

Research Paper

Neuronal-driven glioma growth requires *Gai1* and *Gai3*

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Abstract

Neuroigin-3 (NLGN3) is necessary and sufficient to promote glioma cell growth. The recruitment of *Gai1/3* to the ligand-activated receptor tyrosine kinases (RTKs) is essential for mediating oncogenic signaling.

Methods: Various genetic strategies were utilized to examine the requirement of *Gai1/3* in NLGN3-driven glioma cell growth.

Results: NLGN3-induced Akt-mTORC1 and Erk activation was inhibited by decreasing *Gai1/3* expression. In contrast ectopic *Gai1/3* overexpression enhanced NLGN3-induced signaling. In glioma cells, NLGN3-induced cell growth, proliferation and migration were attenuated by *Gai1/3* depletion with shRNA, but facilitated with *Gai1/3* overexpression. Significantly, *Gai1/3* silencing inhibited orthotopic growth of patient-derived glioma xenografts in mouse brain, whereas forced *Gai1/3*-overexpression in primary glioma xenografts significantly enhanced growth. The growth of brain-metastatic human lung cancer cells in mouse brain was largely inhibited with *Gai1/3* silencing. It was however expedited with ectopic *Gai1/3* overexpression. In human glioma *Gai3* upregulation was detected, correlating with poor prognosis.

Conclusion: *Gai1/3* mediation of NLGN3-induced signaling is essential for neuronal-driven glioma growth.

Key words: Neuron-glioma communication; NLGN3; *Gai1/3*; Signaling

Introduction

Glioma is the most common type of brain tumor of either astrocytic or oligodendroglial origin [1, 2]. High-grade gliomas, such as glioblastoma (GBM) and anaplastic astrocytoma, cause human mortality both in children and adults [3, 4]. Dysregulation of multiple signaling cascades and complex signaling

crosstalk are vital in the tumorigenesis and progression of human glioma [5-9]. These cascades include phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) [10], p53, retinoblastoma (Rb), epidermal growth factor receptor (EGFR) and Wnt/ β -catenin [11] as well as signal

transducer and activator of transcription (STAT), neurotrophic tyrosine receptor kinase (NTRK), fibroblast growth factor receptor (FGFR) and many others [5, 6]. It is therefore extremely important to identify the dominant and key driving signaling molecules of glioma in order to develop efficient targeted therapies [5, 6, 12, 13].

Recent studies have revealed that neuronal activity and neuron-glioma circuits are vital for the malignancy of human glioma, and that the neuronal activity could be a key determinant for glioma tumorigenesis and progression [14-16]. Studies highlighted a crucial role of neuroligin-3 (NLGN3) in the mechanism of glioma progression [17-20]. NLGN3, a synaptic protein cleaved and secreted in an activity-dependent manner, was identified as the primary factor responsible for glioma progression [17-20]. Xenografts derived from high-grade primary human glioma cells failed to grow in a NLGN3-deficient brain [18], demonstrating that NLGN3 is required for glioma cell growth. Furthermore, inhibition of ADAM10 (A Disintegrin And Metalloproteinase 10) blocked activity-dependent NLGN3 secretion to inhibit orthotopic growth of primary glioma xenografts [19].

NLGN3 secreted from neurons was found to induce phosphorylation and activation of several key receptor tyrosine kinases (RTKs) on glioma cells to promote glioma progression [17-19]. Additionally, NLGN3-activated glioma cells produce feedforward expression of NLGN3 to further increase glioma cell growth and proliferation [17-19]. Recent studies found that NLGN3 induces the expression of synaptic genes required for neuron-glioma synapse formation, as well as genes essential for glioma proliferation and invasiveness [14, 15]. These studies identify NLGN3 as a key neuronal-derived factor for promoting activity-dependent glioma growth [17-19].

Gai proteins, the inhibitory α subunits of G proteins (heterotrimeric guanine nucleotide-binding proteins), have three primary family subunits, Gai1, Gai2 and Gai3 [21]. Through binding to GPCRs (G protein-coupled receptors) Gai proteins and the $\beta\gamma$ complexes can suppress adenylate cyclase (AC) to deplete cyclic AMP (cAMP) levels [21]. Gai proteins are inhibited by pertussis toxin. Glider *et al.*, showed that pertussis toxin potently inhibited migration and invasion of high grade glioma cells [22]. In addition, pertussis toxin and temozolomide co-treatment resulted in better anti-glioma efficiency in a rat intracerebral glioma model [23]. Moreover, a D2 dopamine receptor agonist stimulated mitogenesis of C6 glioma cells through activation of pertussis toxin-sensitive Gai proteins [24].

Alternatively, our studies show that Gai1 and

Gai3 proteins also play a key role in mediating the signal transduction of several RTKs, including EGFR (epidermal growth factor receptor) [25], fibroblast growth factor receptor (FGFR) [26], keratinocyte growth factor receptor (KGFR) [27], brain derived neurotrophic factor (BDNF) receptor TrkB [28], and vascular endothelial growth factor receptor 2 (VEGFR2) [29]. Specifically, the recruitment of either Gai1 or Gai3 subunit to these ligand-activated RTKs was found to be essential for mediating PI3K-Akt-mTORC1 and Erk-MAPK signaling [25-29]. As neuron-secreted NLGN3 activates RTKs to promote glioma progression, we explored the potential function of Gai1 and Gai3 in NLGN3-induced signaling and glioma cell progression.

Methods

Ethics. Protocols of this study were approved by the Ethics Committee of Soochow University.

Reagents. From Gibco (Shanghai, China) cell culture reagents were obtained. The antibodies were described early [26]. NLGN3, pertussis toxin (PTX), LY294002 and PD98059 were purchased from Sigma-Aldrich (St. Louis, Mo). All primers, sequences and viral constructs were provided by Shanghai Genechem Co. (Shanghai, China), unless otherwise mentioned.

Cell lines. U251MG glioma cell line was obtained from the Cell Bank of Shanghai Biological Institution (Shanghai, China), cultivated as described. The cell line has previously been tested and authenticated by the Cell Bank of Shanghai Biological Institution. Wild-type (WT), Gai1 and Gai3 double knockout (DKO), Gai1, Gai2 or Gai3 single knockout (SKO) mouse embryonic fibroblasts (MEFs), as well as WT and Gab1 KO MEFs, were cultivated as previously described [25, 27-30]. Cells were starved in 0.5% FBS medium overnight plus 30 min warm PBS for signaling analyses.

Human glioma tissues. According to principles of the Declaration of Helsinki, this study was approved by the Ethics Committee of Soochow University. Human tissues, including the glioma tissues and surrounding normal brain tissues, were described previously [26]. Tissues were obtained from The First, Second, Third Affiliated and Children Hospitals of Soochow University. All participants provided written-informed consent.

Primary culture of human cancer cells. The source and culturing of primary human glioma cells were previously described [26]. From a lung cancer patient with brain metastases (female, 50 years old, brain metastatic lung papillary adenocarcinoma, CK7⁺, CK20⁻, Villin⁻, TTF-1⁺, CDX2⁻, PAX8⁻,

CD10⁻, CAIX⁻, PR⁻, EMA⁺, GFAP⁻), surgical removed brain metastatic cancer tissues were washed, minced into small pieces, and digested with Collagenase I and DNase (Sigma-Aldrich). Single-cell suspensions were then pelleted and washed. Fibroblasts, blood vessel cells, immune cells and other non-cancerous cells were abandoned. Purified brain-metastatic human lung cancer cells (bmLCs) were *ex vivo* cultured in the described medium [31]. The study protocols were reviewed and approved by the Ethics Committee of Soochow University, and conformed to the guidelines of Helsinki declaration. The informed consent was obtained from all subjects before their participation.

Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR). As described, qPCR was carried out by an ABI 7600 Prism equipment through utilizing a SYBR Green PCR kit. *Gai3* and *NLGN3* mRNA levels were quantified by a $\Delta\Delta$ Ct protocol [32], using *GAPDH* as the internal control [26]. Primers are listed in Table 1.

Table 1. Primers and sgRNA sequences utilized in the study.

Genes	qPCR primers
<i>GAPDH</i> Forward	5'-GTCGTGTGAACGGATTG-3'
<i>GAPDH</i> Reverse	5'-AAGATGGTGATGGGCTCC-3'
<i>NLGN3</i> Forward	5'-GTCGTGTTCACTGCGCAACTGG-3'
<i>NLGN3</i> Reverse	5'-CCGTCATTATCCGCTAAGTCCTC-3'
<i>GNAI3</i> Forward	5'-CACTTCACCTGTGCCACAGACA-3'
<i>GNAI3</i> Reverse	5'-GTCGTGTCCAACTCCACAC-3'
sgRNA	Target DNA Sequence
Murine <i>GNAI3</i> sgRNA	GGCTCGTATGATTGCAATGA
Murine <i>GNAI1</i> sgRNA	CCATCATTAGAGCCATGGGG

Western blotting and co-immunoprecipitation. Protocols for Western blotting and co-immunoprecipitation (co-IP), as well as data quantification, have been extensively described previously [25, 28]. For all Western blotting assays, each lane was loaded with exact same amount of quantified protein lysates (30-40 μ g in each treatment). Same set of lysate samples were run in parallel ("sister") gels to test different proteins when necessary. Expression of indicated proteins was quantified via ImageJ software, with results normalized to the equal loadings.

