Cell Cycle 14:13, 2149–2159; July 1, 2015; © 2015 Taylor & Francis Group, LLC

Spy1 induces de-ubiquitinating of RIP1 arrest and confers glioblastoma's resistance to tumor necrosis factor (TNF-α)-induced apoptosis through suppressing the association of CLIPR-59 and CYLD

Zongmei Ding[1,2,#], Yonghua Liu[1,#], Li Yao[3], Donglin Wang[4], Jianguo Zhang[4], Gang Cui[5], Xiaojing Yang[1], Xianting Huang[1], Fang Liu[1,6,7], and Aiguo Shen[1,6,7,*]

[1]Jiangsu Province Key Laboratory for Inflammation and Molecular Drug Target; Nantong University; Nantong, Jiangsu, PR China; [2]Department of Pathology; Subei People's Hospital; Yangzhou, Jiangsu, PR China; [3]Department of Immunology; Wuxi Medical College; Jiangnan University; Wuxi, Jiangsu, PR China; [4]Department of Pathology; Medical College; Nantong University; Nantong, Jiangsu, PR China; [5]Department of Neurosurgery; The First Affiliated Hospital of Soochow University; Suzhou, Jiangsu, PR China; [6]Jiangsu Province Key Laboratory of Neuroregeneration; Nantong University; Nantong, Jiangsu, PR China; [7]Co-innovation Center of Neuroregeneration; Nantong University; Nantong, Jiangsu, PR China

[#]These authors contributed equally to this work.

Keywords: apoptosis, CLIPR-59, Glioma, RIP1, Spy1, TNF-α

Abbreviations: CLIPR-59, CLIP-170-related 59 kDa protein; Spy1, speedy inducer of meiotic maturation; GBM glioblastoma multiforme; TNFR1, TNF-receptor-type 1; TRADD, TNF receptor-associated death domain protein; RIP1, receptor-interacting protein 1; FADD, Fas-associated protein with death domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Glioblastoma multiforme (GBM), a grade-IV glioma, is resistant to TNF-α induced apoptosis. CLIPR-59 modulates ubiquitination of RIP1, thus promoting Caspase-8 activation to induce apoptosis by TNF-α. Here we reported that CLIPR-59 was down-regulated in GBM cells and high-grade glioma tumor samples, which was associated with decreased cancer-free survival. In GBM cells, CLIPR-59 interacts with Spy1, resulting in its decreased association with CYLD, a de-ubiquitinating enzyme. Moreover, experimental reduction of Spy1 levels decreased GBM cells viability, while increased the lysine-63-dependent de-ubiquitinating activity of RIP1 via enhancing the binding ability of CLIPR-59 and CYLD in GBM, thus promoting Caspase-8 and Caspase-3 activation to induce apoptosis by TNF-α. These findings have identified a novel Spy1-CLIPR-59 interplay in GBM cell's resistance to TNF-α-induced apoptosis revealing a potential target in the intervention of malignant brain tumors.

Introduction

Suppression of apoptosis has been recognized as a key element in cancer progression over the past decade, and therapies designed to relieve the apoptosis blockade in cancer cells are currently undergoing clinical trials.[1] Despite its ability to induce apoptosis, several tumors are resistant to TNF-α-mediated apoptosis.[2] Glioma is the most common primary tumor of the brain, and glioblastoma is largely resistant to current therapeutic regimens.[3-5]

Human Speedy A1 (Spy1), as a member of the Speedy/RINGO family, was found to be able to prevent apoptosis in response to UV dependent upon p53.[6] We and others have recently shown that high expression of Spy1 correlates with high glioma cell viability, and Spy1 is highly expressed in GBM.[7] We suspect that the GBM cell's resistance to TNF-α-induced apoptosis may be attributed to the high expression of Spy1.

TNF-α mediates an aggregate of TNF-receptor-type 1 (TNFR1) and induces a Complex-I formation by recruiting adaptor proteins TNF receptor-associated death domain protein (TRADD) and receptor-interacting protein 1 (RIP1) to TNFR1. Nevertheless, the dissociation of these proteins from Complex-I initiates an formation of a second protein complex (Complex-II)-containing Fas-associated protein with death domain (FADD) and Caspase-8.[8-11] Besides, apoptotic signaling mediated through the transition from Complex-I to Complex-II is tightly regulated by de-ubiquitination of RIP1.[11,12] CLIP-170-related 59 kDa protein (CLIPR-59)-a protein important for cell apoptosis and activation of caspases in the context of TNF-α signaling, is 547 amino

*Correspondence to: Aiguo Shen; Email: shenaiguoaiguo@sina.com
Submitted: 01/12/2015; Revised: 04/09/2015; Accepted: 04/11/2015
http://dx.doi.org/10.1080/15384101.2015.1041688

acids long and consists of 3 ankyrin repeats near its N-terminus and 2 CAP-Gly domains (also referred as the microtubule binding domain) in the middle of the molecule.[12,13] CLIPR-59 scaffolds a de-ubiquitinating enzyme-CYLD into the complex containing RIP1 and facilitates de-ubiquitination on RIP1 in TNF-α signaling, thus resulting in the transition from Complex-I to Complex-II. Because of their proapoptotic property, CLIPR-59 can be considered a tumor suppressor.[12] Recent work has shown that CLIPR-59 gene expression is epigenetically silenced in tumor-conditioned human umbilicalvein endothelial cells.[14]

Accordingly, in this work, we investigated whether overexpression of Spy1 could confer resistance to TNF-α-induced apoptosis in glioma cells. We found that CLIPR-59 was downregulated in malignant glioma cells and high-grade glioma tumor samples, which was associated with decreased cancer-free survival. Spy1 interacts with CLIPR-59, which leads to the inhibition of interaction of CLIPR-59 with CYLD in a competitive manner thereby attenuating the lysine-63-dependent de-ubiquitinating activity of RIP1. Consequently, TNF-α-induced apoptosis of glioma cells is suppressed. Therefore, our data have identified a novel Spy1-CLIPR-59 interplay in GBM cell's resistance to TNF-α-induced apoptosis revealing a potential target in the intervention of malignant brain tumors.

Results

Low CLIPR-59 expression level in high grade glioma biopsy samples

Though the antitumor activity of CLIPR-59 has been reported,[12] nothing is known about the status of CLIPR-59 in human glioma samples. We investigated that CLIPR-59 expression was significantly lower in malignant glioma cells (e.g., U87MG) than in less aggressive glioma cells (e.g., H4) (**Fig. 1A**). Importantly, CLIPR-59 levels were reduced in high-grade glioma samples as opposed to low-grade glioma and normal tissue (**Fig. 1A**, **Table 1**). Immunohistochemistry (IHC) also confirmed the results of western blot analysis. CLIPR-59 immunoreactivity was predominantly located in the cytoplasm in a variable number of glial cells (**Fig. 1B**). CLIPR-59 does not correlate with patients' age, gender, KPS score, tumor location, type of surgery, tumor diameter, and vessel density (**Table 1**). However, CLIPR-59 was significantly associated with pathologic stage of the glioma (**Table 1**). We further suggested that Spy1 expression is negatively associated with CLIPR-59 (Spearman's $\gamma = -0.872$, $P = 0.000$) in all 70 cases of glioma analyzed (**Fig. 1C**). Furthermore, Kaplan-Meier survival curves indicated that low CLIPR-59 expression related to a poor survival with statistical significance (**Fig. 1D**a). And patients with the phenotype of Spy1(0–3)/CLIPR-59(4–7) have even longer overall survival than others (**Fig. 1D**b). Therefore, low CLIPR-59 expression is clearly associated with a highly aggressive phenotype of glioma.

