

Regulation of glioma migration and invasion via modification of Rap2a activity by the ubiquitin ligase Nedd4-1

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Abstract. Neuronal precursor cell expressed and developmentally downregulated protein (Nedd4-1) is an E3 ubiquitin ligase with critical roles in the pathogenesis of cancer. Herein, we demonstrated that Nedd4-1 protein was upregulated in glioma tissues vs. that in non-cancerous tissues by western blotting and immunohistochemistry. Scratch migration and Transwell chamber assays indicated that downregulation of Nedd4-1 significantly reduced the migration and invasion of the glioma cell lines U251 and U87. Conversely, overexpression of Nedd4-1 obviously enhanced the migratory and invasive capacities in both cell lines. To investigate the role of Nedd4-1 and the intracellular pathways involved, we performed pull-down and co-immunoprecipitation assays, and recognized that Nedd4-1, TNIK and Rap2a formed a complex. Moreover, Nedd4-1 selectively ubiquitinated its specific substrates, the wild-type Rap2a (WT-Rap2a) and dominant-active Rap2a (DA-Rap2a) rather than the dominant-negative Rap2a (DN-Rap2a) in the U251 cells. Subsequently, we demonstrated that Rap2a was robustly ubiquitinated by Nedd4-1 along with the K63-linked, but not the K48-linked ubiquitin chain, which significantly inhibited GTP-Rap2a activity by GST-RalGDS pull-down assay. To further verify whether the ubiquitination

of Rap2a by Nedd4-1 regulated the migration and invasion of glioma cells, Nedd4-1, HA-tagged ubiquitin and its mutants as well as WT-Rap2a were co-transfected in the U251 and U87 cell lines. The results confirmed that Nedd4-1 inhibited GTP-Rap2a activity, and promoted the migration and invasion of glioma cells. In brief, our findings demonstrated the important role of Nedd4-1 in regulating the migration and invasion of glioma cells via the Nedd4-1/Rap2a pathway, which may qualify Nedd4-1 as a viable therapeutic target for glioma.

Introduction

Gliomas are the most common primary tumors of the central nervous system. Despite recent advances in surgical approaches and available adjuvant therapies, a poor prognosis is imminent due to the highly migratory and invasive nature of glioma cells (1,2). Their active migration and invasion ultimately lead to tumor recurrence and reduced life expectancy (3). Nevertheless, the molecular mechanisms underlying glioma cell migration and invasion remain unclear. Therefore, it is vital to investigate the signaling pathways mediating glioma pathogenesis and progression in order to formulate efficient therapeutic regimens.

Ubiquitination is essential in many biologic processes, while aberrant expression of the enzymes involved in the regulation of the ubiquitination process plays a key role in malignant transformation (4). Protein ubiquitination is achieved in the ubiquitin-conjugating process via the covalent attachment of enzymes E1, E2 and E3 on the specific-target of substrate protein. The E3 ubiquitin ligases are the key regulatory components, which recognize specific substrates (5). Emerging evidence indicates that the E3 ubiquitin ligase Nedd4-1 is involved in cancer pathogenesis (6,7), and may be a target protein for cancer therapy (8-10), as the overexpression of Nedd4-1 in colorectal and gastric cancers has been found to promote malignant cell proliferation (11,12). FoxM1-regulated Nedd4-1 expression was reported to destabilize phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and to promote astrocyte transformation and glioma formation (5). To date, PTEN is the substrate which has received the most attention in Nedd4-1 ubiquitination (6,13,14). PTEN is argued to be dispensable for Nedd4-1 (15,16), which is supported by numerous studies (17-21). In addition, Nedd4-1 has been

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Abbreviations: Nedd4-1, neuronal precursor cell expressed and developmentally downregulated protein; TNIK, Traf2- and Nck-interacting kinase; Ub, ubiquitin

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reported in several cases of human tumors, whereas the functional significance of Nedd4-1 in glioma remains unclear.

Rap, the closest homolog of the small G protein Ras, plays a variety of biological functions in human cells, such as signal transduction, migration and proliferation (22,23). In addition to the recognized multiple mammalian proteins as Nedd4-1 substrates (24), the mono-ubiquitination of Rap2a by Nedd4-1 reportedly controls neurite development and decreases Rap2a activity in mouse neurons (24). Recent studies indicate that upregulation of Rap2a expression is correlated with tumor formation and progressive malignancy in human prostate and follicular thyroid cancer (25,26), whereas our previous study indicated that Rap2a is modestly expressed in glioma (27). Thus, further investigations are warranted to ascertain the effects of Nedd4-1-mediated Rap2a ubiquitination on the migration and invasion of glioma cells.

In the present study, we investigated the role of Nedd4-1 and the involvement of intracellular pathways in glioma. We validated that Nedd4-1 protein was overexpressed in glioma tissues vs. that noted in non-cancerous tissues. Moreover, Nedd4-1 promoted the migration and invasion of glioma cells, mediated by Nedd4-1/Rap2a ubiquitination.

Materials and methods

Glioma and non-cancerous brain tissue samples. The present study was approved by the Institutional Review Boards of The Affiliated Hospital of Xuzhou Medical College. Glioma tissue samples, obtained from patients undergoing resection for glioma tumors at the Department of Neurosurgery of The Affiliated Hospital of Xuzhou Medical College, were histologically confirmed by experienced pathologists based on the World Health Organization (WHO) grading system (28). Non-cancerous brain tissues were collected from patients undergoing decompression surgery for intracranial traumas.

Ethical approval. All procedures performed in the present study involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants or their legally authorized representatives prior to enrollment in the study.

Cell culture. Human glioma cell lines U251 and U87 (Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium and F-12 (DMEM/F-12) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), followed by incubation at 37°C in a humidified incubator with 5% CO₂.

Plasmids and antibodies. The mammalian expression plasmid encoding full-length human Nedd4-1 was a gift from Dr Xuejun Jiang (Memorial Sloan-Kettering Cancer Center, New York, USA). The shNedd4-1 plasmid was constructed by Shanghai GenePharma, and the human Nedd4-1 residues 217-549 was a gift from Dr Nile Brose (Max Planck Institute, Göttingen, Germany). The Myc-tagged Rap2a plasmids,

including wild-type (WT-Rap2a), dominant-active (DA-Rap2a) and dominant-negative (DN-Rap2a), were gifts from Dr Kenichi Kariya (University of the Ryukyus, Okinawa, Japan). DA-Rap2a and DN-Rap2a were constructed by mutating Gly 12 to Val and Ser 17 to Asn, respectively. Hemagglutinin (HA)-tagged ubiquitin and its mutants including K63R-ubiquitin and K48 R-ubiquitin were gifts from Shanghai Institute of Neuroscience, Chinese Academy of Sciences. The bacterial expression plasmid for the glutathione S-transferase (GST)-fusion protein of Ras-binding region of RalGDS was cloned by reverse transcription polymerase chain reaction (RT-PCR) using a human brain cDNA library as a template. The following antibodies were commercial products: rabbit polyclonal antibodies to Nedd4-1 and Rap2a (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-TNIK (BD Biosciences, Franklin Lakes, NJ, USA), mouse monoclonal antibodies P4D1 and FK1 to ubiquitin (Santa Cruz Biotechnology, Inc.), rabbit monoclonal antibodies to ubiquitin Apu2 and Apu3, and mouse monoclonal (9E10) antibody to Myc (Millipore, Billerica, MA, USA).