Endosome fractions. Cells with the applied treatments were harvested and re-suspended in the hypotonic swelling buffer [33], and lysed with 30 strokes in a Dounce homogenizer using a tight pestle, and swelling was stopped by the addition of two fold homogenization buffer [33]. Lysates were centrifuged to obtain the post-nuclear supernatants, and centrifuged [33]. The resulting supernatants were centrifuged, and the pellet solubilized in the homogenization buffer [33]. Insoluble particles were removed by short centrifugation and the supernatant

loaded onto a 5-20% continuous OptiprepTM (Sigma-Aldrich), poured using homogenization buffer. The gradient was further centrifuged at 60,000 g for 24h, with total 10 endosomal fractions collected, and proteins precipitated with 12% TCA for 1h. Fractions were centrifuged at 12,000 g for 1h. The protein pellets, combining all ten endosomal fractions, were dissolved in SDS-sample buffer for analysis by Western blotting.

***Gai1/3* shRNA.** Glioma cells or bmLCs were seeded into six-well plates at 50-60% confluence, treated with *Gai1* shRNA lentiviral particles (sc-105382-V) (Santa Cruz, CA) and *Gai3* shRNA lentiviral particles [29]. After 24h, cells were further cultured in puromycin (1.0 μ g/mL)-containing complete medium for 12-14 days. *Gai1/3* knockdown (over 95% efficiency) in stable cells was confirmed by Western blotting. shRNA-mediated knockdown of *Gai1* and *Gai3* in MEFs was reported previously [27-29]. *Gab1* shRNA lentiviral particles, for both human and mouse, were also obtained from Santa Cruz Biotech.

CRISPR/Cas9 knockout of *Gai1* and *Gai3*. The lentiviral CRISPR/Cas-9 *Gai1* KO construct and the lentiviral CRISPR/Cas-9 *Gai3* KO construct were provided by Shanghai Genechem (Shanghai, China), transfected into MEFs, and selected with puromycin. *Gai1/3* knockout was confirmed by Western blotting. Control cells were treated with the empty vector with nonsense sgRNA (Santa Cruz Biotech). The sgRNA sequences used for *Gai1* and *Gai3* KO are listed in Table 1.

***Gai1/3* overexpression.** The recombinant adenovirus containing full-length *Gai1* ("Ad-*Gai1*", human or mouse) and *Gai3* ("Ad-*Gai3*", human or mouse) were described earlier [29]. Virus was filtered, enriched and added to cultured MEFs, glioma cells or bmLCs. Stable cells were established following selection by puromycin, and *Gai1/3* overexpression confirmed by Western blotting.

Glioma cell functional assays. Cell growth, proliferation, CCK-8 viability and EdU staining assays [26, 34, 35] and cell migration by the "Transwell" assays [29, 36, 37], were carried out using previously described protocols.

The orthotopic primary-derived xenograft assay. For intracranial tumor implantation, primary human glioma cells or bmLCs (5×10^5 cells of each mouse in 200 μ L of Matrigel gel/10% FBS medium, with different genetic treatments) were implanted using the previously described coordinates [26, 38]. Magnetic resonance imaging (MRI) was carried out to visualize tumor xenografts. On the day when the first mouse in any group exhibited symptoms (severe fever, vomiting, or greater than 15% body weight

loss), all groups were sacrificed and tumors isolated through surgery. Tumor volumes were measured by the formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. Immunohistochemistry (IHC) was performed using the previously described procedures [26]. All animal procedures were approved by Soochow University Ethics Review Board.

Statistical analysis. The *in vitro* experiments were replicated three times or more, and data expressed as means \pm standard deviation (SD). To examine statistical differences among different groups one-way ANOVA was carried out with multiple comparisons performed by post hoc Bonferroni test (SPSS 18.0). *P* values < 0.05 were considered statistically significant. A two-tailed unpaired *t* test (Excel 2007) was applied to examine significance between two treatment groups.

Results

Gai1 and Gai3 double knockout inhibits NLGN3-induced Akt, Erk and mTORC1 activation in mouse embryonic fibroblasts (MEFs)

To test whether Gai proteins are required for NLGN3-induced signal transduction, wild-type (WT) and Gai1 and Gai3 double knockout (Gai1/3 DKO) MEFs [28, 29] were treated with NLGN3 (50 ng/mL) for 5-30 min. Western blotting results demonstrated that NLGN3 significantly increased phosphorylation of Akt (Ser-473) and GSK3 α/β (Ser21/9) in WT MEFs, whereas phosphorylation was impaired in Gai1/3 DKO MEFs (Figure 1A). Furthermore, NLGN3-induced Erk activation, tested by p-Erk1/2 at Thr202/Tyr204, was also attenuated in the DKO MEFs (Figure 1A). In WT MEFs NLGN3 activated mTORC1 signaling by inducing phosphorylation of p70S6K1 ("S6K", Thr389) and S6 (Ser-235/236), which was abolished in Gai1/3 DKO MEFs (Figure 1B). Levels of total Akt, Erk1/2, S6K and S6 were equivalent between WT and DKO MEFs (Figure 1A-B). These results show that NLGN3-induced Akt, Erk and mTORC1 activation was significantly inhibited in Gai1/3 DKO MEFs.

In Gai1 or Gai3 single knockout (SKO) MEFs, NLGN3-induced phosphorylation of Akt, Erk1/2, S6K and S6 was partially inhibited (*P* < 0.05 vs. WT MEFs, Figure 1C), indicating that both Gai1 and Gai3 can be utilized for NLGN3-induced signaling. To control for possible epigenetic modifications in the knockout MEFs, the CRISPR/Cas9 gene-editing method was applied to knockout both Gai1 and Gai3 in WT MEFs. As shown, NLGN3-induced Akt, Erk and mTORC1 activation was almost completely blocked in Gai1/3 KO MEFs ("CRISPR-Gai1/

3-DKO") (Figure 1C).

Gai1 and Gai3 are required for NLGN3-induced Akt, Erk and mTORC1 activation in MEFs

To further confirm that Gai1/3 are required for NLGN3-induced signaling the shRNA method was utilized. As described previously [29], Gai1 shRNA lentiviral particles and/or Gai3 shRNA lentiviral particles were added to WT MEFs to silence Gai1 and/or Gai3. As shown, shRNA-mediated single knockdown of Gai1 or Gai3 resulted in partial inhibition of Akt, GSK3 α/β and Erk1/2 phosphorylation in response to NLGN3 (*P* < 0.05 vs. MEFs with control shRNA, Figure 2A). Significantly, Gai1 and Gai3 double knockdown by targeted shRNAs ("Gai1/3 DshRNA") led to further inhibition of NLGN3-induced signaling in MEFs (Figure 2A).

To determine whether exogenously expressed Gai proteins can rescue signaling, Gai proteins were re-expressed in Gai1/3 DKO MEF cells by infection with an adenovirus Gai1 construct ("Ad-Gai1", no Tag) [28, 29] or adenovirus Gai3 construct ("Ad-Gai3", no Tag) [28, 29]. As shown, Ad-Gai1 or Ad-Gai3 restored NLGN3-induced Akt, GSK3 α/β and Erk1/2 in DKO MEFs (Figure 2B), further demonstrating that Gai1 and Gai3 are required for NLGN3-induced signaling. Conversely overexpression of Ad-Gai1 (adenovirus-packed Gai1) and Ad-Gai3 in WT MEFs significantly increased NLGN3-induced signaling. Stable Gai1 and Gai3 overexpressing MEFs ("OE-Gai1/3") were established. Protein levels of Gai1 and Gai3 (but not Gai2) were significantly increased in OE-Gai1/3 MEFs (*P* < 0.05 vs. vector control MEFs, Figure 2C), where NLGN3-induced Akt, Erk1/2 and S6K phosphorylation were significantly increased (*P* < 0.05 vs. vector control MEFs, Figure 2C). Therefore, Gai1/3 overexpression in WT MEFs enhanced NLGN3-induced downstream signaling activation.

Gai1 and Gai3 are essential for NLGN3 signaling in glioma cells

To silence Gai1 and Gai3 in glioma cells, primary human glioma cells ("P1") were utilized. The pathology of P1 patients (female, 69) was: WHO grade IV, GFAP⁺, S100⁺, IDH1⁻, Nestin⁺, Olig2⁺, CD34⁺, Ki67⁺, p53-mutant, SOX10⁺, ATRX⁺, H3K27M⁻ and MGMT⁻. Cells were infected with Gai1 shRNA lentivirus plus Gai3 shRNA lentivirus [29]. Following puromycin selection, stable glioma cells were established with depleted Gai1 and Gai3 ("Gai1/3 DshRNA", Figure 3A). In control P1 glioma cells (expressing a scramble nonsense shRNA/"scr-shRNA") NLGN3 potently induced phosphorylation

of Akt, Erk1/2 and S6K, whereas signaling was almost blocked in P1 glioma cells transduced with *Gai1/3* DshRNA (Figure 3A). In U251MG glioma cells, shRNA-mediated silencing of *Gai1/3* (“*Gai1/3* DshRNA”) similarly inhibited NLGN3-induced Akt and Erk1/2 phosphorylation (Figure 3A).