Spy1 interacts with CLIPR-59 in GBM cells

Because Spy1 is overexpressed in malignant gliomas and critically regulates the proliferation of glioma cells,[7] and we observed that Spy1 expression is negatively associated with CLIPR-59 in glioma analyzed, we sought to tackle the possible relationship between Spy1 and CLIPR-59. Spy1 was found to interact with CLIPR-59 in reciprocal coimmunoprecipitations in HEK293T cells transfected with hemag-glutinin (HA)-tagged Spy1 and myc-tagged CLIPR-59 (**Fig. 2A**). More importantly, endogenous Spy1 and CLIPR-59 could interact with each other in H4 and U87MG cells (**Fig. 2B**). The interaction between Spy1 and CLIPR-59 was further supported by the co-localization of these 2 proteins in H4 and U87MG cells by immunofluorescence microscopy (**Fig. 2C**).

Next, to identify the domain that is involved in the interaction, we generated different deletion constructs of Spy1 and CLIPR-59. As shown in **Figure 2D**, the Speedy/Ringo box and amino-terminal domains seemed to be required for Spy1 to interact with CLIPR-59, as deletion of the domain abrogated the interaction. Conversely, the C-terminal domain of CLIPR-59 mediated the Spy1 and CLIPR-59 interaction, whereas the amino-terminal, ANK and MTB domains were neither sufficient nor necessary (**Fig. 2E**).

Spy1 does not affect the amount of the transduction proteins while supresses the interaction of CLIPR-59 with CYLD during TNF-α signaling

Previous work has shown that CLIPR-59 is important for controlling cell apoptosis in the TNF-α signal transduction pathway,[12] and we observed that CLIPR-59 expression is significantly lower in GBM, whereas Spy1 is overexpressed in malignant gliomas. Though CLIPR-59 trigger apoptosis through the formation of Complex-I and II, containing TNFR1, CYLD, RIP1, FADD, and Caspase-8,[12] CLIPR-59 does not affect the amount of these proteins in the TNF-α signal transduction pathway. CLIPR-59 regulates TNF-α-induced apoptosis by controlling ubiquitination of RIP1. Similarly, we found that Spy1 also has no effect on the expression of these proteins in H4 and U87MG glioma cells, including CLIPR-59. Neither down-regulation nor overexpression of CLIPR-59 did affect the expression of Spy1 (**Fig. 3A**). These results suggest that it does not display a clear upstream-downstream relationship between Spy1 and CLIPR-59 in this experiment. We concluded that the way of Spy1 participated in the development of GBM is not by regulating the amount of these mediators.

Previous work suggested that CLIPR-59 scaffolds CYLD into the complex containing RIP1 and thus facilitates de-ubiquitination of RIP1 in TNF-α signaling.[12] CLIPR-59 regulates TNF-α-induced apoptosis by controlling ubiquitination of RIP1. Co-IP assays showed that the binding ability of CLIPR-59 and CYLD was decreased when Spy1 was overexpressedwhile increased when Spy1 was knockdown in HEK293T cells transfected with myc-CYLD and Flag-CLIPR-59 (**Fig. 3B and C**). The corresponding results were obtained in vivo using U87MG and H4 cells. However, overexpression of Spy1 did not impair CLIPR-59 binding to RIP1 (**Fig. 3D and E**). This indicated that Spy1 may supress the binding ability of CLIPR-59 and CYLD during TNF-α signaling in vitro and vivo.

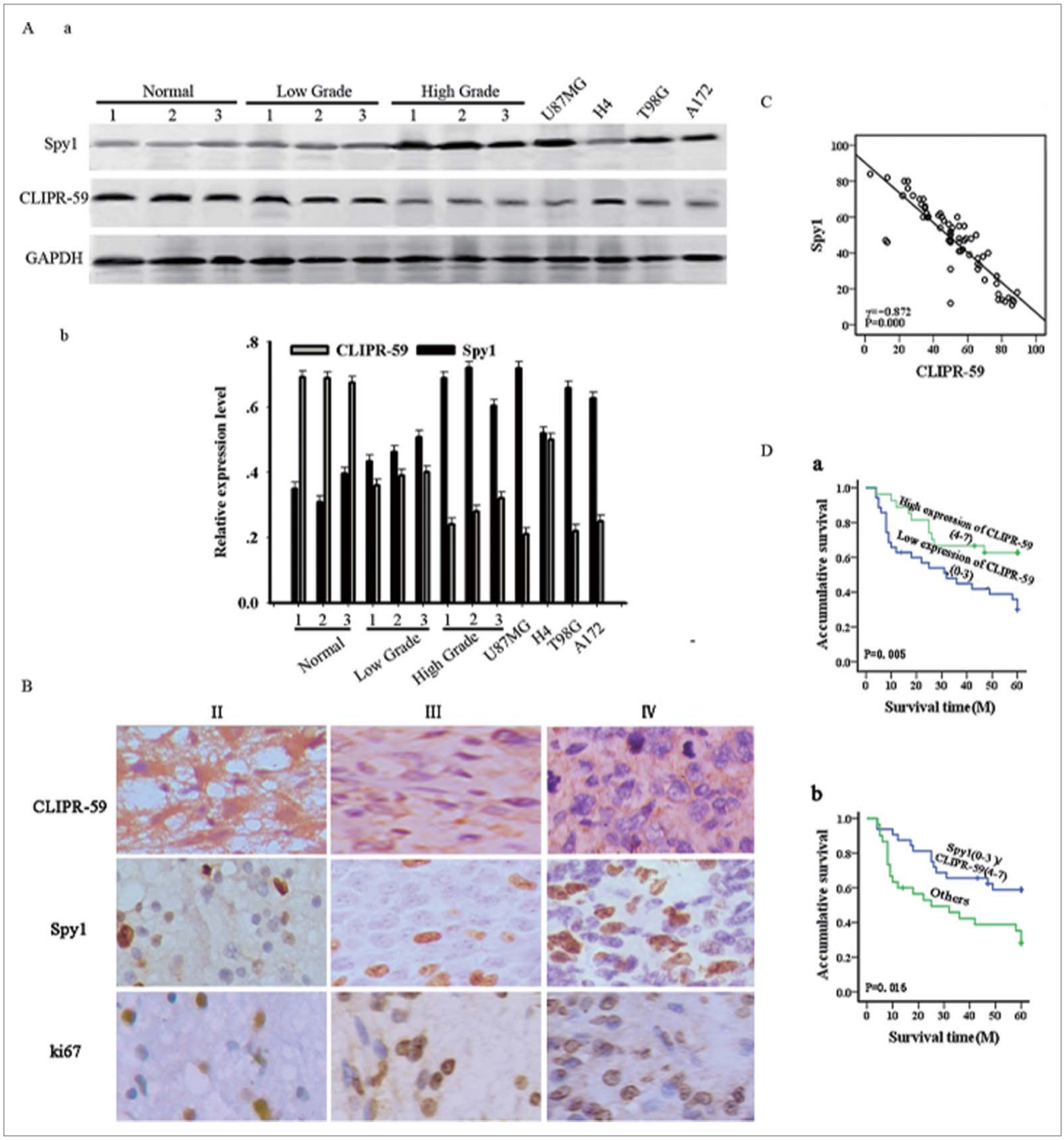

Figure 1. CLIPR-59 directly correlates with the grade of glioma and confers a better survival. (**A**) a. Western blots of brain normal tissue samples from 3 subjects and samples of glioma tissues (grades II, III, and IV) from 9 subjects and 4 glioma cell lines (U87MG, T98G, A172 and H4) using antibodies against CLIPR-59 and Spy1. GAPDH was used as a control for protein load and integrity. b. Relative density of immunoblotting bands was determined using a gel documentation system. Data were normalized to GAPDH. The data are means ± SD. (**B**) Immunohistochemical staining for CLIPR-59, Spy1 and ki-67 was performed as described in materials and methods. (**C**) Graphic representation of relationship between CLIPR-59 and Spy1 expression in glioma by Spearman's analysis. (**D**) Kaplan-Meier analysis of the correlation between CLIPR-59 and Spy1 expression and the overall survival of glioma patients. a. Patients in the low expression CLIPR-59 group were significantly associated with short overall survival. b. Patients with the phenotype of Spy1(0–3)/CLIPR-59(4–7) have even shorter overall survival than others.