Reverse transcription-PCR. Total RNA was extracted from U251 and U87 cells using TRIzol reagent (Tiangen, Beijing, China). The RT-PCR protocol was performed according to the manufacturer's instructions using reverse transcription reagents (Takara, Dalian, China). Primers (Invitrogen, Shanghai, China) for Nedd4-1 were as follows: sense, 5'-ACTT TATCCATTACCGACAG-3' and antisense, 5'-TGGTGGCTT CATCTTCTC-3', yielding a 320-bp product. Primers for the internal standard β -actin were: sense, 5'-GCGCGGCTACAGC TTCAC-3' and antisense, 5'-GGGGCCGGACTCGTCATA-3', yielding a 500-bp product. PCR conditions were as follows: the first-strand DNA template was heated at 94°C for 3 min before amplification, a total of 30 cycles (for Nedd4-1) or 23 cycles (for β -actin) were used including 94°C for 30 sec (denaturation), 63°C for 30 sec (annealing), 72°C for 35 sec (extension), and 72°C for 7 min (extension).

Western blotting. Standard western blotting was performed using antibodies against Nedd4-1, Rap2a, TNIK, Myc and β -actin. Cell extracts were prepared 48 h after transfection and subjected to 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transference to 0.45- μ m pore size polyvinylidene fluoride membranes (Millipore) and blotted with the antibodies as indicated.

Immunohistochemistry. Immunohistochemistry was performed as previously described, with minor modifications (29). Paraffin-embedded tissue sections were obtained from the Department of Pathology, The Affiliated Hospital of Xuzhou Medical College, including 11 cases of WHO grade II astrocytomas, 12 cases of WHO grade III anaplastic astrocytomas, and 12 cases of WHO grade IV glioblastomas, with non-cancerous brain tissues as controls. Slices were incubated at 4°C overnight with the anti-Nedd4-1 antibody (1:100 dilution; Abcam, Cambridge, MA, USA). Subsequent to microphotography, the percentages of glioma cells with positive staining were presented and the Nedd4-1 expression levels were thereby categorized.

Transfection assay. Cell transfection was performed using PolyJet™ *In Vitro* DNA Transfection Reagent (SignaGen,

Rockville, MD, USA) when U251 cells reached 90% confluence on 10-cm plates. Plasmids encoding shRNA-Nedd4-1, full-length Nedd4-1, HA-tagged ubiquitin, and Myc-tagged Rap2a and its mutants were transfected in the respective experiments. Forty-eight hours after transfection, the cells were harvested, rinsed with phosphate-buffered saline (PBS) and lysed in 1% SDS or 1% NP40 buffer.

In vitro scratch assay. Cell motility was examined by scratch assay as previously described, except for minor modifications (30). Twelve hours after transfection, an artificial gap was created on the confluent cell monolayer using a plastic pipette tip. Migrated cells were quantified at 48 h (magnification, x200) in order to compare with the cell counts at base line with a computer-aided microscopy imaging system. All experiments were performed in triplicates.

Migration and invasion assays. The capabilities of cell migration were assessed by Transwell chamber assay. The invasion assay was performed as previously described with minor modifications (31). Glioma cells were harvested and resuspended in serum-free medium at a concentration of 1×10^5 cells/ml, and 200 μ l was added to the top chamber. The chambers were incubated for 24 h at 37°C in an incubator with 5% CO₂. The noninvasive cells on the upper surface of the membrane were removed by wiping with cotton-tipped swabs. The 8- μ m pore-size membrane was fixed and stained, and the population of the invaded cells was counted by light microscopy. All the experiments were performed in triplicates.

Affinity pull-down assay with GST-Nedd4-1. The pull-down assay was performed as previously described with minor modifications (24). Total proteins were extracted from a 1% NP-40 extract of U251 cells and separated by 12% SDS-PAGE followed by western blotting using anti-TNIK (1:500) and anti-Rap2a (1:500) antibodies.

Chemical cross-linking of the endogenous Nedd4-1/TNIK/Rap2 complex. U251 cells in 10-cm culture dishes were incubated with 1 mM dithiobis(succinimidyl propionate) (DSP) (Pierce, Rockford, IL, USA) for 3 min on ice. Excess DSP was quenched with 20 mM Tris-Cl (pH 7.5 at 4°C) for 15 min at room temperature, and then the proteins were extracted with RIPA buffer [50 mM Tris-Cl (pH 8.0) at 4°C, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin and 0.5 μ g/ml leupeptin]. Nedd4-1 was immunoprecipitated with anti-Nedd4-1 polyclonal antibodies coupled to protein A-sepharose beads (GE Healthcare, Milwaukee, WI, USA) at 4°C for 14 h. The beads were rinsed with RIPA buffer, and then the precipitated proteins were eluted and boiled in 2X loading buffer containing 50 mM DTT, and separated by SDS-PAGE followed by western blotting using anti-Nedd4-1, anti-TNIK and anti-Rap2 antibodies, respectively.

GST-RalGDS pull-down assay. A total of 40 μ g of GST fusion proteins of RalGDS encoding the Ras-binding domain (RBD) was purified and immobilized on GST-sepharose beads to pull down the GTP-bound form of Myc-Rap2a extracted from the U251 cells transfected with Myc-Rap2a, Nedd4-1

and HA-ubiquitin. After extensive washing at 4°C overnight with a buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2.5 mM MgCl₂, 10% glycerol and 1% NP-40, the bound proteins were eluted by boiling in 2X loading buffer followed by SDS-PAGE and western blotting.

Statistical analysis. Data are presented as mean \pm standard error of mean (SEM). Statistical significance was determined using Student's t-tests for comparisons between two groups and ANOVA for intergroup comparisons. P-values of <0.05 were considered statistically significant (P<0.05).

Results

Nedd4-1 expression in human glioma tissue samples. To investigate the possible effect in glioma pathogenesis, we determined Nedd4-1 protein expression in clinical glioma tissues and non-cancerous brain samples. A representative immunoblot is shown in Fig. 1A. Analysis of the band density revealed that Nedd4-1 was significantly upregulated in the glioma tissues vs. that observed in the non-cancerous samples (Fig. 1B). We further verified Nedd4-1 expression by immunohistochemistry. As shown in Fig. 1C, Nedd4-1 immunoreactivity was predominant in the cytoplasm. As shown in Fig. 1D, the percentage of Nedd4-1-positive cells reached $57.35 \pm 2.02\%$ (WHO grade II) and $75.82 \pm 3.06\%$ (WHO grade III-IV) in glioma tissues vs. the percentage of $22.30 \pm 2.17\%$ in non-cancerous brain samples. Thus, Nedd4-1 expression was significantly correlated with glioma malignancy. Fig. 1 shows significant upregulation of Nedd4-1 in glioma tissues vs. non-cancerous brain samples. These results indicated the potential involvement of Nedd4-1 in the pathogenesis of human glioma.

Effects of Nedd4-1 on cell migration. Given the overexpression of Nedd4-1 in glioma in contrast to non-cancerous tissues, we employed a gain-of-function strategy to assess the effect of Nedd4-1 on glioma cell migration. As shown in Fig. 2A and B, exogenous shNedd4-1 and Nedd4-1 were added to interfere with or overexpress Nedd4-1 in the U251 and U87 cell lines. The effect of Nedd4-1 on cell migration was assessed by scratch assay. It was shown that the migratory capacity of glioma U251 cells was significantly reduced upon downregulation of Nedd4-1 as compared with the scramble group. Conversely, the scratch in the Nedd4-1-overexpressing group was rapidly replenished compared with the vector group (Fig. 2C and D). We also performed cell migration assays using Transwell chambers to investigate the migratory capacities of the U251 and U87 cells. Likewise, the shNedd4-1 group showed significantly reduced migration compared with that noted in the scramble group (Fig. 2E and F), whereas the migratory behavior in the Nedd4-1-overexpressing group was evidently enhanced compared with that noted in the vector group in both the U251 and U87 cell lines (Fig. 2G and H). Collectively, we demonstrated that either the downregulation or overexpression of Nedd4-1 exerted relevant causality on the migratory capacity of both cell lines.