Gai1/3 association is essential for RTK endocytosis and endosomal translocation, a key step for downstream signal activation [28, 29]. In response

to NLGN3, multiple RTKs (VEGFR2, EGFR and FGFR1) as well as *Gai1* and *Gai3* were enriched in the endosomal fraction in P1 glioma cells (Figure 3B), but not in *Gai1/3*-silenced cells (Figure 3B). RTKs expression and NLGN3-induced RTKs phosphorylation were not affected by *Gai1/3* silencing (Figure 3C). These results indicated that *Gai1/3* are required for NLGN3-induced RTK endocytosis and downstream signaling transduction.

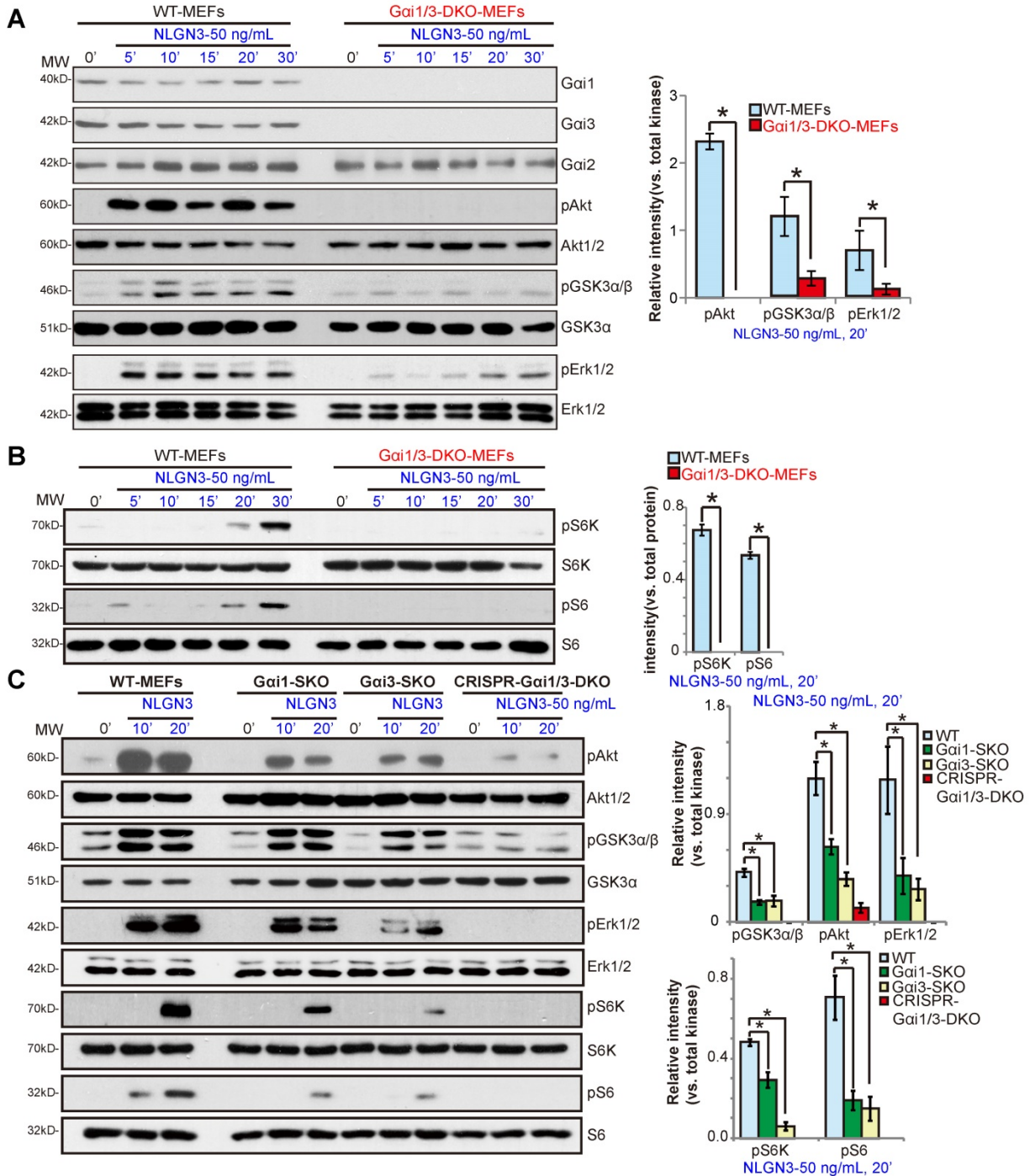


Figure 1. *Gai1* and *Gai3* double knockout largely inhibits NLGN3-induced Akt, Erk and mTORC1 activation in MEFs. Wild-type (WT), *Gai1* and *Gai3* double knockout (DKO) (A-B), *Gai1*, or *Gai3* single knockout (SKO) (C) mouse embryonic fibroblasts (MEFs), or WT MEFs with *Gai1* plus *Gai3* CRISPR/Cas9 KO constructs (“CRISPR-*Gai1/3*-DKO”, C), were treated with NLGN3 (50 ng/mL) for applied time, tested by Western blotting of listed proteins in total cell lysates. “MW” stands for molecular weight (Same for all Figures). Data were expressed as mean ± standard deviation (SD, same for all Figures). Quantifications were from five replicate blot data. *P < 0.05.

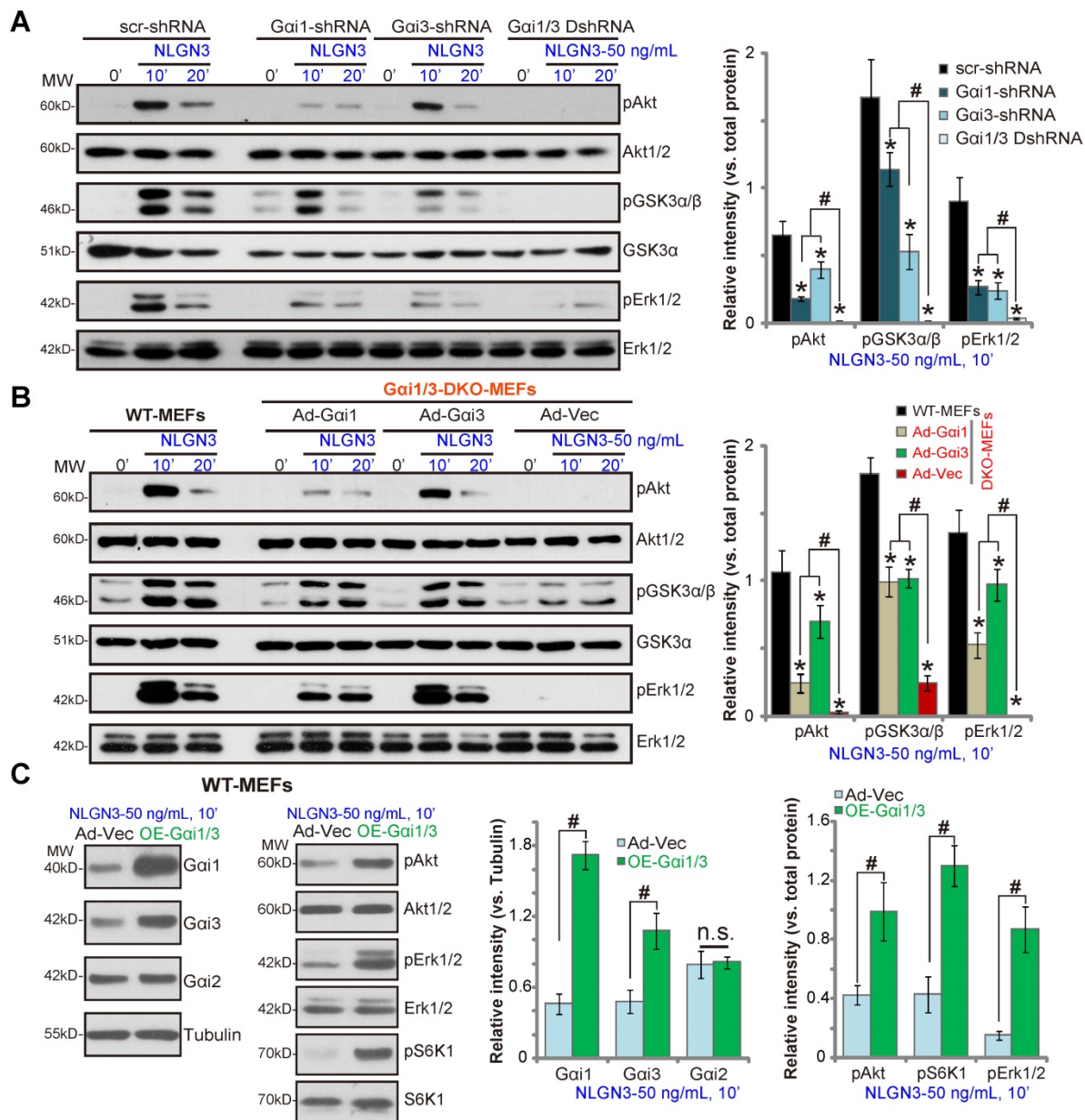


Figure 2. Gai1 and Gai3 are required for NLGN3-induced Akt, Erk and mTORC1 activation in MEFs. WT MEFs with the scramble control shRNA (“scr-shRNA”), the lentiviral Gai1 shRNA, the lentiviral Gai3 shRNA, or both (“Gai1/3 DshRNA”), were treated with NLGN3 (50 ng/mL) for applied time periods, and tested by Western blotting of listed proteins in total cell lysates (**A**). Gai1/3 DKO MEFs (**B**) or WT MEFs (**C**) were transfected with the adenovirus Gai1 construct (“Ad-Gai1”), the adenovirus Gai3 construct (“Ad-Gai3”) or the empty vector (“Ad-Vec”), treated with NLGN3 (50 ng/mL) for applied time periods, and tested of listed proteins. Quantifications were from five replicate blot data. **P* < 0.05 vs. “scr-shRNA” (**A**) or WT MEFs (**B**). #*P* < 0.05. “n.s.” stands for *P* > 0.05 (no statistical differences, **C**).