Spy1 inhibits the lysine-63-dependent ubiquitination of RIP1 by suppressing the association of CLIPR-59 and CYLD

Interestingly, ubiquitination of RIP1 switches its function from pro-apoptotic to anti-apoptotic.[15,16] Constitutive RIP1 ubiquitination is detected in several tumor cell lines, but was not observed in primary cells such as MEFs.[17] CLIPR-59 appears to interact with CYLD and modulates de-ubiquitination of RIP1.[12] For an ubiquitination assay HA-Ub and Flag-RIP1 were transfected together with/without GFP-CLIPR-59 and myc-Spy1 or SiSpy1 in HEK293T cells. Western blotting against all

Table 1. Correlation between CLIPR-59 and Spy1 expression and clinicopathologic variables

Variable	Total	CLIPR-59 expression				P*	Spy1 expression				P*
		0–3,(n) %		4–7,(n) %			0–3,(n) %		4–7,(n) %		
Gender											
Female	31	13	(41.94)	18	(58.06)	0.148	15	(48.39)	16	(51.61)	0.228
Male	39	24	(61.54)	15	(38.46)		13	(33.33)	26	(66.67)	
Age											
<40	26	12	(46.15)	14	(53.85)	0.461	14	(53.85)	12	(46.15)	0.082
≥40	44	25	(56.82)	19	(43.18)		14	(31.82)	30	(68.18)	
KPS											
<80	16	9	(56.25)	7	(43.75)	0.886	6	(37.50)	10	(62.50)	0.973
≥80	49	25	(51.02)	24	(48.98)		20	(40.82)	29	(59.18)	
Unknown	5	3	(60.00)	2	(40.00)		2	(40.00)	3	(60.00)	
Tumor location											
Frontal	26	15	(57.69)	11	(42.31)	0.688	9	(34.62)	17	(65.38)	0.186
Parietal	6	2	(33.33)	4	(66.67)		3	(50.00)	3	(50.00)	
Occipital	3	1	(33.33)	2	(66.67)		3	(100.00)	0	(0.00)	
Temporal	15	7	(46.67)	8	(53.33)		7	(46.67)	8	(53.33)	
Unknown	20	12	(60.00)	8	(40.00)		6	(30.00)	14	(70.00)	
Surgery											
Biopsy	12	6	(50.00)	6	(50.00)	0.465	4	(33.33)	8	(66.67)	0.670
Partial resection	21	9	(42.86)	12	(57.14)		10	(47.62)	11	(52.38)	
Gross total resection	37	22	(59.46)	15	(40.54)		14	(37.84)	23	(62.16)	
Vessel density											
Normal	39	25	(64.10)	14	(35.90)	0.053	14	(35.90)	25	(64.10)	0.470
Increased	31	12	(38.71)	19	(61.29)		14	(45.16)	17	(54.84)	
Tumor diameter											
<4 cm	36	19	(52.78)	17	(47.22)	1.000	13	(36.11)	23	(63.89)	0.626
≥4 cm	34	18	(52.94)	16	(47.06)		15	(44.12)	19	(55.88)	
WHO grade											
II	34	11	(32.35)	23	(67.65)	0.004	22	(64.71)	12	(35.29)	0.000
III	24	17	(70.83)	7	(29.17)		5	(20.83)	19	(79.17)	
IV	12	9	(75.00)	3	(25.00)		1	(8.33)	11	(91.67)	

*Correlation was analyzed by use of Fisher's exact test. KPS Kar-nofsky performance scale, CLIPR-59 CLIP-170-related 59 kDa protein.

HA-ubiquitin conjugated proteins in whole cell lysates revealed that, in the group cells overexpressing Spy1 remarkably decreased de-ubiquitination of RIP1 compared with the other groups (**Fig. 4A**). On the other hands, in the cells treated with small interfering RNAs (siRNAs) against Spy1 (SiSpy1), the ubiquitination of RIP1 was low as the conrol group (**Fig. 4B**). We also obtained the similar results in TNF-α treated U87MG glioma cells which highly expressed Spy1 and found that the level of de-ubiquitination of RIP1 reversed when Spy1 and CLIPR-59 were simultaneously knockout (**Fig. 4E**). These findings suggested that Spy1 may supress the de-ubiquitination of RIP1 via CLIPR-59 in GBM.

For further analysis, we used different deletion constructs of Spy1 in HEK293T cells suffering similar treatment as **Figure 4A and B** to determine the mechanism through which Spy1 regulates the de-ubiquitination of RIP1. Fragment 69–199 of Spy1 decreased de-ubiquitination of RIP1 compared with the other Spy1 fragments (**Fig. 4C**). In addition, fragment 488–547 of CLIPR-59 is needed for regulating de-ubiquitination of RIP1 in TNF-α signaling.[12] We noticed a marked reduction in RIP1 de-ubiquitination in the group of cells transfected with fragment 69–199 of Spy1 and 488–547 of CLIPR-59 compared with group of the fragment 488–547 of CLIPR-59 lonely (**Fig. 4D**), indicating that the effect of Spy1 on de-ubiquitination of RIP1 was mediated by the interaction of Spy1 with CLIPR-59. Similar results were also obtained in TNF-α treated H4 glioma cells which highly expressed CLIPR-59 as shown in **Figure 4F**.

CLIPR-59 should specifically regulate lysine-63-dependent ubiquitination on RIP1, and once K63 ubiquitinated, RIP1 functions as a scaffold that assembles signaling complexes that activate prosurvival signaling pathways.[18-20] Next we investigated the influence of Spy1 in this signaling pathway by using ubiquitin mutant, K63R or K48R (lysine at the position 63 or 48 is mutated to arginine) respectively. In agreement with the data, Spy1 should specifically supress lysine-63-dependent de-ubiquitination of RIP1 (**Fig. 4G**). Taken together, our results indicated that the interaction between Spy1 and CLIPR-59 may specifically regulate the lysine-63-dependent ubiquitination of RIP1 by suppressing the association of CLIPR-59 and CYLD.

