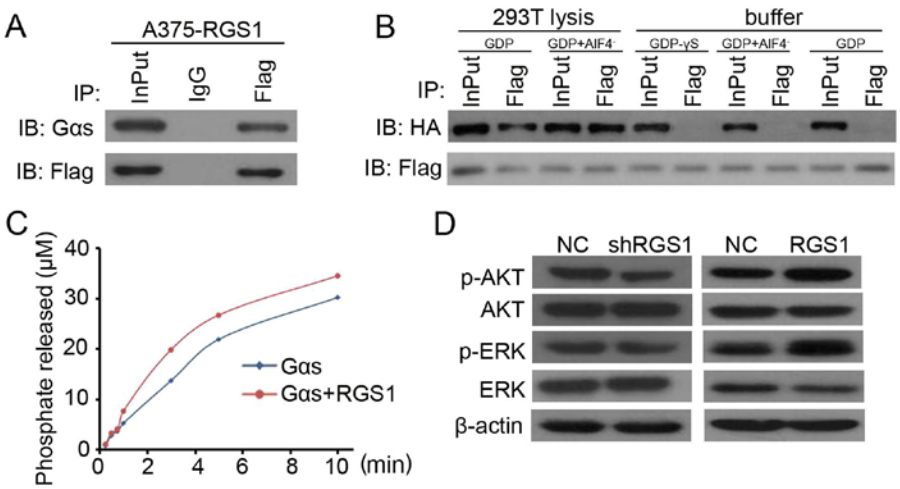


Figure 3. RGS1 directly binds to Gαs in an endogenous environment and regulates AKT and ERK activation. (A) We used Flag-tagged RGS1 to pull down the proteins in RGS1-overexpressing A375 cell lysis. Western blotting was performed to detect HA and Gαs. (B) We used Flag-tagged RGS1 to pull down the proteins in 293T cell lysis (Flag-RGS1 and HA-Gαs transfected) added together with GDP or GDP+AlF4- to the buffer (inactive, active and transitive). Western blotting was performed to detect HA and Flag. (C) Assessment of RGS1 accelerating the GTPase (released phosphate by time) rate of Gαs in a single catalytic turnover at room temperature. (D) Western blot analysis of p-AKT, AKT, p-ERK and ERK expression in NC (negative control)-transfected, shRGS1-transfected and RGS1-transfected A375 cells. β-actin was used as a loading control.

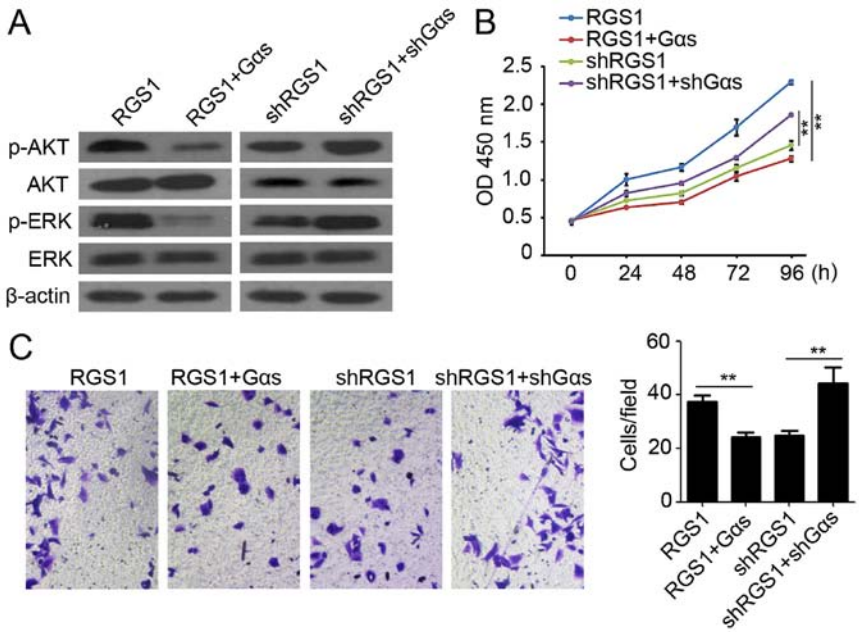


Figure 4. Gαs plays a necessary role during RGS1-mediated promotion of melanoma proliferation and migration. (A) Western blot analysis of p-AKT, AKT, p-ERK and ERK expression in A375 cells after RGS1, RGS1+Gαs, shRGS1 and shRGS1+shGαs transfection. β-actin was used as a loading control. (B) CCK-8 assay was utilized to analyze A375 cell proliferation at 0, 24, 48, 72 and 96 h post RGS1, RGS1+Gαs, shRGS1 and shRGS1+shGαs transfection. **P<0.01. (C) Matrigel-based invasion assay was performed to determine A375 cell invasion ability post RGS1, RGS1+Gαs, shRGS1 and shRGS1+shGαs transfection. **P<0.01.