Effects of Nedd4-1 on cell invasion. In addition to the regulation of cell migration by Nedd4-1, we investigated the effect of

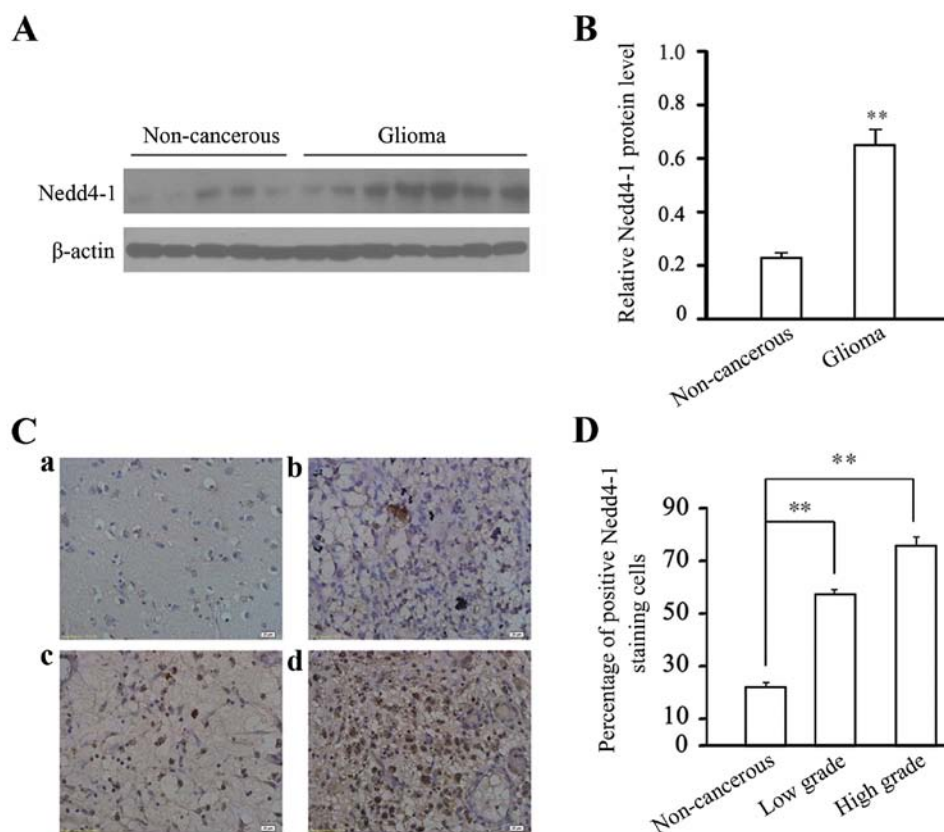


Figure 1. Expression profile of Nedd4-1 in human glioma tissues. (A) Representative western blotting of the total protein extracts isolated from human glioma (7 samples shown) specimens and human non-cancerous (5 samples shown) brain using rabbit Nedd4-1 antibody. β -actin served as a loading control. (B) Relative protein levels of Nedd4-1 in glioma specimens ($n=35$) and non-cancerous brain tissue ($n=15$). Data are expressed as the mean \pm SEM from three independent experiments; ** $P<0.01$ compared with non-cancerous brain tissue. (C) Representative IHC analyses of Nedd4-1 expression in non-cancerous brain and human glioma tissues of various (WHO) grades. a, Normal brain tissue; b, diffuse astrocytoma, grade II; c, anaplastic astrocytoma, grade III; and d, GBM, grade IV are shown. Scale bar, 20 μ m. (D) Quantitative analysis of the percentage of Nedd4-1-positive cells; ** $P<0.01$ compared with non-cancerous brain tissue. Grade II, low-grade glioma. Grade III-IV, high-grade glioma.

Nedd4-1 on cell invasion with Matrigel-precoated Transwell chambers. The results indicated that the invasion of U251 and U87 cells was significantly diminished by the downregulation of Nedd4-1 vs. the scramble group subsequent to 24 h of incubation (Fig. 3A and B), while the invasion in the Nedd4-1-overexpressing group was markedly enhanced vs. that noted in the empty vector group in both cell lines (Fig. 3C and D). Thus, both downregulation and overexpression of Nedd4-1 regulated the invasive capacities of the cell lines.

Rap2a as a Nedd4-1 substrate. To verify the role of Nedd4-1 targets in glioma cells, we selected the Nedd4-1 WW domains (32) (residue 217-547) as an affinity matrix and U251 cell lysate (1% NP40) to pull down TNIK and Rap2a. The results exhibited the presence of TNIK and Rap2a in the affinity-purified complex, confirming the binding of Nedd4-1 to either of the substrates (Fig. 4A). We further identified that endogenous Nedd4-1, TNIK and Rap2a constituted a complex (Fig. 4B). U251 cell lysate was incubated with thiol-cleavable cross-linker prior to immunoprecipitation with an anti-Nedd4-1 antibody. Under this condition, TNIK and Rap2a were coimmunoprecipitated with Nedd4-1.

Rap2a rather than TNIK was found to be ubiquitinated by Nedd4-1 in HEK cells, which decreased the activity of the GTP-bound form of Rap2a (24). To ascertain the potential

effect of Nedd4-1 on Rap2a ubiquitination in glioma cells, expression plasmids for the full-length Nedd4-1, HA-tagged ubiquitin as well as Myc-tagged WT-Rap2a, DA-G12V Rap2a or DN-S17N Rap2a were co-transfected in the U251 cells, with lysates extracted under denaturing conditions (100°C, 1% SDS) and Myc-tagged proteins immunoprecipitated from cell extracts using anti-Myc antibodies. As shown in Fig. 4C, immunoprecipitates from WT and DA-G12V instead of DN-S17N-expressing U251 cells showed a gradient of protein bands visible to an anti-ubiquitin antibody (P4D1) only in the presence of Nedd4-1 overexpression. These data demonstrated that Nedd4-1 selectively ubiquitinated WT-Rap2a and DA-Rap2a, but not DN-Rap2a.

To investigate the exact form of ubiquitination of Rap2a (i.e. mono-ubiquitination, di-ubiquitination or poly-ubiquitination), expression plasmids for full-length Nedd4-1, HA-tagged ubiquitin and Myc-tagged WT-Rap2a were co-transfected in U251 cells. Four types of anti-ubiquitin monoclonal antibodies which discriminate between mono-ubiquitinated and poly-ubiquitinated proteins were employed: P4D1 for poly-ubiquitin and mono-ubiquitin conjugates; FK1 only for poly-ubiquitin conjugates; Apu2 and Apu3 for K48-linked and K63-linked poly-ubiquitin chains, respectively. As shown in Fig. 4D, the escalation of ubiquitinated Rap2a was only identified by P4D1, indicating mono-ubiquitination