Spy1 regulates glioma cell viability and confers resistance to TNF-α-induced apoptosis possiblely by suppressing the association of CLIPR-59 and CYLD

U87MG cells are resistant to TNF-α-mediated apoptosis, while H4 and T98G glioma cells are not.[21] As Spy1 levels were elevated in GBM samples, we investigated its role in glioma cell survival and resistance to apoptosis. Treatment with TNF-α alone increased the viability of U87MG cells, while co-treatment

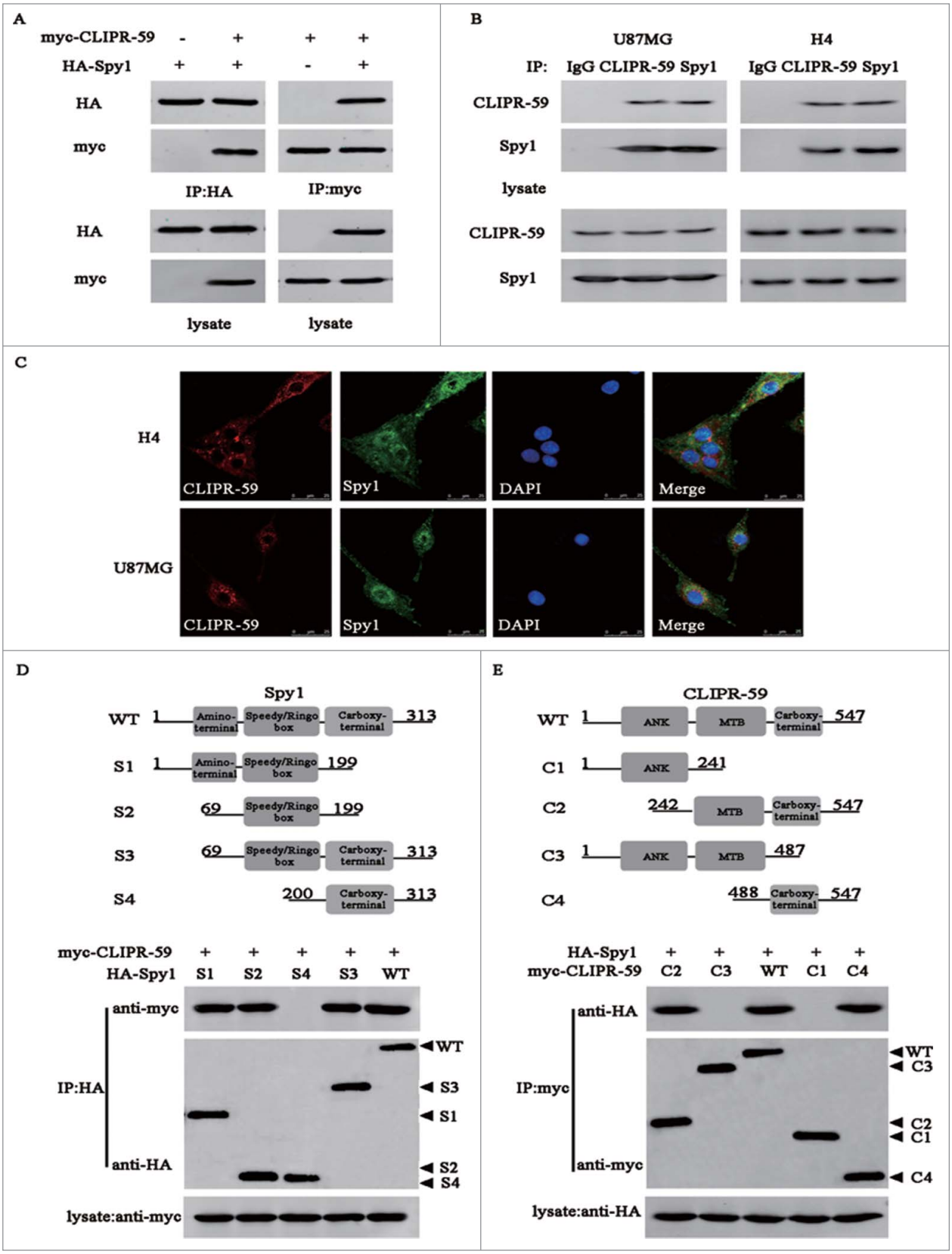

Figure 2. For figure legend, see page 6.

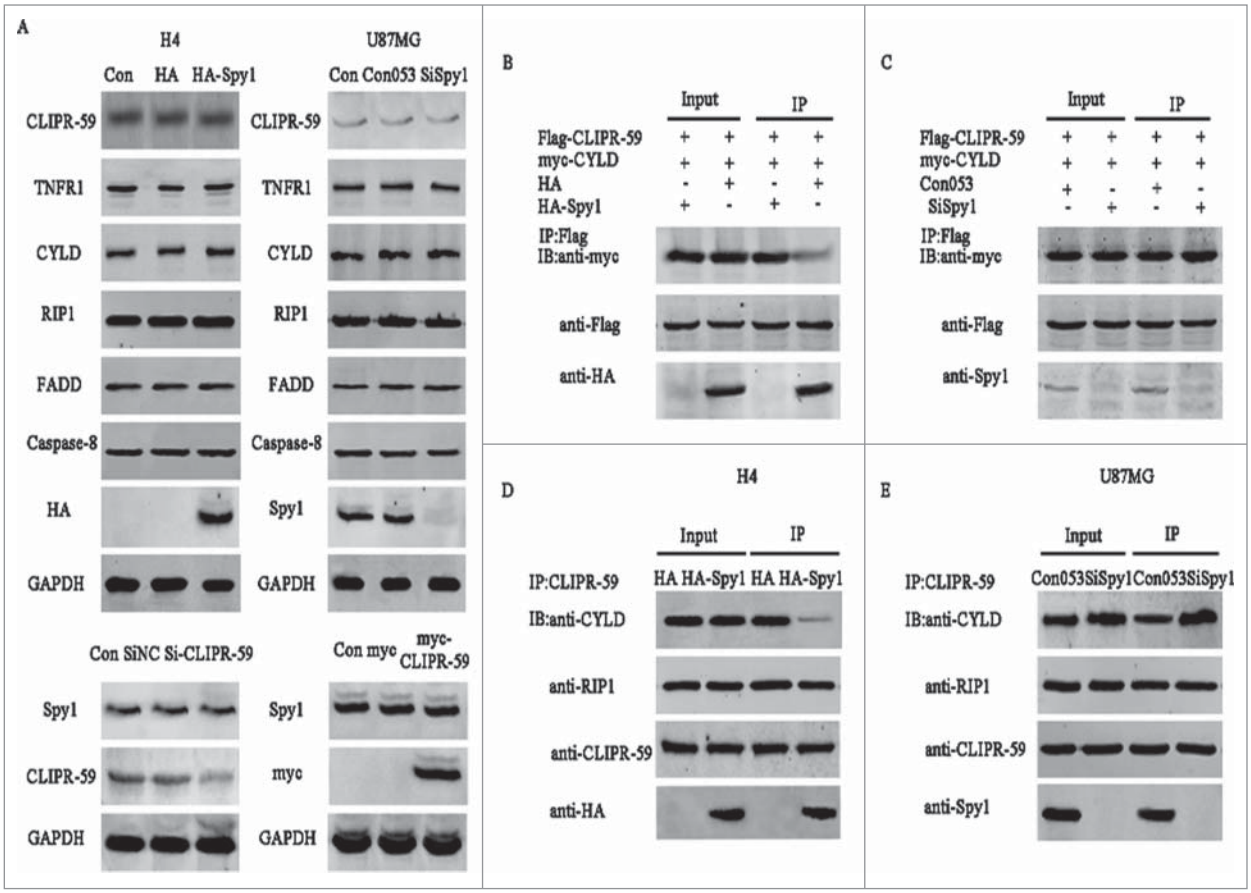

Figure 3. Spy1 did not affect the amount of the transduction proteins while supress the interaction of CLIPR-59 with CYLD during TNF-α signaling. (**A**) To determine which was the specificity target of Spy1 in the present study, U87MG and H4 glioma cells were transfected with the control vector (Con053), Spy1 siRNA (SiSpy1) or HA-Spy1 expression vector as indicated. At 72 hr after the transfection, cells were lysed and analyzed by SDS-PAGE and immunoblot using the indicated antibodies. (**B, C**) HEK293T cells were co-transfected with Flag-CLIPR-59, myc-RIP1, HA-Spy1 or SiSpy1 vector. Cell lysates were immunoprecipitated with anti-Flag antibody and analyzed by Western as indicated. (**D, E**) H4 cells were transfected with HA empty vector (HA), HA-Spy1 expression vector (**D**), and U87MG cells were transfected with the control vector (Con053), SiSpy1 vector (**E**). Cells were lysed and immunoprecpitated using anti-CLIPR-59-specific Ab. Precipitated materials were analyzed by IB using the indicated Abs.