to determine the potential for direct targeting of RGS1 to Gαs. As shown in Fig. 3A, RGS1 was found to directly target Gαs in the RGS1-overexpressing A375 cells. Next, the binding of RGS1 protein and Gαs was assessed in the following three types of exogenous environments: Gαs-GDP, Gαs-GDP-$AlF_4^-$, and nothing added to the buffer. The result indicated that RGS1 did not bind to Gαs in any state (Fig. 3B). In the circumstance of the 293T cell lysis with added GDP or GDP+$AlF_4^-$, RGS1 binding to Gαs was detected in both environments (Fig. 3B). Binding was also detected in the endogenous experiment performed with A375 cells (Fig. 3B). The above results demonstrated the direct targeting of RGS1 and Gαs in the endogenous environment.

GTPase activity was evaluated using a specific kit testing GTPase activity in the exogenous environment (no other molecules added). The result showed that the GTPase activity was not significantly elevated for Gαs after the combination with RGS1 (Fig. 3C). This indicated that the binding of RGS1 to Gαs might not accelerate the GTP hydrolysis process (Fig. 3C). Further western blotting demonstrated the stimulatory role of

RGS1 on AKT and ERK activation in A375 cells (Fig. 3D). Collectively, the above results suggest that RGS1-induced AKT and ERK phosphorylation is dependent on the non-GAP function of Gαs.

*Gαs plays a necessary role during RGS1-mediated promotion of melanoma proliferation and migration*. To confirm that Gαs regulates the RGS1-mediated promotion of AKT and ERK activation involved in A375 cell proliferation and invasion, Gαs overexpression and knockdown systems were utilized. As shown in Fig. 3D, the increased expression of RGS1 significantly promoted AKT and ERK phosphorylation. Using the Gαs overexpression system, the phosphorylation of AKT and ERK was reduced (Fig. 4A). In contrast to RGS1 overexpression, knockdown of RGS1 significantly decreased AKT and ERK phosphorylation (Fig. 3D). Using the knockdown system to reduce the expression of Gαs, the phosphorylation of AKT and ERK was enhanced (Fig. 4A). The above results demonstrated that Gαs plays a critical role during RGS1-mediated promotion of AKT and ERK phosphorylation in melanoma.

Our CCK-8 assay also demonstrated the stimulatory role of RGS1 in melanoma proliferation (Fig. 2C), but increased expression of Gαs reduced the cell proliferation (Fig. 4B). In contrast, knockdown of Gαs reversed the shRGS1-mediated inhibition of proliferation (Figs. 2D and 4B). Furthermore, RGS1 was found to function as a promoter of melanoma invasion (Fig. 2E and F). Overexpression of Gαs abrogated RGS1-mediated promotion of A375 invasion (Fig. 4C), while knockdown of Gαs promoted A375 invasion of the shRGS1-transfected cells (Fig. 4C). Collectively, the above results suggest that Gαs plays a critical role during RGS1-mediated promotion of melanoma proliferation and migration.

## Discussion

In the present study, we identified differential RGS1 expression levels between melanoma and nevus samples, as well as a significant role for RGS1 in promoting melanoma cell invasion and proliferation. In addition, RGS1 expression was found to be negatively correlated with patient disease-specific survival (DSS). Further mechanistic investigation indicated that RGS1 directly targets Gαs in the endogenous environment and promotes AKT and ERK activation through the non-GAP function of Gαs. Rescue experiments established the critical role of Gαs during RGS1-mediated promotion of melanoma proliferation and invasion.