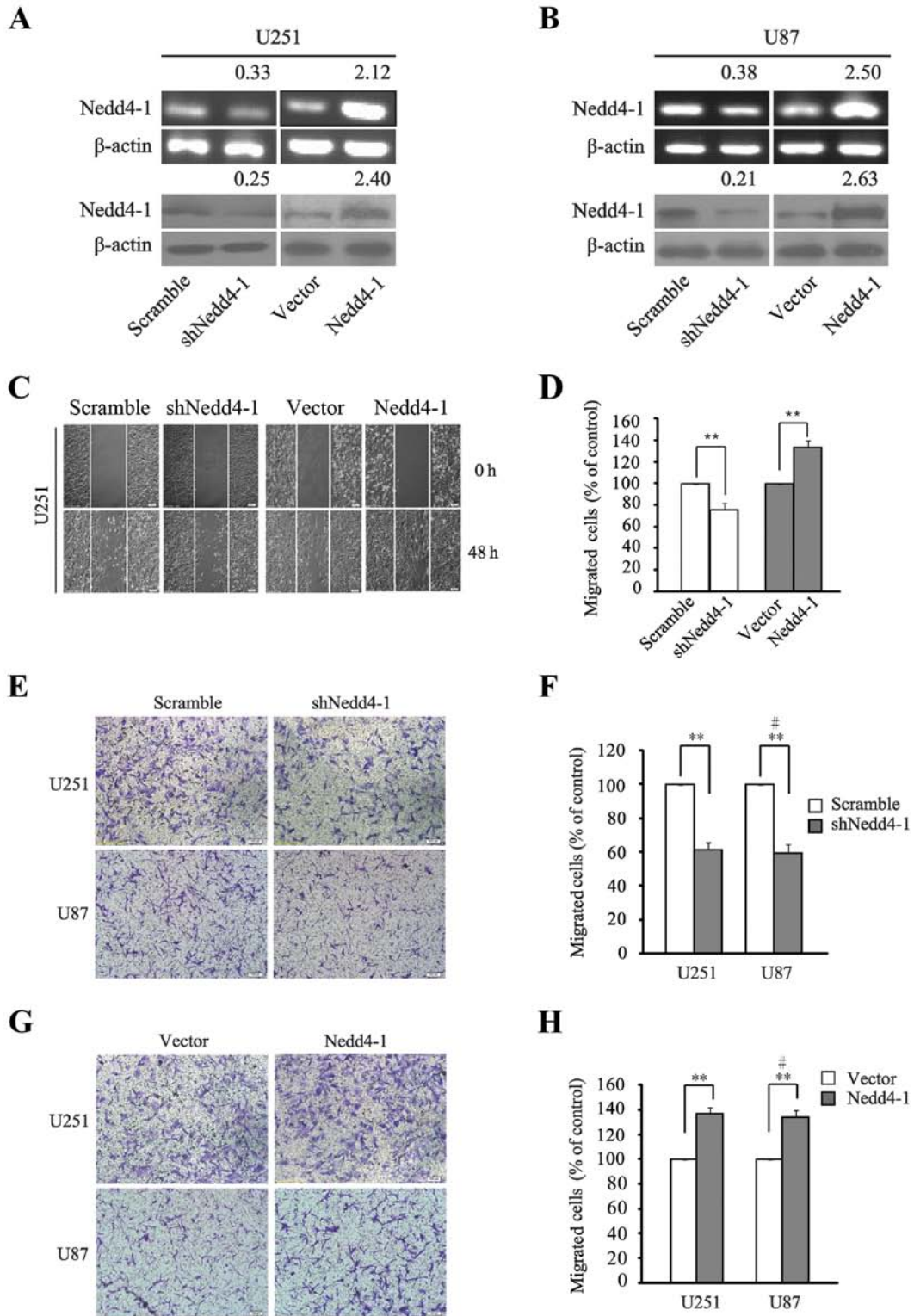


Figure 2. Effects of Nedd4-1 on cell migration. (A) Exogenous shNedd4-1 and Nedd4-1 were markedly expressed 48 h after transient transfection of U251 cells as assessed by RT-PCR and western blotting. (B) Exogenous shNedd4-1 and Nedd4-1 were markedly expressed 48 h after transient transfection of U87 cells as assessed by RT-PCR and western blotting. (C) Scratch migration assay of U251 cells at time 0 and 48 h after transient transfection with the plasmids shNedd4-1 and Nedd4-1. Scale bar, 100 μm. (D) Cell migration was quantified after 48 h; **P<0.01 compared with the scramble group; #**P<0.01 compared with the vector group. (E and G) U251 and U87 cell migration was assessed by Transwell assay 24 h after transient transfection with the shNedd4-1 and Nedd4-1 plasmids. Scale bar, 100 μm. (F and H) Quantification of cell migration calculated from the Transwell assays; **P<0.01 compared with the scramble group; #**P<0.01 compared with the vector group. The number of migratory cells was normalized to that of the NC group. Data are expressed as the means values ± SEM of three independent experiments.

of Rap2a by Nedd4-1. Apu3 recognized a single faint band representing ubiquitinated Rap2a by Nedd4-1 via K63-linked

ubiquitination. No bands were determined by Apu2, exempting the Rap2a ubiquitination by Nedd4-1 from K48-linked

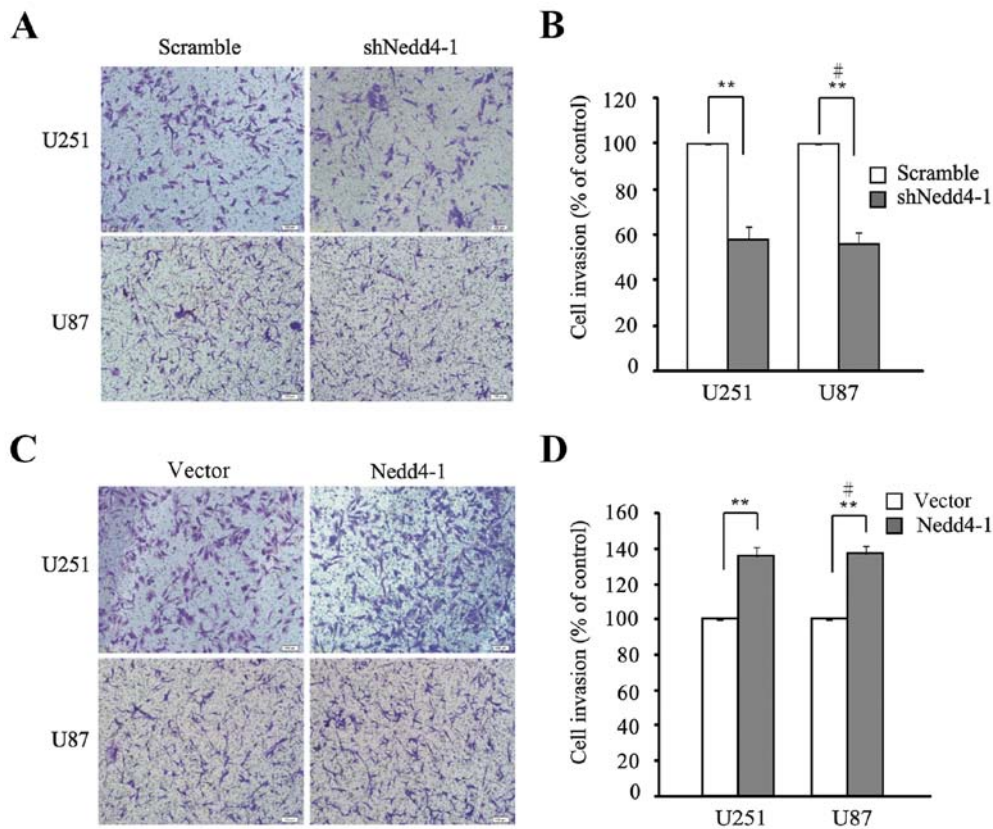


Figure 3. Effects of Nedd4-1 on cell invasion. (A) Representative micrographs of U251 and U87 cell invasion 24 h after Nedd4-1 downregulation. Invaded cells were fixed in formaldehyde and stained with crystal violet. Scale bar, 100 μ m. (B) Cell invasion was calculated from Transwell chambers precoated with Matrigel. (C) Representative micrographs of U251 and U87 cell invasion 24 h after Nedd4-1 overexpression. (D) Cell invasion was calculated from Transwell chambers precoated with Matrigel. The number of invasive cells was normalized to that of the NC group. Data are expressed as the means values \pm SEM of three independent experiments; ** P <0.01 compared with the scramble group; #** P <0.01 compared with the vector group.

polyubiquitin chain, which may lead to proteasome-dependent degradation. A total lysate of Nedd4-1-expressing U251 cells was immunoblotted using these antibodies as controls with blurred bands.