with SiSpy1 resulted in approximately 51–83% decrease in viability of U87MG cells, as compared with control (**Fig. 5A**). To further assess the effects of Spy1 on apoptosis in TNF-α signaling, FACS assay was performed. The 1.62% increase in apoptosis cells observed upon Spy1 inhibited was further elevated to 10.55% when Spy1 inhibited was co-administered with TNF-α treated for 24 h in U87MG cells (**Fig. 5B and C**). This ability of Spy1 inhibited to reverse TNF-α mediated resistance to apoptosis indicated the involvement of Spy1 in CLIPR-59-induced cell apoptosis in TNF-α signaling. In addition, SiSpy1-treatment induced the activation of Caspase-8 and the following activation of Caspase-3 (**Fig. 5D**) stimulated by TNF-α, further suggesting that Spy1 confers resistance to TNF-α-induced apoptosis. Thus, Spy1 expression is required for the resistance to TNF-α-induced apoptosis, and this process may be mediated by suppressing the association of CLIPR-59 and CYLD.

Figure 2 (**See previous page**). Spy1 interacts with CLIPR-59 in vivo and in vitro. (**A**) HA-Spy1 and myc-CLIPR-59 vectors (1.5 μg each) were co-transfected into HEK293T cells. Extracts with equal amount of protein were immunoprecipitated with anti-HA or anti-myc antibody and analyzed by Western Blot. (**B**) U87MG and H4 cells were lysed and equal amount of lysates were co-immunoprecipitated with anti-CLIPR-59 or anti-Spy1 antibody. The normal rabbit IgG was used as a negative control. (**C**) U87MG and H4 cells were fixed and immunofluorescence microscopy was performed using indicated antibodies to show co-localization of endogenous CLIPR-59 and Spy1. The nucleus was counterstained by DAPI. Scale Bar=25 μm. (**D**) Spy1 contains a amino-terminal domain (1–68), a Speedy/Ringo box domain (69–199), and a C-terminal domain (200–313). HEK293T cells were transfected with myc-CLIPR-59 and HA-Spy1 mutants (1.5 μg each). Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by Western. (**E**) CLIPR-59 contains a ANK domain (1–241), a MTB (242–487) domain, and a C-terminal domain (488–547). HEK293T cells were transfected with myc-CLIPR-59 mutants and HA-Spy1 (1.5 μg each). Cell lysates were immunoprecipitated with anti-myc antibody and analyzed by western blot.

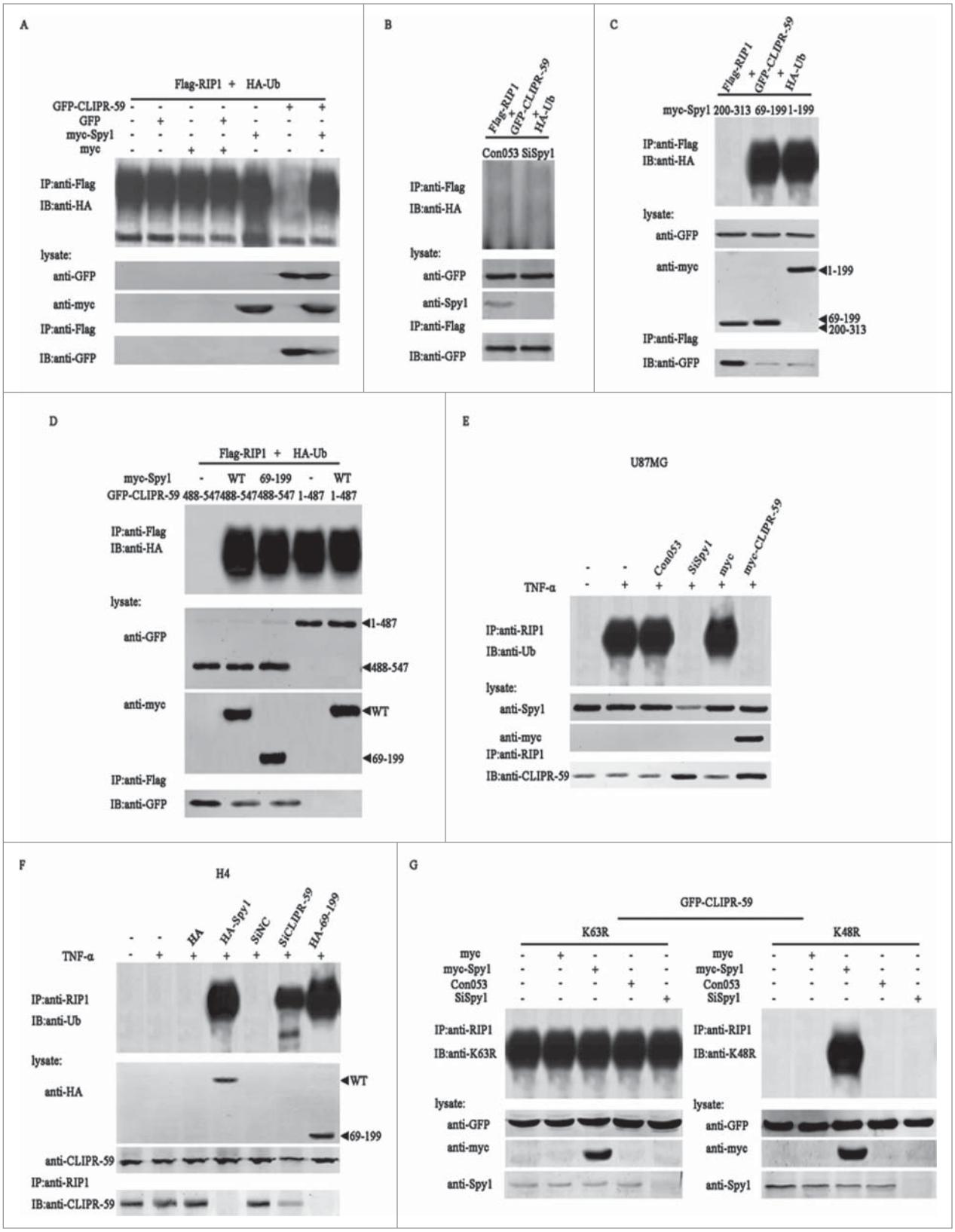

Figure 4. For figure legend, see page 8.