Conversely ectopic overexpression of Gai1 and Gai3 in P1 glioma cells significantly increased NLGN3-induced phosphorylation of Akt and S6K (Figure 3D), without affecting their expression (Figure 3D). For these experiments, Ad-Gai1 (no tag) and Ad-Gai3 (no tag) were transduced into P1 glioma cells, establishing Gai1 plus Gai3-overexpressed cells (“OE-Gai1/3”, two stable cell lines, sL1/sL2) (Figure 3D). Similarly, ectopic overexpression of Gai1 and Gai3 in U251MG cells (“OE-Gai1/3”) enhanced NLGN3-induced Akt, S6K1 and Erk1/2 phosphorylation (Figure 3D). Together, these results

show that Gai1 and Gai3 are essential proteins for NLGN3 signaling in human glioma cells.

Gab1 is a key adaptor protein for NLGN3 signaling in human glioma cells

Gab1 is a key Gai1/3 adaptor protein required for RTK signal transduction [25, 27-29]. We tested whether Gab1 is a required adaptor protein in NLGN3 signaling. NLGN3 was found to induce Gab1 activation, indicated by increased phosphorylation at Tyr-627 and Tyr-307 [39] in P1 primary human glioma cells (Figure 4A) and U251MG cells (Figure 4B). Significantly, shRNA-mediated stable knockdown of

Gab1 inhibited NLGN3-induced Akt, Erk1/2 and S6K phosphorylation in U251 and primary glioma cells (Figure 4A and B).

In MEFs Gab1 KO abolished NLGN3 signaling by blocking downstream Akt-mTORC1 and Erk1/2 activation (Figure 4C). Co-IP results in U251MG cells, Figure 4D, demonstrated that, in response to NLGN3, Gab1 associated with Gai1, Gai3, SHP2 and p85. As Gab1-p85 association mediates PI3K-Akt activation and Gab1-SHP2 association is vital for Erk-MAPK cascade activation [39], we propose that Gab1 is a key adaptor protein for NLGN3 signaling in human glioma cells.

Importantly, NLGN3-induced Gab1 activation (p-Tyr at 627) was completely blocked in Gai1/3 DKO MEFs (Figure 4E) and in CRISPR-Cas9-induced Gai1/3 DKO MEFs (Figure 4F), being partially inhibited with Gai1 or Gai3 SKO (Figure 4F). Additionally, shRNA-induced Gai1/3 double knockdown also attenuated NLGN3-induced Gab1 activation in MEFs (Figure 4G). Moreover, in DKO MEFs, re-expression of Gai1 or Gai3 restored NLGN3-induced Gab1 activation (Figure 4H). These results demonstrate that Gab1, lying downstream of Gai1/3, mediates NLGN3-induced Akt-mTORC1 and Erk activation.

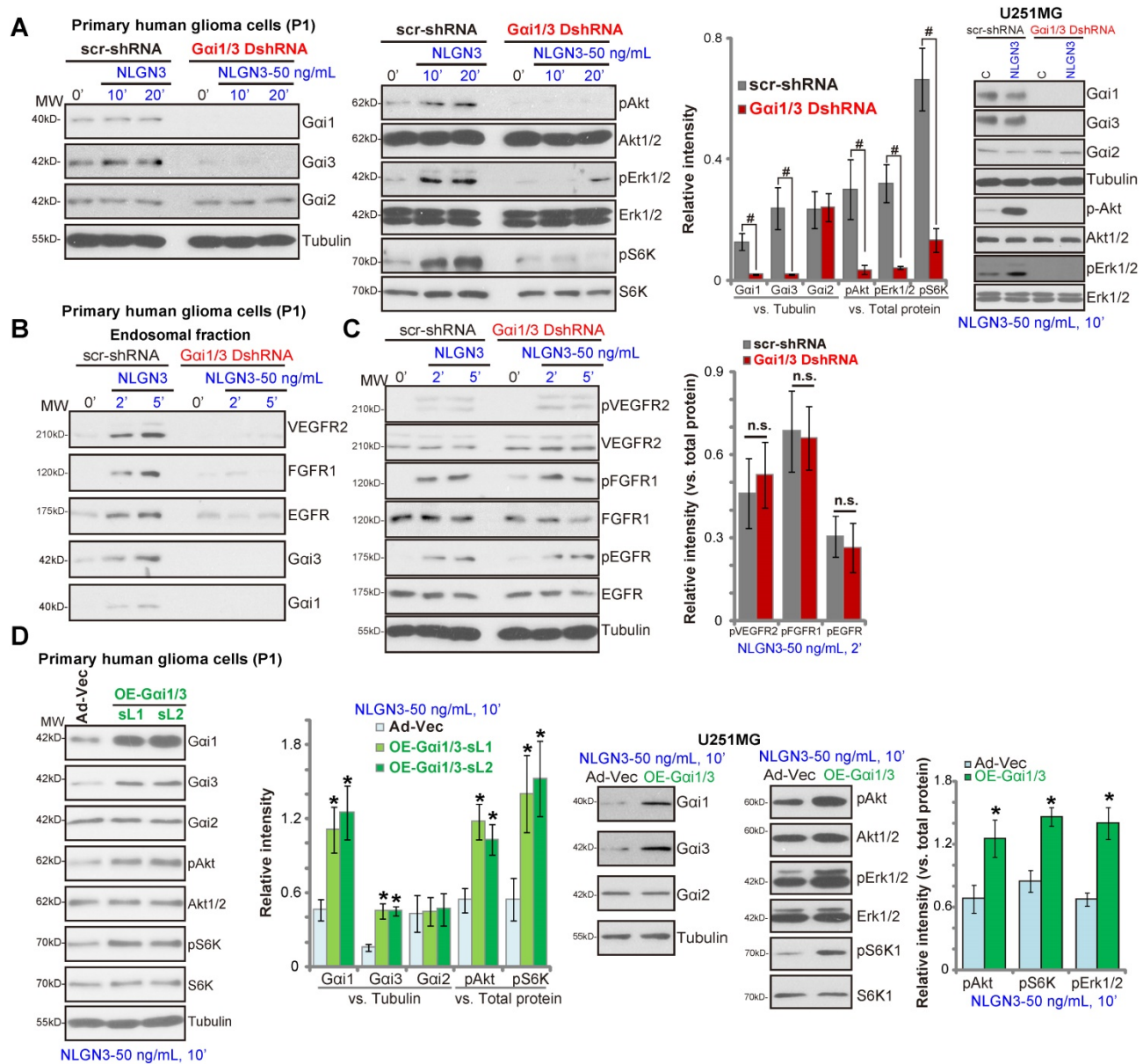


Figure 3. Gai1 and Gai3 are essential for NLGN3 signaling in glioma cells. The primary human glioma cells (“P1”) or U251MG cells, with Gai1 shRNA plus Gai3 shRNA (“Gai1/3 DshRNA”) or the scramble control shRNA (“scr-shRNA”), were treated with NLGN3 (50 ng/mL) for applied time periods, listed proteins in total cell lysates or endosomal fractions were tested by Western blotting assays (A-C). P1 glioma cells or U251MG cells were transfected with the adenovirus Gai1 construct plus the adenovirus Gai3 construct (“OE-Gai1/3”, two lines: “sL1/sL2”) or the empty vector (“Ad-Vec”), treated with NLGN3 (50 ng/mL) for 10 min, and tested by Western blotting assays of listed proteins (D). Quantifications were from five replicate blot data. #P < 0.05 (A). *P < 0.05 vs. “Ad-Vec” cells (D). “n.s.” stands for P > 0.05 (no statistical differences, C).