We found that the two recombinant proteins did not bind in pure buffer with either GDP+$AlF_4^-$ or GDP added, while they did bind in 293T cells lysed with the addition of either GDP+$AlF_4^-$ or GDP. We also detected the binding in A375 cells lysed with nothing added. These results indicated that their binding requires an environment containing specific molecules. Previous studies demonstrated that the binding of Gαs to RGS proteins is controversial and varies in different conditions. Gαs binds directly to the RGS domain of axin in its transition-state in human colon cancer cells (23). Its inactive state also binds PX1 (RGS domain) (23), albeit to a much lesser extent, as observed for other RGS proteins *in vitro* (1). In *Magnaporthe* pathogenesis, RGS1 regulates MagA, the Gαs subunit, during surface signaling (24), and this result was based on a cell function experiment instead of a binding assay. Gαs does not bind to any RGS in its GTP-bound active state (23). The binding of RGS1 to Gαs in different states was not fully investigated in previous studies (1,25). Our exogenous binding assay in buffer agrees with the previous research, but the 'half-exogenous' binding assay, the recombinant proteins in 293T cell lysis, showed a positive result. This is not necessarily that different from the negative result found by Moratz *et al* (25), for in that research the total Gαs expression in HS-Sultan cells was extremely low. Our half-exogenous binding assay showed a much higher expression of total HA-Gαs protein. The binding was not based on the state of Gαs. Considering all these binding assays, RGS1 is able to bind to Gαs in a different way from the traditional RGS-Gα binding pattern involving specific and indispensable molecules in cells.

The acceleration of GTP hydrolysis by RGS occurs through the stabilization of Gα proteins' transition state upon binding. We did not find clear evidence of RGS1 accelerating the GTPase activity of Gαs. From current information, the GAP function of RGS upon binding to Gαs is also controversial. The RGS domain in RGS-PX1 acts as a Gαs-specific GAP, which is the only example of RGS promoting GTPase activity (26). In another study, neither the RGS domain of axin nor the full-length axin purified from baculovirus-infected Sf9 cells demonstrated the GTPase activity of Gαs (23). In the case mentioned above, additional accessory molecules or other modifications of axin could be required for its GAP activity, as is the case for other RGS proteins (27). Similarly, our GTPase activity experiment was performed in a pure chemical environment containing only artificial buffer, Gαs protein, and RGS1 protein. Due to the limits of the method, the real interaction and effects are difficult to confirm. Not merely accessory molecules or modifications need to be taken into consideration. It is also possible the RGS domain of axin is used as a scaffold protein that can interact with and act as an effector for Gαs, as do the RGS domain-containing RhoGEFs, which are effectors for G proteins of the Ga12/13 family (27). Therefore, RGS1 could either act as an effector, antagonize the effector of Gαs, or potentially target PKA and receptor kinases (28). Further rescue experiments confirmed the critical role of the function of RGS1 through the interaction with Gαs in melanoma progression.

We found that RGS1 promoted the activation of AKT and ERK by regulating the non-GAP function of Gαs. Previous research has demonstrated that all G-protein pathways may either stimulate or inhibit one or more of the MAPK signaling pathways (29). For example, in Gαs signaling pathways, MEK can be stimulated or inhibited through different paths in different conditions (29). A possible mechanism is that RGS1 enhances some receptor signaling through RGS or non-RGS domains and motifs (28). These functions depend on intact cells and physiological systems. Another potential mechanism worth noting is that RGS1 may have promoted melanoma progression with the heterotrimeric G-protein derived Gβγ-mediated signaling or protein-protein interactions upon the binding of Gαs and RGS1 proteins. According to the competitive mechanism in which GAP (including RGS1) competes with Gβγ, the two surfaces of Gα that interact with

Gβγ and RGS proteins overlap substantially. High expression of RGS1 may influence the binding of Gβγ to Gαs, therefore causing some downstream effects (30). For example, among the downstream effectors of Gβγ are the class I PI 3-kinases, PI3Kβ and PI3Kγ (31-33). Gβγ activates these PI3K isoforms by directly binding to the p110β and p110γ catalytic subunits (34,35). It is possible that the binding of RGS1 to Gαs maintained the function of Gβγ by activating PI3K, which consequently increased the phosphorylation of AKT. In our rescue experiment, the increased and decreased expression of Gαs may have abolished and elevated the AKT and ERK expression, respectively.

Our study offers a novel finding to explain the tumor-enhancing mechanism of RGS1 in melanoma progression. The binding with Gαs in melanoma is confirmed and meaningful. When RGS binds to Gαs, it carries with it other functional units providing a great diversity of protein-protein interactions (28), which may also influence the downstream effectors of Gαs. The present study provides new insights into the regulation and functional diversity of G-protein signaling in tumor progression.

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**Availability of data and materials**

The datasets used during the present study are available from the corresponding author upon reasonable request.

**Authors' contributions**

SMY and WYC conceived and designed the study. SMY, WYC and ZJ performed the experiments and wrote the paper. LC, WK, WXW and XCY reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

All experimental protocols were approved by the Medical Ethics Committee of Second Military Medical University (Shanghai, China).

**Consent for publication**

Not applicable.

**Competing interests**

All the authors declare that there are no conflicts of interest.

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