Effect of Nedd4-1 ubiquitination on Rap2a function. To further investigate the effect of Nedd4-1-mediated ubiquitination on Rap2a function in glioma U251 cells, we purified and immobilized a GST fusion of the Rap2a-binding region of RalGDS, which served to pull down active GTP-bound Rap2a. Nedd4-1 and Myc-tagged Rap2a were thereby co-transfected in the U251 cells, and the lysate was loaded onto GST-RalGDS or onto GST as a control. Only active GTP-bound Rap2a was pulled down, indicating that ubiquitination blocked Rap2a function by inhibiting the activity of the GTP-bound Rap2a (Fig. 4E and F).

Effects of Nedd4-1-mediated Rap2a ubiquitination on cell migration and invasion. To verify the role of Nedd4-1-mediated mono-ubiquitination of Rap2a in cell migration and invasion, expression plasmids for full-length Nedd4-1, HA-tagged WT-ubiquitin and its mutants (including HA-tagged K63R-ubiquitin, HA-tagged K48R-ubiquitin, HA-tagged ubiquitin and Myc-tagged WT-Rap2a) were co-transfected in the U251 and U87 cell lines, with MG-132 added to exclude the interference of multiple-ubiquitination in all groups. We assessed migration and invasion parameters in each group of

both U251 and U87 cells. As shown in Fig. 5A and B, Nedd4-1 and Rap2a were overexpressed in the U251 and U87 cell lines. The Transwell assay results demonstrated that the migration of glioma cells was significantly enhanced in the WT-ubiquitin and K48R-ubiquitin groups vs. the vector and K63R-ubiquitin groups (Fig. 5C and D). The invasive capacity of glioma cells was also significantly increased in the WT-ubiquitin and K48R-ubiquitin groups vs. the vector and K63R-ubiquitin groups in the Matrigel Transwell assay (Fig. 5E and F). Furthermore, as illustrated (Fig. 5G), the results of immunoprecipitation revealed the Rap2a mono-ubiquitination of Nedd4-1 via K63-ubiquitin. These results indicated that Nedd4-1 may regulate the migration and invasion of glioma cells via the inhibition of GTP-Rap2a.

Discussion

In the present study, we confirmed that Nedd4-1 promotes the migration and invasion of glioma cells via the inhibition of GTP-Rap2a, a member of the Ras family of small G proteins. This finding clarified the role of Nedd4-1 in migration, invasion and other important pathogenetic processes in glioma.

Nedd4-1, an E3 ligase, plays essential regulatory roles in physiological processes, which is exemplified by its direct binding to and ubiquitination of activated FGFR1, thus regulating the endocytosis and signaling pathways of FGFR1 in neural differentiation and embryonic development (32).