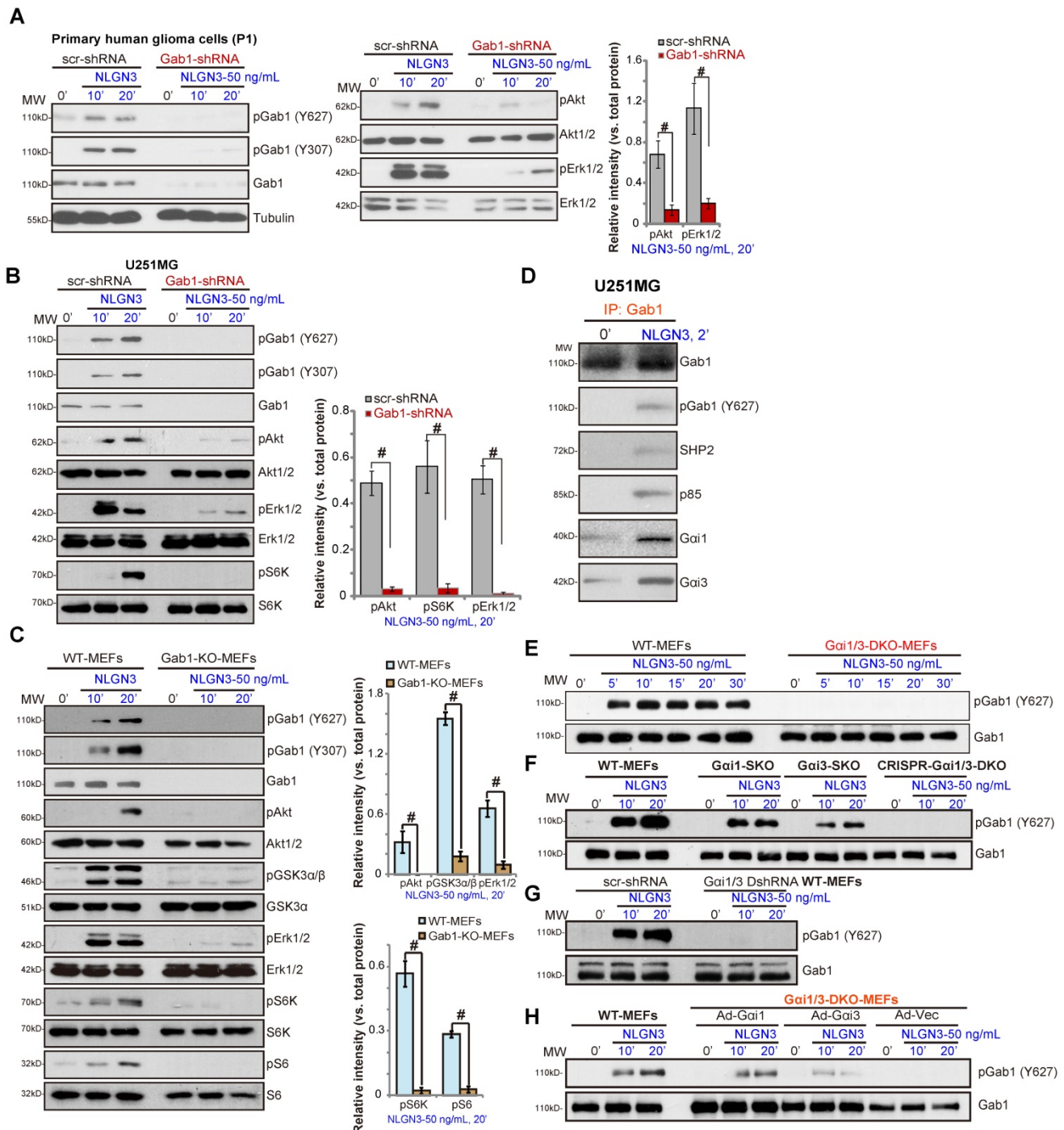


Figure 4. Gab1 is a key adaptor protein of NLGN3 signaling. The primary human glioma cells (“P1”) (A) or U251MG cells (B), with Gab1 shRNA or the scramble control shRNA (“scr-shRNA”), were treated with or without NLGN3 (50 ng/mL) for applied time periods, listed proteins in total cell lysates were tested by Western blotting. Wild-type (WT) and Gab1 knockout (Gab1-KO) MEFs were treated with NLGN3 (50 ng/mL) for applied time, tested by Western blotting of listed proteins in total cell lysates (C). U251MG cells were treated with NLGN3 (50 ng/mL) for 5 min, association of Gai1/3-Gab1-SHP2-p85 was tested by co-IP assays (D). WT, Gai1/3 DKO (E), Gai1 or Gai3 SKO (F), as well as WT MEFs with Gai1 plus Gai3 CRISPR/Cas9 DKO constructs (F) or Gai1 shRNA plus Gai3 shRNA (“Gai1/3 DshRNA”) (G) were treated with NLGN3 (50 ng/mL) for applied time, total- and p-Gab1 were tested. Gai1/3 DKO MEFs were transfected with the adenovirus Gai1 construct (“Ad-Gai1”), the adenovirus Gai3 construct (“Ad-Gai3”) or the empty vector (“Ad-Vec”), treated with NLGN3 (50 ng/mL) for applied time periods, total- and p-Gab1 were tested (H). Quantifications were from five replicate blot data. #P < 0.05.

Gai1 and Gai3 mediates NLGN3-induced glioma cell progression *in vitro*.

We next examined the role of Gai1/3 proteins in glioma cell function. NLGN3 treatment promoted the growth of P1 primary glioma cells (with “scr-

shRNA”), evidenced by increased EdU incorporation (Figure 5A, at 48h) and cell number (Figure 5B, at 72h). Significantly, NLGN3-induced effects were completely blocked by Gai1 and Gai3 shRNA (“Gai1/3 DshRNA”) (Figure 5A and B). Notably,

basal glioma cell growth (by complete medium, no NLGN3) was also inhibited by Gai1/3 DshRNA (Figure 5A and B). Moreover, NLGN3 significantly induced glioma cell migration, evidenced by an increased number of migrated cells in “Transwell” tests (Figure 5C), which was reversed by Gai1/3 DshRNA (Figure 5C). To confirm the efficiency of the applied shRNA, we showed that Gai1 and Gai3 proteins were silenced in Gai1/3 DshRNA-expressing P1 glioma cells (with NLGN3 treatment, at 72h) (Figure 5D). In U251MG cells, NLGN3-induced cell proliferation (EdU-positive nuclei ratio, Figure 5D) and migration (“Transwell” cell number, Figure 5E) were significantly inhibited by Gai1/3 DshRNA, which also inhibited medium-induced basal cell proliferation and migration (Figure 5E). These results demonstrated that Gai1/3 silencing inhibited NLGN3-induced cell growth, proliferation and migration *in vitro*.

Conversely, ectopic overexpression of Gai1 and Gai3 (“OE-Gai1/3”, see Figure 3) in P1 glioma cells promoted NLGN3-induced cell proliferation (increased EdU incorporation, Figure 5F), growth (Figure 5G), and migration (“Transwell” assay, Figure

5H). Western blotting results, Figure 5I, confirmed Gai1/3 overexpression in OE-Gai1/3 glioma cells (72h after NLGN3 treatment). Notably, basal glioma cell proliferation (by complete medium, no NLGN3) and migration were enhanced with OE-Gai1/3 (Figure S1A-B). Akt and Erk1/2 phosphorylation was augmented as well (Figure S1C).

Gai1 and Gai3 are required for orthotopic growth of primary glioma xenografts in mouse brain

We performed orthotopic primary human glioma xenograft experiments using a previously-described protocol [26]. P1 glioma cells, derived from a GBM patient (see MRI diagnosis in Figure 6A), were intracranially (using the described parameters [38]) injected into brains of nude mice. At experimental day-26, when the first mouse in the control group exhibited obvious symptoms, all the mice were sacrificed and tumors isolated [26]. MRI imaging results confirmed that the growth of orthotopic primary glioma xenografts (Figure 6B, yellow boxes) was significantly inhibited when expressing Gai1 shRNA plus Gai3 shRNA (“Gai1/3 DshRNA”). Gai1