Discussion

Deregulation of apoptosis in cancer promotes disease progression, and drugs that restore apoptotic signaling pathways may have substantial therapeutic benefit. GBM is the most common primary malignant brain tumor in adults and is resistant to TNF-α induced apoptosis.[22,23] Our data as summarized here allude to the interplay between Spy1 and CLIPR-59 as a key determinant of resistance of glioma cells to TNF-α induced apoptosis, thus shedding light on the understanding of the pathogenesis of GBM.

CLIPR-59 has been shown to promote Caspase-8/Caspase-3 activation, sensitizing tumor cells to TNF-α-induced cell apoptosis.[12] Moreover, we indicated that CLIPR-59 is low-expressed in high-grade glioma samples as compared with low-grade glioma and normal tissues, and confers a preferable prognosis in this disease. Spy1 enhances mammalian cell survival in response to a number of genotoxic agents, including UV irradiation where it prevents caspase activation and apoptosis dependent on the gene regulatory protein p53.[6,24,25] Our previous investigation has also demonstrated that aberrant Spy1 expression directly affects the proliferation of human gliomas.[7] Interestingly, here we show that CLIPR-59 interacts with Spy1 via the C-terminal domain in glioma, which is needed for de-ubiquitinating function of CLIPR-59.[12] We show that inhibition of Spy1 increases the association of CLIPR-59 with CYLD and reduces the ubiquitination of RIP1. However, overexpression of Spy1 did not impair CLIPR-59 binding to RIP1. It has been broadly vertified that the C-terminal region of CLIPR-59 is essential for its interaction with CYLD whereas it did not destroy the combination with RIP1. [12] Our results are coincident with the viewpoint.

Loss of RIP1 ubiquitination, allows RIP1 to bind caspase-8, and thereby activates apoptosis.[15,17] In this study, we have shown that Spy1 is constitutively high expressed in GBMs and is attenuated by treatment with SiSpy1. Spy1 interrupts the de-ubiquitination of RIP1 mediated by CLIPR-59. We also showed that increased de-ubiquitination of RIP1 evoked by SiSpy1 deteriorates GBM's resistance to TNF-α–induced apoptosis and reported that SiSpy1-induced activation of a caspase 8- caspase 3 cascade is required for its apoptotic activity. We conclude that the interaction between Spy1 and CLIPR-59 is the central mechanism participated in GBM's resistance to TNF-α–induced apoptosis. Besides, the low expression of CLIPR-59 which hinted a better survival may be related to GBM's resistance to TNF-α–induced apoptosis. Accordingly, future investigations exploiting the more precise mechanism will be likely needed.

Materials and Methods

Processing of tissue and IHC

The study was conducted after informed consent was obtained from all subjects and the protocol was approved by the Partners Human Research Committee at the Affiliated Hospital of Nantong University. IHC was performed on tissue samples collected from patients with histologically confirmed gliomas to determine Spy1 and CLIPR-59 expression as described.[26,27] Non-neoplastic brain tissue were acquired from patients undergoing surgery for epilepsy and were reviewed to verify the absence of tumor.

Cell culture and treatment

The human glioma cell lines U87MG, A172, T98G and H4, which were derived from patients with glioblastoma, HEK293T is a human embryonic kidney cell line with stably incorporated SV40 large T antigen. All cell lines were cultured in DMEM (GibCo BRL, Grand Island, NY, USA) with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin mixture (GibCo BRL) at 37°C with 5% CO_2. Transient transfections were performed using lipofectamine 2000 following manufacturer's recommendation. For the creation of stable cells, transfected cells were exposed to 0.5mg/ml neomycin (for overexpression vector) or 0.01 mg/ml blasticidin (for siRNA vector) for 2 weeks.

Immunoprecipitation and protein gel blotting

The interaction between Spy1 and CLIPR-59 was assessed by co-immunoprecipitation assays as previously reported.[28] Briefly, cells were collected in PBS and lysed by RAPI buffer. After preclearing with protein G sepharose beads, cell lysates were incubated with specific antibody bound to either protein A/G sepharose beads (Roche) for 12 hours at 4°C. Precipitated immunecomplexes were washed with RIPA buffer 4 times, eluted by boiling in 2×SDS sample buffer, resloved by SDS-PAGE gel, and analyzed by immunoblotting as previously reported.[29]

Figure 4 (**See previous page**). Spy1 inhibits the lysine-63-dependent de-ubiquitination of RIP1 by suppressing the association of CLIPR-59 and CYLD. (**A, B**) HEK293T cells were transfected with/without the expression plasmids for GFP, GFP-CLIPR-59, myc, myc-Spy1, the control vector (Con053) or SiSpy1 vector (1 μg/well) with the combination of the plasmids for FLAG-RIP1 (1 μg/well) and HA-ubiquitin WT (HA-Ub; 0.1 μg/well) followed by treatment with or without MG132 (50 μM) for 2 hours. At 24 h after transfection, cell lysates were immunoprecipitated with anti-Flag antibody and analyzed by Western as indicated. (**C**) HEK293T cells were transfected with the expression plasmids for myc-Spy1 mutants (1–199, 69–199, 200–313, respectively; 1 μg/well) with the combination of the plasmids for GFP-CLIPR-59, FLAG-RIP1 (1 μg/well) and HA-ubiquitin WT (HA-Ub; 0.1 μg/well). Cells were analyzed as described in **Figure 4A, B**. (**D**) HEK293T cells were transfected with/without the expression plasmids for myc-Spy1 WT, myc-Spy1 mutants (69–199), GFP-CLIPR-59 WT or GFP-CLIPR-59 mutants (1–487, 488–547, respectively; 1 μg/wel) with the combination of the plasmids for FLAG-RIP1 (1 μg/well) and HA-ubiquitin WT (HA-Ub; 0.1 μg/well). Cells were analyzed as described in **Figure 4A, B**. (**E, F**) U87MG and H4 cells transfected with/without SiSpy1, myc-CLIPR-59, myc-Spy1 WT, myc-Spy1 mutants (69–199) or CLIPR-59 siRNA (SiCLIPR-59) were pretreated with proteasome inhibitor MG132 (21 mM) for 10 min before stimulation with TNF-α (10 ng/ml) or medium for additional 10 min. After cell lysing, the cell lysates were subjected to sequential IP methods for detecting ubiquitination of RIP1. (**G**) HEK293T cells were transfected with/without the expression plasmids for myc, myc-Spy1, Con053 or SiSpy1 (1 μg/well) with the combination of the plasmids for GFP-CLIPR-59 (1 μg/well), FLAG-RIP1 (1 μg/well) and HA-ubiquitin K63R or K48R mutant (K48R or K63R, respectively; 0.1 mg/well). Cells were analyzed as described in **Figure 4A, B**.