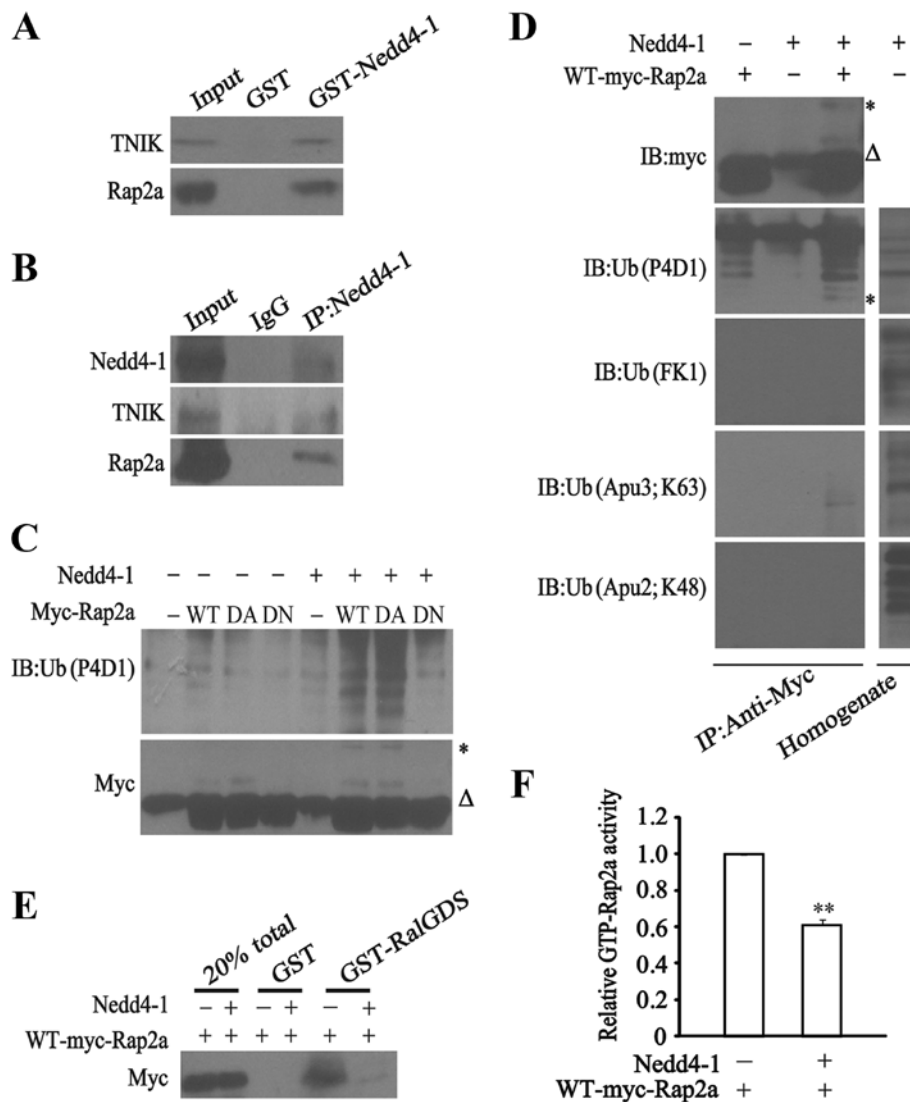


Figure 4. Rap2a is a Nedd4-1 substrate. (A) Complex of Rap2a with TNIK and GST-Nedd4-1 (residues 217-549). Protein was extracted from U251 cells and blotted for TNIK (top) and Rap2a (bottom) after washing GST-Nedd4-1 beads. GST beads were used as a negative control. (B) Endogenous Rap2a, TNIK and Nedd4-1 form a complex. Nedd4-1 was immunoprecipitated using a rabbit polyclonal anti-Nedd4-1 antibody from U251 cell extract after treatment with a thiol-cleavable chemical cross-linker. Western blot analyses were probed with anti-Nedd4-1, anti-TNIK and anti-Rap2a antibodies. Rap2a and TNIK co-immunoprecipitated with Nedd4-1, but no band was observed with the negative control IgG. (C) Nedd4-1 selectively ubiquitinates Rap2a. Nedd4-1 (+) or (-) was co-expressed with WT, dominant-active (DA), or dominant-negative (DN) mutants of Myc-tagged Rap2a. Myc-Rap2a was immunoprecipitated using a mouse monoclonal anti-Myc antibody. Immunoprecipitates were blotted for Myc (bottom panel) and ubiquitin (P4D1, top panel). Note that Nedd4-1 selectively ubiquitinated WT and DA mutants of Myc-tagged Rap2a but not the DN mutant of Myc-Rap2a. (D) Mono- and di-ubiquitination of Rap2a by Nedd4-1. Myc-Rap2a was immunoprecipitated from U251 cells expressing Myc-Rap2a (WT) with or without Nedd4-1. Myc-Rap2a was eluted from anti-Myc antibody-coupled beads and immunoblotted with different monoclonal mouse or rabbit anti-ubiquitin antibodies: P4D1, FK1, Apu3 and Apu2. P4D1 recognizes both poly- and mono-ubiquitin-conjugated proteins, and FK1 recognizes only poly-ubiquitin-conjugated ones. Apu3 and Apu2 recognize K63- and K48-linked poly-ubiquitin chains, respectively. Lysate from U251 cells overexpressing Nedd4-1 was also blotted with the antibodies described above to show that the antibody titers are comparable. Asterisks in (C and D) indicate bands corresponding to Myc-Rap2a conjugated with two ubiquitin moieties. Triangular symbols in (C and D) indicate the light chain of the anti-Myc used for immunoprecipitation. (E) The function of Rap2a by Nedd4-1 ubiquitination. WT Myc-Rap2a expressed in U251 cells with or without overexpressed Nedd4-1 was precipitated using anti-Myc antibodies. GTP-bound Rap2a was assessed with GST-RalGDS pull-down assays compared with 20% total and GST as negative control. (F) Relative GTP-Rap2a activity. Note that GTP-bound active Rap2a was obviously decreased in the overexpressed Nedd4-1 condition (sixth lane). Data are expressed as the means \pm SEM of three independent experiments; **P<0.01 compared without Nedd4-1 overexpression.

Nevertheless, increasing evidence shows that Nedd4-1 is also involved in a variety of cancers such as bladder, lung and brain carcinomas (5,6,33). In the present study, we identified that the level of Nedd4-1 protein in glioma tissues was approximately 2- to 3-fold higher than that in non-cancerous tissues (Fig. 1A and C). The downregulation of Nedd4-1 reduced the migration and invasion of human glioma cell lines U251 and U87, while Nedd4-1 overexpression reversed these

phenomena (Figs. 2C-F and 3A-D). These findings suggest a pivotal role of Nedd4-1 in glioma pathogenesis.

Nedd4-1 reportedly serves as a ubiquitin ligase for multiple proteins (6,12,34,35), with several different domains including C2, two to four WW domains and a HECT domain. Nedd4-1 is reported to bind rather than ubiquitinate TNIK in regulating the ubiquitination of GTP-Rap2a in neuron development. In addition, Rap2a, in lieu of Rap1a, H-Ras

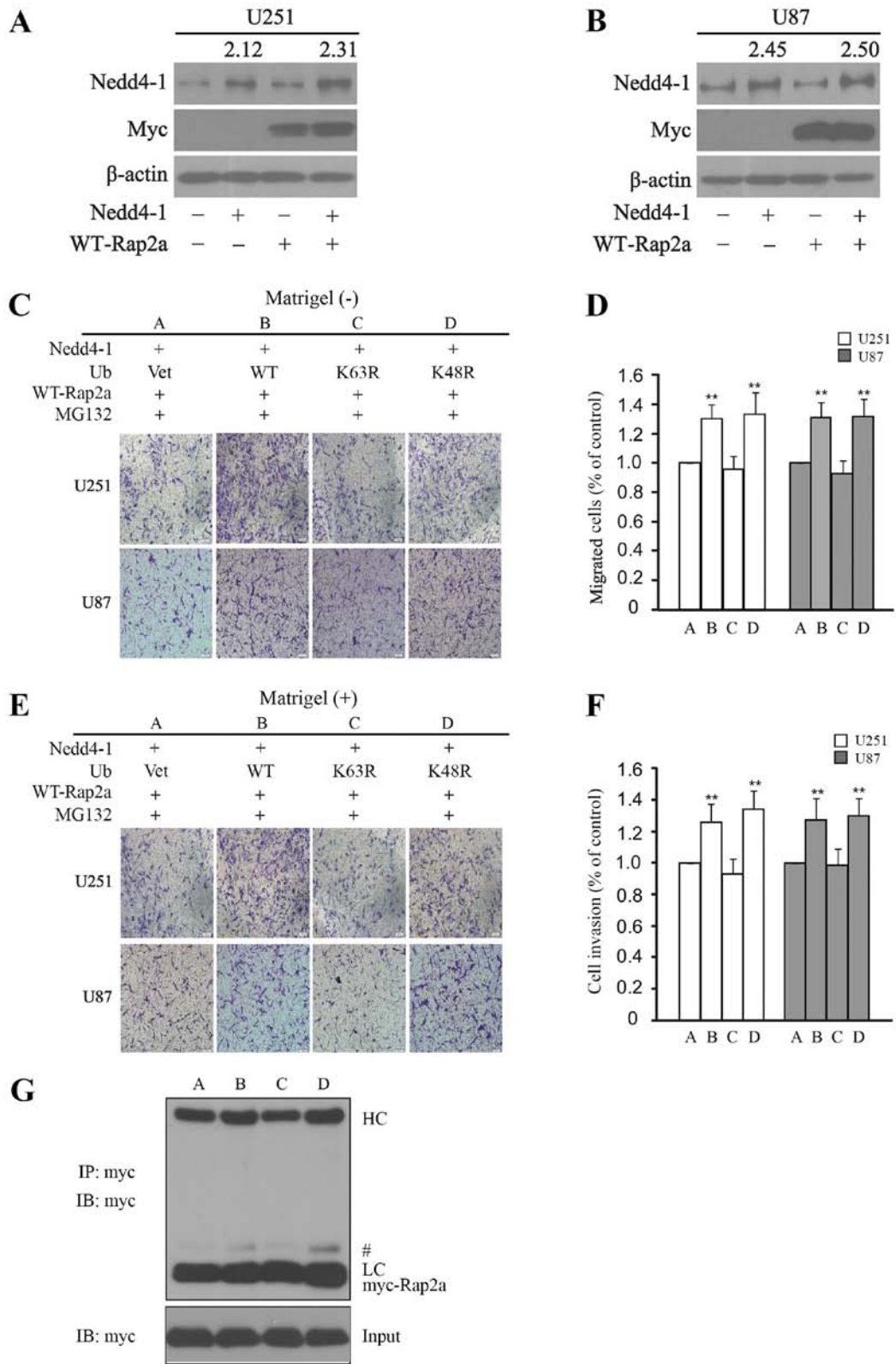


Figure 5. Effects of the ubiquitination of Rap2a by Nedd4-1 on cell migration and invasion. (A) Relative protein levels of Nedd4-1 and Rap2a in the U251 cell line. (B) Relative protein levels of Nedd4-1 and Rap2a in the U87 cell line. (C) U251 and U87 cell migration 24 h after transient transfection with the indicated plasmids. Scale bar, 100 μ m. (D) Quantification of cell migration calculated from Transwell assays. (E) Invasion of U251 and U87 cells 24 h after transient transfection with the indicated plasmids. Scale bar, 100 μ m. (F) Quantification of cell invasion was calculated from Transwell chambers precoated with Matrigel. (G) Rap2a mono-ubiquitination by Nedd4-1 was determined by immunoprecipitation combined with western blotting. #Indicates the mono-ubiquitinated Myc-Rap2a. Data are expressed as the means \pm SEM of three independent experiments; **P<0.01 compared with the vector group.