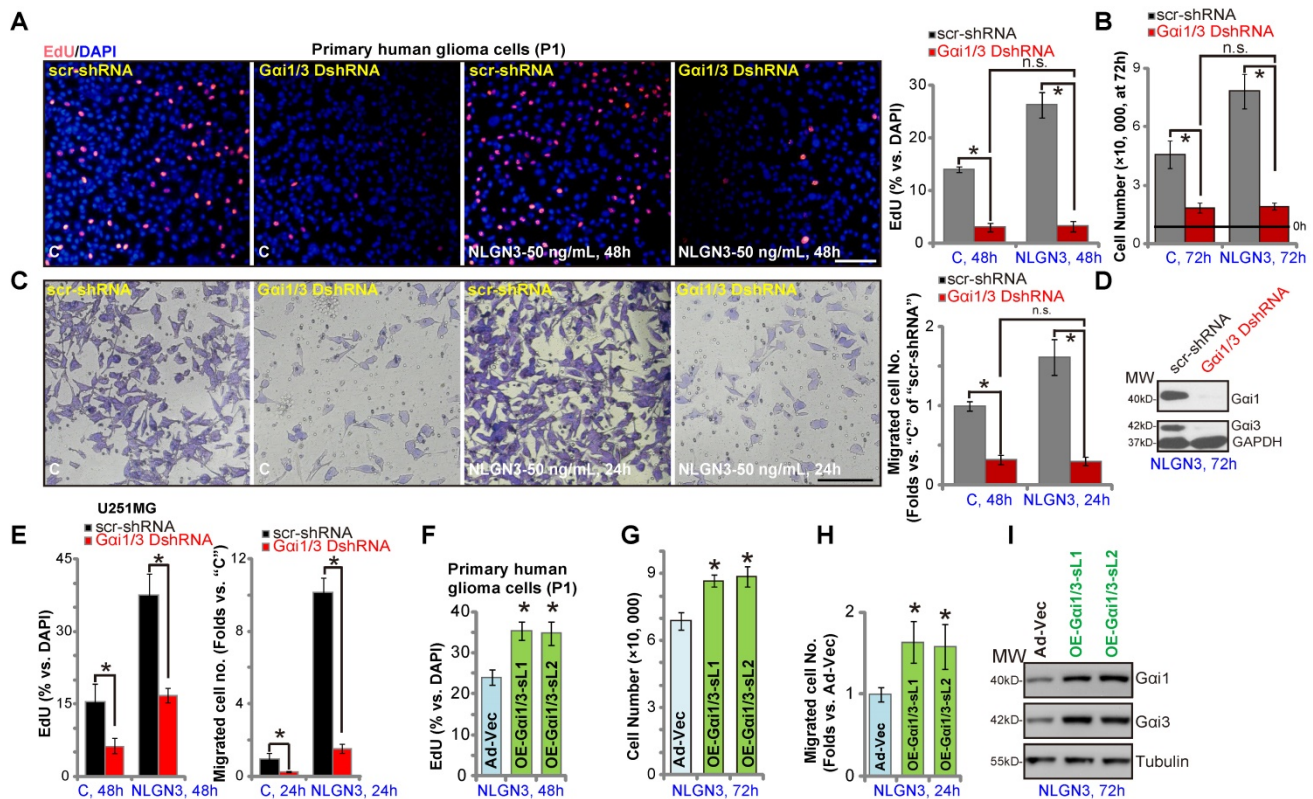


Figure 5. Gai1 and Gai3 mediate NLGN3-induced glioma cell progression *in vitro*. The P1 primary glioma cells or U251MG cells, with Gai1 shRNA plus Gai3 shRNA (“Gai1/3 DshRNA”) or the scramble control shRNA (“scr-shRNA”), were treated with or without NLGN3 (50 ng/mL) for applied time periods, cell proliferation (A and E), growth (B), and migration (C and E) were examined by the assays mentioned in the text, with Gai1 and Gai3 expression tested as well (D, for P1 glioma cells). P1 glioma cells, with the adenovirus Gai1 construct plus the adenovirus Gai3 construct (“OE-Gai1/3”, two lines: “sL1/sL2”) (F-I), were treated with or without NLGN3 (50 ng/mL) for applied time period, cell proliferation (F), growth (G), migration (H) and Gai1/3 expression (I) were tested, with results quantified. For the EdU staining assay, ten random views were included to calculate EdU/DAPI ratios. For the “Transwell” assays, ten random views of each condition were included to calculate the average number of migrated cells. For all functional assays exact same number of viable cells of different genetic treatments were initially seeded into each well/dish (at 0h). “C” stands for medium control. Blotting data was repeated five times (D and I). *P < 0.05 (A-E). *P < 0.05 vs. “Ad-Vec” cells (F-H). “n.s.” stands for non-statistical difference (A-C). Scale bar=100 μm (A and C).

and *Gai3* double silencing resulted in an 80% reduction of orthotopic glioma xenograft growth (Figure 6C), and was significantly more effective than *Gai1* single silencing (50-60% inhibition, see our previous study [26]). Mouse body weights were equivalent between the *Gai1/3* DshRNA and control shRNA groups (Figure 6D). *Gai1/3* protein expression levels were significantly decreased in orthotopic primary xenografts with *Gai1/3* DshRNA, and levels of p-Akt, p-S6K and p-Erk1/2 inhibited (Figure 6E). Immunohistochemistry (IHC) results, shown in Figure 6F, further confirmed reductions in p-Akt and p-S6K in *Gai1/3*-silenced orthotopic tumors (vs. control tumors). Therefore *Gai1/3* silencing significantly inhibited orthotopic growth of primary glioma xenografts.

In contrast, ectopic *Gai1/3* overexpression promoted orthotopic growth of primary glioma xenografts. Primary glioma cells transduced with Ad-*Gai1* plus Ad-*Gai3* ("OE-*Gai1/3*") or the vector control ("Ad-Vec") were injected into the brains of the nude mice. At experimental day-20, when the first

mouse in the OE-*Gai1/3* group exhibited obvious symptoms, all mice were sacrificed and the tumors isolated. MRI images showed that *Gai1/3*-overexpressing primary glioma xenografts grew faster than the control tumors (with Ad-Vec) (Figure 6G), presenting with higher tumor volumes (Figure 6H), and higher tumor volumes (Figure 6H). Mouse body weights were unchanged (Figure 6I). Western blotting of tumor lysates confirmed *Gai1/3* overexpression in OE-*Gai1/3*-tumor tissues, where levels of p-Akt, p-S6K and p-Erk1/2 increased (Figure 6J). Taken together, *Gai1* and *Gai3* are required for orthotopic primary glioma xenograft growth in mouse brain.

Orthotopic growth of brain-metastatic human lung cancer cells requires *Gai1* and *Gai3*

Next we tested whether *Gai1* and *Gai3* were also required for the growth of metastatic brain tumor. Brain-metastatic lung cancer cells, bmLCs, from a lung cancer patient with brain metastasis (see the MRI image of brain metastatic tumor, Figure 7A) were obtained and primary-cultured. The bmLCs were

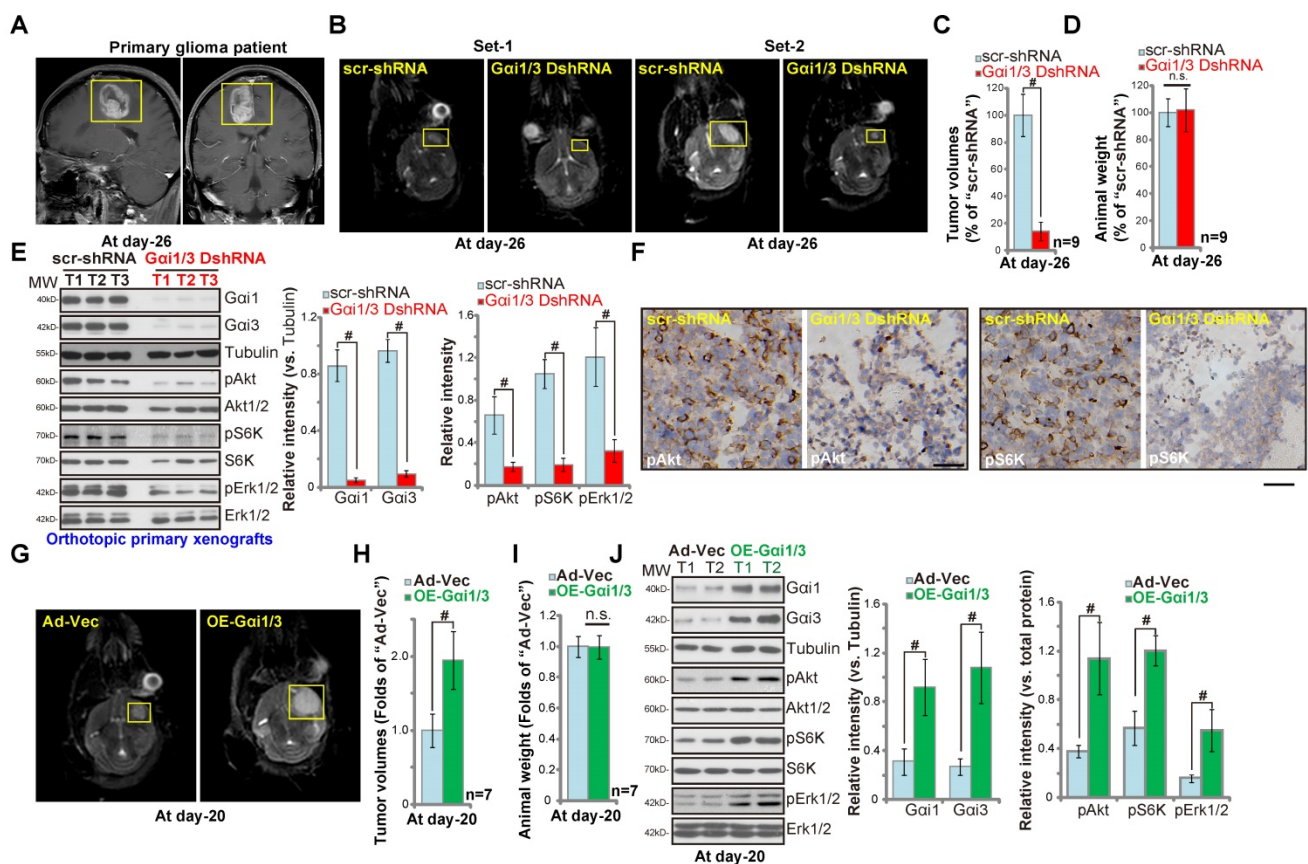


Figure 6. *Gai1* and *Gai3* are required for orthotopic growth of primary glioma xenografts in mouse brain. Brain MRI images of a primary glioma patient providing primary human glioma cells ("P1" cells, **A**). The exact same amount of glioma cells (5×10^5 cells of each mouse), expressing *Gai1* shRNA plus *Gai3* shRNA ("*Gai1/3* DshRNA"), scramble non-sense shRNA ("scr-shRNA"), the adenovirus *Gai1* plus *Gai3* constructs ("OE-*Gai1/3*"), or the empty vector ("Ad-Vec"), were intracranially injected to brains of nude mice (5-6 week old), after 26/20 days, representative MRI images of orthotopic glioma xenografts were presented (**B** and **G**); Animals were decapitated and tumors were isolated by surgery, tumor volumes (**C** and **H**) and mice body weights (**D** and **I**) were recorded. Tumor tissue lysates were tested by Western blot assay of listed proteins (**E** and **J**). Immunohistochemistry (IHC) images of p-Akt and p-S6K were shown (**F**). Western blotting quantifications were from five replicate blot data. IHC experiments were repeated in three pairs of tissues. # $P < 0.05$. "n.s." stands for non-statistical difference (**D** and **I**). Scale bar=100 μ m (**F**).

infected with the lentivirus encoding Gai1 shRNA plus Gai3 shRNA ("Gai1/3 DshRNA") or scramble control shRNA ("scr-shRNA"), and stable bmLCs established after puromycin selection. *In vivo* cultured scr-shRNA control bmLCs, NLGN3 (50 ng, 10 min) increased phosphorylation of Akt, S6K and Erk1/2 (Figure S2). Gai1 and Gai3 double silencing by Gai1/3 DshRNA potently inhibited NLGN3-induced signaling (Figure S2) in bmLCs.