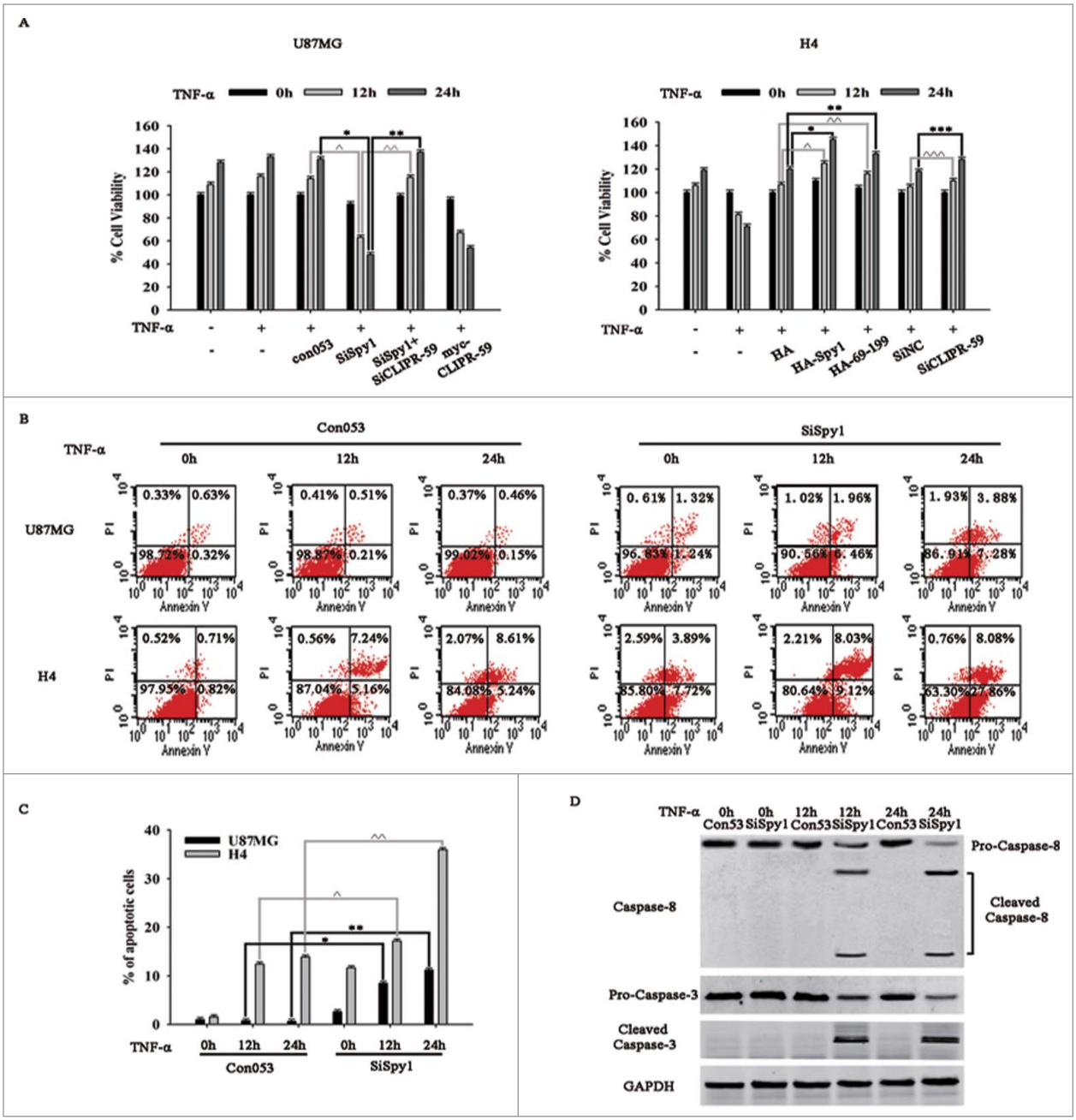

Figure 5. Spy1 regulates glioma cell viability and confers resistance to TNF-α–induced apoptosis in vitro and vivo. (**A**) Viability of glioma cells stably transfected with/without SiSpy1, SiCLIPR-59, myc-CLIPR-59 or HA-Spy1 in the presence or absence of TNF-α was determined by CCK8 assay. The graphs represent the viable glioma cells, percentage of control, upon treatment with 10 ng/ml TNF-α for 24 h. *^**^^***^^^, $P < 0.05$, are identified in comparison with the control group. Only one of 3 representative experiments is shown here. Error bars represent SEM. (**B, C**) Stably transfected glioma cells were treated as indicated and cultured in a humidified (37°C, 5% CO_2) incubator. Cells were then harvested and measured for apoptosis using Annexin V-FITC apoptosis detection kit followed by flow cytometry analysis. * ^ ** ^^, $P < 0.05$, are identified in comparison with the control group. Only one of 3 representative experiments is shown here. Error bars represent SEM. (**D**) Increase in caspase-3 and caspase-8 activity in glioma cells treated with both SiSpy1 and TNF-α, as determined by western.

Immunofluorescence staining

H4 and U87MG cells were seeded on coverslips in 24-well plate and cultured overnight. Subsequently cells were washed with PBS, fixed with 4% paraformaldehyde (1 hours), permeabilized with 0.1% Triton X-100 (15 minutes), and blocked for nonspecific binding in phosphate-buffered saline (PBS), 3% milk for 30 minutes. H4 and U87MG were incubated with a primary antibody at 4°C overnight. After three washes with PBS, samples were incubating with secondary antibody for 30 minutes at 37°C. For DNA staining, samples were incubated with Hoechst 33342 dye (1 μg/ml, 10 minutes) after incubation with secondary antibodies. The fluorescence was detected by use of a Leica

fluorescence microscope (Germany). All assays were performed 3 times in duplicate.

Ubiquitination assays and protein purification

Various plasmids coding for Spy1, CLIPR-59, Flag-RIP1, HA-ubiquitin (WT), HA-ubiquitin (K48) or HA-ubiquitin (K63) were transfected in HEK293T, U87MG and H4 cells. 24 h post-transfection, cells were treated with MG132 or with TNF-α. Cells were harvested, lysed and boiled 5 min in 1% SDS (v/v) to remove all non-covalently associated proteins. Lysates were diluted 1 :10 in RIPA buffer supplemented by 2× protease inhibitor mixture (Sigma). Flag-RIP1 or RIP1 was immunoprecipitated using an anti-Flag (Sigma; F7425) or an anti-RIP1 antibody (Abcam; ab42125) as described above. Proteins bound to the beads were then eluted in Laemmli buffer and subjected to immunoblot analysis. All antibodies for Western were used at a dilution ratio of 1:1000.

Cell viability assay

To evaluate cell viability, cells were seeded on a 96-well cell culture cluster (Corning Inc., Corning, NY, USA) at a concent ration of 2×10^4/well in a volume of 100 μL. Twenty-four hours later, each well was transfected for different times. Cell numbers were measured colorimetrically using the Cell Counting Kit (Dojindo, Kumamoto, Japan) by ImmunoMini NJ-2300 (NJ InterMed, Tokyo, Japan) at a test wave length of 450 nm.

Quantification of cell apoptosis

Cells treated as indicated were washed 3 times in ice-cold PBS, resuspended in lysis buffer (0.1% sodium citrate and 0.1% Triton X-100 in PBS) and incubated with Annexin V-FITC (Bestbio, China) for 15 min at 4°C in the dark, according to the manufacturer's instructions. After staining, cells were incubated with propidium iodide (PI, Sigma, 20 μg/ml) for 5 min at 4°C in the dark and then analyzed using a flow cytometer (Beckman, USA).

Statistical analysis

All comparisons between groups were performed using 2-tailed paired student's *t*-test. All numerical data were described as mean ± SEM. All *P*-values less than 0.05 were taken as significant. All experiments were repeated at least 3 times. The non-parametric Spearman's rank correlation coefficient was applied to evaluate the strength of the relationship between Spy1 and CLIPR-59 expression. χ^2 and Fisher's exact tests were used to compare the expression of all proteins as groups (positive versus negative) with various clinicopathological parameters. Survival analysis was undertaken using the Kaplan-Meier method, and curves were compared using the log-rank test. All computations were carried outusing the SPSS 13.0 (SPSS, Inc., Chicago, IL) statistical programs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by National Basic Research Program of China (973 Program, No. 2012CB822104, 2011CB910604); grants from the National Natural Science Foundation of China (Nos. 314440037, 31270802, 81272789, 81372687 and 31300902); A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); the Colleges and Universities in Natural Science Research Project of Jiangsu Province (Nos.11KJA310002 and 13KJB310009).