and K-Ras, is a specific substrate of Nedd4-1 (24). It is unclear whether Nedd4-1 ubiquitinates Rap2a in glioma. In

the present study, Nedd4-1 was found to bind to TNIK and Rap2a, forming a ternary complex in both the GST-Nedd4-1

pull-down assays and co-immunoprecipitation in cell line U251 (Fig. 4A and B). These results suggest that the ubiquitination of Rap2a by Nedd4-1 may be involved in the regulation of migration and invasion of glioma cells. Moreover, the fate of the ubiquitinated proteins is often determined by the chains of ubiquitin conjugation (36). Poly-ubiquitination of substrate proteins by K48-linked ubiquitin chains leads to degradation within the 26S proteasome, whereas K63-linked poly-ubiquitin chains, mono-ubiquitination and multiple mono-ubiquitination usually regulate protein function, gene transcription, endocytosis and DNA repair (36,37). Additionally, there is a wealth of evidence that selective mono-ubiquitination or alternative ubiquitin chains can regulate protein activity (38-42). Herein, WT-Rap2a and DA-Rap2a, but not DN-Rap2a (Fig. 4C) were robustly ubiquitinated by Nedd4-1 by K-63-linked ubiquitin chains (Fig. 4D), with inhibition of GTP-Rap2a activity (Fig. 4E). The present study was supported by the study of Kawabe *et al* (24), in which Nedd4-1 ubiquitinated Rap2a and inhibited GTP-Rap2a activity in neurons. Based on these findings, we speculate that Rap2a ubiquitination by Nedd4-1 may contribute to glioma pathogenesis.

Rap proteins belong to the Ras superfamily and have been implicated in cell cycle control, cell adhesion and cell migration (25,43,44). To date, the role of Rap1 in tumorigenesis and progression remains controversial, with some researchers hypothesizing that aberrant Rap1 activation promotes cancer cell proliferation and tumorigenesis (45-47) and others reporting that inactivation of DOCK4, a Rap1 activator, rendered osteosarcoma cells with a higher invasiveness (48), and the expression of Rap1GAP was correlated with increased invasion *in vitro* in squamous cell carcinoma (49). Similar to Rap1, the function of Rap2a remains elusive despite its history of cloning (50). In the present study, overexpression of Nedd4-1 promoted the migration and invasion of human glioma cell lines U251 and U87 via Rap2a ubiquitination (Fig. 5C and D). Furthermore, only WT-ubiquitin and K48R exhibited mono-ubiquitination (Fig. 5G). Taken together, these findings confirm the hypothesis that Nedd4-1 regulates the migration and invasion of glioma cells via Rap2a ubiquitination. Three Rap2 proteins (Rap2a/b/c) have been cloned, but we only focused on the function of Rap2a protein. Additionally, E3 ubiquitin ligase was also found to be involved in the regulation of cell cycle, apoptosis and differentiation (51,52), which may illuminate our forthcoming investigation of glioma.

In summary, our findings suggest that Nedd4-1 plays a pivotal role in promoting the migration and invasion of glioma cell lines U251 and U87 via the inhibition of Rap2a activity, and may qualify as a candidate therapeutic target in glioma.

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References

1. Watkins S and Sontheimer H: Hydrodynamic cellular volume changes enable glioma cell invasion. *J Neurosci* 31: 17250-17259, 2011.
2. Bush NA, Chang SM and Berger MS: Current and future strategies for treatment of glioma. *Neurosurg Rev* 40: 1-14, 2017.
3. Demuth T, Rennert JL, Hoelzinger DB, Reavie LB, Nakada M, Beaudry C, Nakada S, Anderson EM, Henrichs AN, McDonough WS, *et al*: Glioma cells on the run - the migratory transcriptome of 10 human glioma cell lines. *BMC Genomics* 9: 54, 2008.
4. Nakayama KI and Nakayama K: Ubiquitin ligases: Cell-cycle control and cancer. *Nat Rev Cancer* 6: 369-381, 2006.
5. Dai B, Pieper RO, Li D, Wei P, Liu M, Woo SY, Aldape KD, Sawaya R, Xie K and Huang S: FoxM1B regulates NEDD4-1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. *Cancer Res* 70: 2951-2961, 2010.
6. Wang X, Trotman LC, Koppie T, Alimonti A, Chen Z, Gao Z, Wang J, Erdjument-Bromage H, Tempst P, Cordon-Cardo C, *et al*: NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* 128: 129-139, 2007.
7. Chen Y, van de Vijver MJ, Hibshoosh H, Parsons R and Saal LH: PTEN and NEDD4 in human breast carcinoma. *Pathol Oncol Res* 22: 41-47, 2016.
8. Sun Y: Targeting E3 ubiquitin ligases for cancer therapy. *Cancer Biol Ther* 2: 623-629, 2003.
9. Hoeller D and Dikic I: Targeting the ubiquitin system in cancer therapy. *Nature* 458: 438-444, 2009.
10. Yang Y, Kitagaki J, Wang H, Hou DX and Perantoni AO: Targeting the ubiquitin-proteasome system for cancer therapy. *Cancer Sci* 100: 24-28, 2009.
11. Eide PW, Cekaite L, Danielsen SA, Eilertsen IA, Kjenseth A, Fykerud TA, Ågesen TH, Bruun J, Rivedal E, Lothe RA, *et al*: NEDD4 is overexpressed in colorectal cancer and promotes colonic cell growth independently of the PI3K/PTEN/AKT pathway. *Cell Signal* 25: 12-18, 2013.
12. Kim SS, Yoo NJ, Jeong EG, Kim MS and Lee SH: Expression of NEDD4-1, a PTEN regulator, in gastric and colorectal carcinomas. *APMIS* 116: 779-784, 2008.
13. Drinjakovic J, Jung H, Campbell DS, Strohlic L, Dwivedy A and Holt CE: E3 ligase Nedd4 promotes axon branching by downregulating PTEN. *Neuron* 65: 341-357, 2010.
14. Kwak YD, Wang B, Pan W, Xu H, Jiang X and Liao FF: Functional interaction of phosphatase and tensin homologue (PTEN) with the E3 ligase NEDD4-1 during neuronal response to zinc. *J Biol Chem* 285: 9847-9857, 2010.
15. Cao XR, Lill NL, Boase N, Shi PP, Croucher DR, Shan H, Qu J, Sweezer EM, Place T, Kirby PA, *et al*: Nedd4 controls animal growth by regulating IGF-1 signaling. *Sci Signal* 1: ra5, 2008.
16. Fouladkou F, Landry T, Kawabe H, Neeb A, Lu C, Brose N, Stambolic V and Rotin D: The ubiquitin ligase Nedd4-1 is dispensable for the regulation of PTEN stability and localization. *Proc Natl Acad Sci USA* 105: 8585-8590, 2008.
17. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliareis C, Rodgers L, McCombie R, *et al*: *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275: 1943-1947, 1997.
18. Carico C, Nuño M, Mukherjee D, Elramsisy A, Dantis J, Hu J, Rudnick J, Yu JS, Black KL, Bannykh SI, *et al*: Loss of PTEN is not associated with poor survival in newly diagnosed glioblastoma patients of the temozolomide era. *PLoS One* 7: e33684, 2012.
19. Zainuddin N, Jaafar H, Isa MN and Abdullah JM: Malignant glioma: The involvement of loss of allelic heterozygosity and PTEN mutations in a group of Malay patients. *Southeast Asian J Trop Med Public Health* 36: 748-756, 2005.
20. Wei Q, Clarke L, Scheidenhelm DK, Qian B, Tong A, Sabha N, Karim Z, Bock NA, Reti R, Swoboda R, *et al*: High-grade glioma formation results from postnatal pten loss or mutant epidermal growth factor receptor expression in a transgenic mouse glioma model. *Cancer Res* 66: 7429-7437, 2006.
21. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, Cachola KE, Murray JC, Tihan T, Jensen MC, *et al*: Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 13: 84-88, 2007.
22. Itoh M, Nelson CM, Myers CA and Bissell MJ: Rap1 integrates tissue polarity, lumen formation, and tumorigenic potential in human breast epithelial cells. *Cancer Res* 67: 4759-4766, 2007.