Thereafter, cells were intracranially injected into brains of nude mice. At experimental day-25, when the first mouse in the control group exhibited obvious symptoms, all the mice were sacrificed. MRI image results confirmed that orthotopic bmLCs xenografts bearing Gai1/3 DshRNA grew significantly slower than control bmLCs xenografts with scr-shRNA (Figure 7B). Gai1 and Gai3 double shRNA led to over

80% reduction of orthotopic bmLCs xenograft growth (Figure 7C). The mice body weights were indifferent between the two groups (Figure 7D). Next, signaling proteins in orthotopic bmLCs xenografts were examined. Western blotting assay results, Figure 7E, confirmed Gai1 and Gai3 silencing in bmLCs xenografts with Gai1/3 DshRNA, while Gai2 expression was unchanged (Figure 7E). Importantly, phosphorylations of Akt, S6K and Erk1/2 were largely decreased in Gai1/3 DshRNA-expressing orthotopic bmLCs xenografts (Figure 7E). IHC images further confirmed decreased Akt-S6K phosphorylations in orthotopic bmLCs xenografts with Gai1/3 DshRNA (Figure 7F). These results showed that silencing of Gai1 and Gai3 inhibited orthotopic growth of bmLCs in mouse brain.

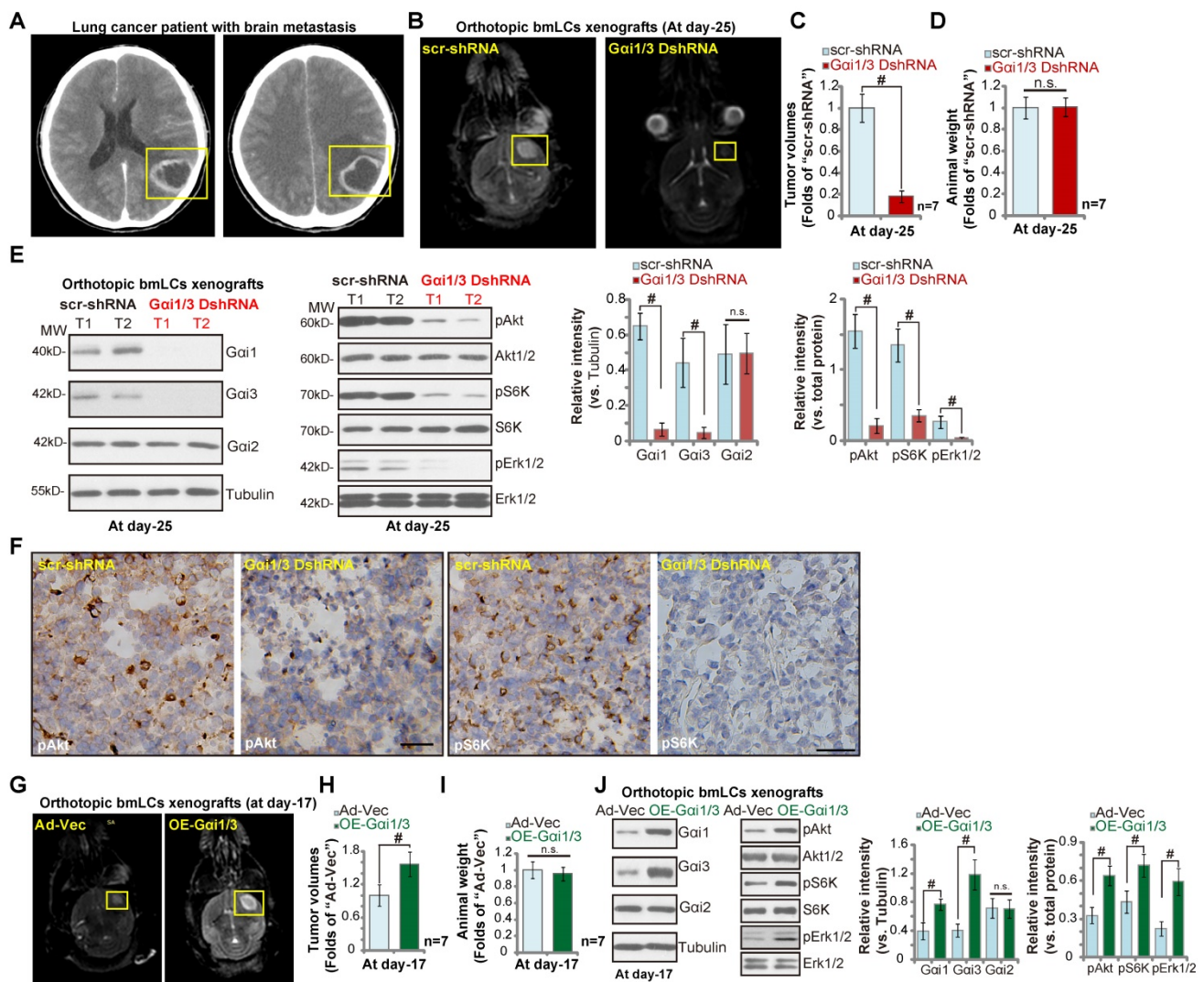


Figure 7. Orthotopic growth of brain-metastatic human lung cancer cells requires Gai1 and Gai3. Brain MRI images of a primary lung cancer patient with brain metastasis (A) providing the brain-metastatic human lung cancer cells ("bmLCs"). bmLCs (5×10^5 cells of each mouse), expressing Gai1 shRNA plus Gai3 shRNA ("Gai1/3 DshRNA"), scramble non-sense shRNA ("scr-shRNA"), the adenovirus Gai1 plus Gai3 constructs ("OE-Gai1/3"), or the empty vector ("Ad-Vec"), were intracranially injected to brains of nude mice (5-6 week old); After 25/17 days, representative MRI images of orthotopic bmLCs xenografts were presented (B and G); Animals were decapitated and tumors were isolated by surgery, tumor volumes (C and H) and mice body weights (D and I) were recorded. The orthotopic bmLCs xenograft tissue lysates were tested by Western blot assay of listed proteins (E and J). Immunohistochemistry (IHC) images of p-Akt and p-S6K were shown (F). Western blotting quantifications were from five replicate blot data. IHC experiments were repeated in three pairs of tissues. # $P < 0.05$. "n.s." stands for non-statistical difference (D and I). Scale bar=100 μ m (F).

To further support our hypothesis, primary bmLCs, stably transduced with Ad-Gai1 plus Ad-Gai3 ("OE-Gai1/3"), as well as the control cells with the vector control ("Ad-Vec"), were injected into the brains of the nude mice. At day-17, the first mice in the OE-Gai1/3 group exhibited obvious neurological symptoms. MRI images showed that the orthotopic bmLCs xenografts with OE-Gai1/3 were larger than the control xenografts (Figure 7G). Tumor volumes of OE-Gai1/3 bmLCs xenografts were significantly higher than those of Ad-Vec xenografts (Figure 7H). The mice body weights were indifferent (Figure 7I). Western blotting assaying of orthotopic bmLCs xenograft tissues confirmed Gai1/3 overexpression as well as increased phosphorylations of Akt-S6K1 and Erk1/2 in OE-Gai1/3 xenograft tissues (Figure 7J). Together, these results show the orthotopic growth of bmLCs requires Gai1 and Gai3.

Gai3 upregulation in human glioma tissues

We have previously shown that *Gai1* mRNA and protein levels are upregulated in human glioma, correlating with high tumor grade [26]. To examine Gai3 expression in human glioma, we first consulted the TCGA database to examine RNA-Seq data. As shown, in human glioma tissues *Gai3* mRNA expression is significantly upregulated [$P < 0.05$ vs. normal tissues] (Figure 8A). The overall survival of the low grade glioma (LGG) patients with *Gai3*-high gliomas is significantly lower than those with

Gai3-high gliomas ($P < 0.001$, Figure 8B). There is however no significant difference in the overall survival of *Gai3*-low GBM patients and *Gai3*-high GBM patients (Figure 8B). This could be due to the extremely low overall survival of GBM patients (Figure 8B). When combining all glioma data, we showed that the average survival of *Gai3*-high glioma patients is significantly lower than those with *Gai3*-low gliomas ($P < 0.001$, Figure 8B). These results indicate that *Gai3* upregulation in human glioma is correlated with a poor overall survival.