References

1. Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat Clin Pract Oncol 2006; 3:388-98; PMID:16826219; http://dx.doi.org/10.1038/ncponc0538
2. Tsujimoto M, Yip YK, Vilcek J. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. Proc Natl Acad Sci U S A 1985; 82:7626-30; PMID:2999773; http://dx.doi.org/10.1073/pnas.82.22.7626
3. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007; 21:2683-710; PMID:17974913; http://dx.doi.org/10.1101/gad.1596707
4. Holland EC. Gliomagenesis: genetic alterations and mouse models. Nat Rev Genet 2001; 2:120-9; PMID:11253051; http://dx.doi.org/10.1038/35052535
5. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, DePinho RA. Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001; 15:1311-33; PMID:11390353; http://dx.doi.org/10.1101/gad.891601
6. McAndrew CW, Gastwirt RF, Donoghue DJ. The atypical CDK activator Spy1 regulates the intrinsic DNA damage response and is dependent upon p53 to inhibit apoptosis. Cell Cycle 2009; 8:66-75; PMID:19106603; http://dx.doi.org/10.4161/cc.8.1.7451
7. Zhang L, Shen A, Ke Q, Zhao W, Yan M, Cheng C. Spy1 is frequently overexpressed in malignant gliomas and critically regulates the proliferation of glioma cells. J Mol Neurosci 2012; 47:485-94; PMID:22447439; http://dx.doi.org/10.1007/s12031-012-9709-5
8. Bhardwaj A, Aggarwal BB. Receptor-mediated choreography of life and death. J Clin Immunol 2003; 23:317-32; PMID:14601641; http://dx.doi.org/10.1023/A:1025319031417
9. Reed JC. Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med 2001; 7:314-9; PMID:11425640; http://dx.doi.org/10.1016/S1471-4914(01)02026-3
10. Green DR. Overview: apoptotic signaling pathways in the immune system. Immunol Rev 2003; 193:5-9; PMID:12752665; http://dx.doi.org/10.1034/j.1600-065X.2003.00045.x
11. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003; 114:181-90; PMID:12887920; http://dx.doi.org/10.1016/S0092-8674(03)00521-X
12. Fujikura D, Ito M, Chiba S, Harada T, Perez F, Reed JC, Uede T, Miyazaki T. CLIPR-59 regulates TNF-alpha-induced apoptosis by controlling ubiquitination of RIP1. Cell Death Dis 2012; 3:e264; PMID:22297296; http://dx.doi.org/10.1038/cddis.2012.3
13. Lallemand-Breitenbach V, Quesnoit M, Braun V, El Marjou A, Pous C, Goud B, Perez F. CLIPR-59 is a lipid raft-associated protein containing a cytoskeleton-associated protein glycine-rich domain (CAP-Gly) that perturbs microtubule dynamics. J Biol Chem 2004; 279:41168-78; PMID:15262990; http://dx.doi.org/10.1074/jbc.M406482200
14. Hellebrekers DM, Melotte V, Vire E, Langenkamp E, Molema G, Fuks F, Herman JG, Van Criekinge W, Griffioen AW, van Engeland M. Identification of epigenetically silenced genes in tumor endothelial cells. Cancer Res 2007; 67:4138-48; PMID:17483324; http://dx.doi.org/10.1158/0008-5472.CAN-06-3032
15. O'Donnell MA, Legarda-Addison D, Skountzos P, Yeh WC, Ting AT. Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Curr Biol 2007; 17:418-24; PMID:17306544; http://dx.doi.org/10.1016/j.cub.2007.01.027
16. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 2006; 22:245-57; PMID:16603398; http://dx.doi.org/10.1016/j.molcel.2006.03.026
17. Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1

ubiquitination. Mol Cell 2008; 30:689-700; PMID:18570872; http://dx.doi.org/10.1016/j.molcel.2008.05.014
18. Lee TH, Shank J, Cusson N, Kelliher MA. The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. J Biol Chem 2004; 279:33185-91; PMID:15175328; http://dx.doi.org/10.1074/jbc.M404206200
19. Li H, Kobayashi M, Blonska M, You Y, Lin X. Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem 2006; 281:13636-43; PMID:16543241; http://dx.doi.org/10.1074/jbc.M600620200
20. Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 2004; 430:694-9; PMID:15258597; http://dx.doi.org/10.1038/nature02794
21. Sharma V, Tewari R, Sk UH, Joseph C, Sen E. Ebselen sensitizes glioblastoma cells to Tumor Necrosis Factor (TNFalpha)-induced apoptosis through two distinct pathways involving NF-kappaB downregulation and Fas-mediated formation of death inducing signaling complex. Int J Cancer 2008; 123:2204-12; PMID:18709644; http://dx.doi.org/10.1002/ijc.23771
22. Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2006; 2:494-503; quiz 1 p following 16; PMID:16932614; http://dx.doi.org/10.1038/ncpneuro0289
23. Tewari R, Choudhury SR, Ghosh S, Mehta VS, Sen E. Involvement of TNFalpha-induced TLR4-NF-kappaB and TLR4-HIF-1alpha feed-forward loops in the regulation of inflammatory responses in glioma. J Mol Med (Berl) 2012; 90:67-80; PMID:21887505; http://dx.doi.org/10.1007/s00109-011-0807-6
24. Barnes EA, Porter LA, Lenormand JL, Dellinger RW, Donoghue DJ. Human Spy1 promotes survival of mammalian cells following DNA damage. Cancer Res 2003; 63:3701-7; PMID:12839962
25. Gastwirt RF, Slavin DA, McAndrew CW, Donoghue DJ. Spy1 expression prevents normal cellular responses to DNA damage: inhibition of apoptosis and checkpoint activation. J Biol Chem 2006; 281:35425-35; PMID:16951407; http://dx.doi.org/10.1074/jbc.M604720200
26. Xie F, Liu H, Zhu YH, Qin YR, Dai Y, Zeng T, Chen L, Nie C, Tang H, Li Y, et al. Overexpression of GPR39 contributes to malignant development of human esophageal squamous cell carcinoma. BMC Cancer 2011; 11:86; PMID:21352519; http://dx.doi.org/10.1186/1471-2407-11-86
27. Ding Z, Liu X, Liu Y, Zhang J, Huang X, Yang X, Yao L, Cui G, Wang D. Expression of far upstream element (FUSE) binding protein 1 in human glioma is correlated with c-Myc and cell proliferation. Mol Carcinog 2013; 54:405-15; PMID:24347226 ; http://dx.doi.org/10.1002/mc.22114
28. Zhou L, Ma Q, Shi H, Huo K. NUMBL interacts with TRAF6 and promotes the degradation of TRAF6. Biochem Biophys Res Commun 2010; 392:409-14; PMID:20079715; http://dx.doi.org/10.1016/j.bbrc.2010.01.037
29. Tao T, Ji Y, Cheng C, Yang H, Liu H, Sun L, Qin Y, Yang J, Wang H, Shen A. Tumor necrosis factor-alpha inhibits Schwann cell proliferation by up-regulating Src-suppressed protein kinase C substrate expression. J Neurochem 2009; 111:647-55; PMID:19694904; http://dx.doi.org/10.1111/j.1471-4159.2009.06346.x