23. Kooistra MR, Dubé N and Bos JL: Rap1: A key regulator in cell-cell junction formation. *J Cell Sci* 120: 17-22, 2007.
24. Kawabe H, Neeb A, Dimova K, Young SM Jr, Takeda M, Katsurabayashi S, Mitkovski M, Malakhova OA, Zhang DE, Umikawa M, *et al*: Regulation of Rap2A by the ubiquitin ligase Nedd4-1 controls neurite development. *Neuron* 65: 358-372, 2010.
25. Bigler D, Gioeli D, Conaway MR, Weber MJ and Theodorescu D: Rap2 regulates androgen sensitivity in human prostate cancer cells. *Prostate* 67: 1590-1599, 2007.
26. Prabhakaran I, Grau JR, Lewis R, Fraker DL and Guvakova MA: *Rap2A* is upregulated in invasive cells dissected from follicular thyroid cancer. *J Thyroid Res* 2011: 979840, 2011.
27. Wang L, Zhan W, Xie S, Hu J, Shi Q, Zhou X, Wu Y, Wang S, Fei Z and Yu R: Over-expression of Rap2a inhibits glioma migration and invasion by down-regulating p-AKT. *Cell Biol Int* 38: 326-334, 2014.
28. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P: The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 114: 97-109, 2007.
29. Zhou X, Hua L, Zhang W, Zhu M, Shi Q, Li F, Zhang L, Song C and Yu R: FRK controls migration and invasion of human glioma cells by regulating JNK/c-Jun signaling. *J Neurooncol* 110: 9-19, 2012.
30. Liang CC, Park AY and Guan JL: In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2: 329-333, 2007.
31. van Golen KL, Risin S, Staroselsky A, Berger D, Tainsky MA, Pathak S and Price JE: Predominance of the metastatic phenotype in hybrids formed by fusion of mouse and human melanoma clones. *Clin Exp Metastasis* 14: 95-106, 1996.
32. Persaud A, Alberts P, Hayes M, Guettler S, Clarke I, Sicheri F, Dirks P, Ciruna B and Rotin D: Nedd4-1 binds and ubiquitylates activated FGFR1 to control its endocytosis and function. *EMBO J* 30: 3259-3273, 2011.
33. Yim EK, Peng G, Dai H, Hu R, Li K, Lu Y, Mills GB, Meric-Bernstam F, Hennessy BT, Craven RJ, *et al*: Rak functions as a tumor suppressor by regulating PTEN protein stability and function. *Cancer Cell* 15: 304-314, 2009.
34. Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J and Rotin D: WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na⁺ channel deleted in Liddle's syndrome. *EMBO J* 15: 2371-2380, 1996.
35. Murdaca J, Treins C, Monthouël-Kartmann MN, Pontier-Bres R, Kumar S, Van Obberghen E and Giorgetti-Peraldi S: Grb10 prevents Nedd4-mediated vascular endothelial growth factor receptor-2 degradation. *J Biol Chem* 279: 26754-26761, 2004.
36. Shearwin-Whyatt L, Dalton HE, Foot N and Kumar S: Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *BioEssays* 28: 617-628, 2006.
37. Haglund K and Dikic I: Ubiquitylation and cell signaling. *EMBO J* 24: 3353-3359, 2005.
38. Lai Z, Ferry KV, Diamond MA, Wee KE, Kim YB, Ma J, Yang T, Benfield PA, Copeland RA and Auger KR: Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. *J Biol Chem* 276: 31357-31367, 2001.
39. Dong L and Xu CW: Carbohydrates induce mono-ubiquitination of H2B in yeast. *J Biol Chem* 279: 1577-1580, 2004.
40. Brenkman AB, de Keizer PL, van den Broek NJ, Jochemsen AG and Burgering BM: Mdm2 induces mono-ubiquitination of FOXO4. *PLoS One* 3: e2819, 2008.
41. Torres MP, Lee MJ, Ding F, Purbeck C, Kuhlman B, Dokholyan NV and Dohlman HG: G protein mono-ubiquitination by the Rsp5 ubiquitin ligase. *J Biol Chem* 284: 8940-8950, 2009.
42. Wu K, Yan H, Fang L, Wang X, Pflieger C, Jiang X, Huang L and Pan ZQ: Mono-ubiquitination drives nuclear export of the human DCN1-like protein hDCN1. *J Biol Chem* 286: 34060-34070, 2011.
43. McLeod SJ, Shum AJ, Lee RL, Takei F and Gold MR: The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J Biol Chem* 279: 12009-12019, 2004.
44. Price LS, Hajdo-Milasinovic A, Zhao J, Zwartkruis FJ, Collard JG and Bos JL: Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J Biol Chem* 279: 35127-35132, 2004.
45. Zhang L, Chenwei L, Mahmood R, van Golen K, Greenson J, Li G, D'Silva NJ, Li X, Burant CF, Logsdon CD, *et al*: Identification of a putative tumor suppressor gene *Rap1GAP* in pancreatic cancer. *Cancer Res* 66: 898-906, 2006.
46. Gao L, Feng Y, Bowers R, Becker-Hapak M, Gardner J, Council L, Linette G, Zhao H and Cornelius LA: Ras-associated protein-1 regulates extracellular signal-regulated kinase activation and migration in melanoma cells: Two processes important to melanoma tumorigenesis and metastasis. *Cancer Res* 66: 7880-7888, 2006.
47. Ishida D, Kometai K, Yang H, Kakugawa K, Masuda K, Iwai K, Suzuki M, Itohara S, Nakahata T, Hiai H, *et al*: Myeloproliferative stem cell disorders by deregulated Rap1 activation in *SPA-1*-deficient mice. *Cancer Cell* 4: 55-65, 2003.
48. Yajnik V, Paulding C, Sordella R, McClatchey AI, Saito M, Wahrer DC, Reynolds P, Bell DW, Lake R, van den Heuvel S, *et al*: *DOCK4*, a GTPase activator, is disrupted during tumorigenesis. *Cell* 112: 673-684, 2003.
49. Mitra RS, Goto M, Lee JS, Maldonado D, Taylor JM, Pan Q, Carey TE, Bradford CR, Prince ME, Cordell KG, *et al*: Rap1GAP promotes invasion via induction of matrix metalloproteinase 9 secretion, which is associated with poor survival in low N-stage squamous cell carcinoma. *Cancer Res* 68: 3959-3969, 2008.
50. Pizon V, Chardin P, Lerosey I, Olofsson B and Tavittian A: Human cDNAs rap1 and rap2 homologous to the *Drosophila* gene *Dras3* encode proteins closely related to ras in the 'effector' region. *Oncogene* 3: 201-204, 1988.
51. Chantry A: WWP2 ubiquitin ligase and its isoforms: New biological insight and promising disease targets. *Cell Cycle* 10: 2437-2439, 2011.
52. Tuoc TC and Stoykova A: Roles of the ubiquitin-proteasome system in neurogenesis. *Cell Cycle* 9: 3174-3180, 2010.