To confirm the significance of the bioinformatics observations, we examined Gai3 expression in human glioma specimens ("T") and surrounding normal brain ("N") tissues, from sixteen (16) low-grade (Grade I-II) tumors and another sixteen high-grade (Grade III-IV) tumors [26]. qPCR results, Figure 8C, demonstrated that *Gai3* mRNA levels are significantly upregulated in human glioma tissue (*vs.* normal brain tissue, Figure 8C). In late-stage gliomas (Grade III-IV, $n=16$) *Gai3* mRNA is more highly upregulated than in early-stage gliomas (Grade I-II, $n=16$) (Figure 8C). In the late-stage gliomas (Grade III-IV, $n=16$) *Gai3* mRNA is highly upregulated than that in early-stage gliomas (Grade I-II, $n=16$) (Figure 8C). Western blotting quantification results confirmed Gai3 protein upregulation in human glioma tissues (Figure 8D), being more significant in the high-grade tumors (Figure 8D).

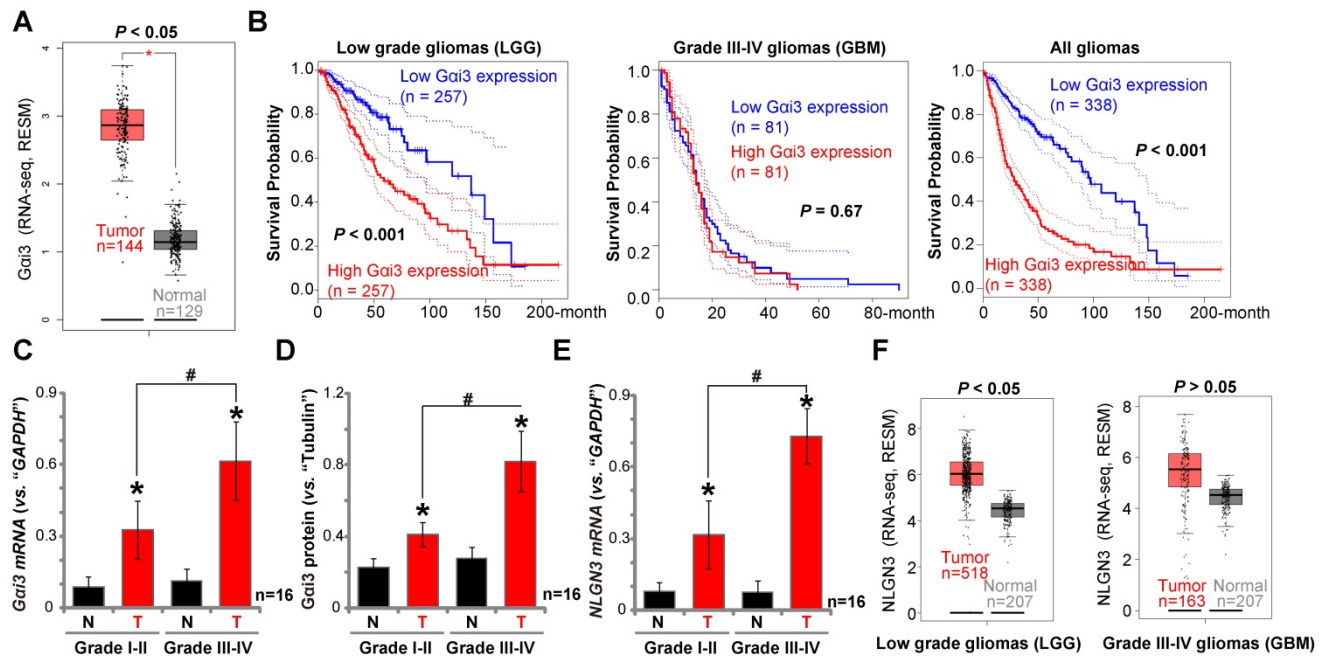


Figure 8. Gai3 upregulation in human glioma tissues. TCGA database shows *Gai3* and *NLGN3* expression (RNASeq, RSEM) in glioma tissues and normal brain tissues (A and F). Kaplan Meier Survival analyses of *Gai3*-low and *Gai3*-high glioma (LGG and GBM) patients (B). Human glioma tissues ("T", 16 high-grade and 16 low-grade) and the paired normal brain tissues ("N") were homogenized and dissolved in the tissue lysis buffer. *Gai3* mRNA and protein levels were tested by qPCR (C) and Western blotting (D), respectively, with results quantified. *NLGN3* mRNA was tested by qPCR (E). * $P < 0.05$ vs. "N" group. # $P < 0.05$.

In line with previous studies [18, 19, 40], *NLGN3* mRNA levels were increased in human glioma tissues (Figure 8E), and levels were higher in the late-stage gliomas (Figure 8E). TCGA cohorts showed that *NLGN3* transcripts are higher in glioma tissues when compared to normal brain tissues (Figure 8F), being significant in LGG tissues (Figure 8F). Therefore, *Gai3*, similar to *Gai1* [26], is upregulated in human glioma tissues, correlating with poor survival, high tumor grade and *NLGN3* upregulation.

Discussion

GPCR components, including GPR56 [41], GPR55 [42] and many others, are dysregulated in human glioma and in other brain tumors. cAMP contents and the adenylyl cyclase (AC) activity were decreased in brain tumors [43]. He *et al.*, showed that *Gas* subunit was downregulated in medulloblastoma, functioning as a tumor suppressor by blocking Sonic hedgehog signaling [44]. Lelievre *et al.*, indicated that AC-mediated cAMP regulation plays an important cooperating role in the genesis of medulloblastoma [45]. Importantly, *Gai*-coupled GPCRs, including cannabinoid receptors [46], CXCR4 [47, 48], dopamine D2 receptors [24] and melatonin receptor II (MTII) [49], were reported to be important for glioma progression. Pharmacological inhibition or genetic silencing of the *Gai*-coupled GPCRs has potent anti-tumor effects [46-49]. Similarly, Urotensin II-activated Urotensin II receptor coupled to both *Gai/o* and *Gα13* to activate PI3K-PIP3-GEF-Rac-Cdc42 signaling cascade [50]. Le Joncour *et al.*, reported that Urotensin II and its receptor were overexpressed in human glioma, important for angiogenesis of glioma [51]. Consistently, CXCR4-mediated *Gai* activation and cAMP suppression is stimulated by the Sonic hedgehog pathway [52].

Our results show that *Gai1* and *Gai3* are essential players in *NLGN3* signaling. In MEFs, *Gai1/3* DKO blocked *NLGN3*-induced Akt-mTORC1 and Erk-MAPK activation, with *Gai1* or *Gai3* SKO resulting in partial inhibition. Furthermore, in WT MEFs shRNA-mediated silencing of *Gai1* and *Gai3* attenuated Akt-mTORC1 and Erk-MAPK activation by *NLGN3*. Ectopic *Gai1/3* expression restored *NLGN3*-induced Akt-mTORC1 and Erk-MAPK activation in *Gai1/3* DKO MEFs, and enhanced *NLGN3*-induced signaling in WT MEFs. Similarly in established (U251MG) or primary human glioma cells, *NLGN3*-induced Akt-mTORC1 and Erk-MAPK activation was inhibited by *Gai1/3* shRNA, but enhanced with ectopic *Gai1/3* overexpression. These results show that *Gai1* and *Gai3* are required for *NLGN3*-induced mitogenic signal transduction.

Sustained and constitutive activation of multiple

key RTKs is a primary driving force for glioma cell progression [53, 54], causing hyperactivation of downstream mitogenic pathways, including PI3K-Akt-mTOR and Erk-MAPK [54, 55]. There are reports of GPCR-RTKs crosstalk in glioma cells. For example, Huang *et al.*, found that activation of the formylpeptide receptor induced trans-activation of EGFR via *Gai* proteins, exacerbating malignant behaviors in glioblastoma cells [56]. Ghosh *et al.*, found that *Gai*-GIV (a novel non-receptor GEF for *Gai* [57]) complex directly associated with and activated EGFR to promote cancer cell migration and proliferation [57].

Studies have shown that *NLGN3* induces phosphorylations of multiple RTKs as well as downstream PI3K-Akt-mTOR and Erk-MAPK cascades, responsible for glioma cell growth and proliferation [19]. We found that *Gai1/3* proteins associate with activated RTKs (VEGFR and TrkB ([28, 29]) and are required for their endocytosis and downstream signaling. Here in glioma cells, *Gai1/3* silencing, by targeted shRNAs, blocked *NLGN3*-induced RTKs endosomal translocation and subsequent *Gab1* activation, without affecting RTK phosphorylation and expression. Importantly, *Gab1* silencing inhibited *NLGN3*-induced downstream signaling activation in glioma cells. These results suggest that *Gai1* and *Gai3* mediate *NLGN3*-induced signal transduction by promoting RTK endocytosis and adaptor protein *Gab1* activation.

Functional studies demonstrate that *Gai1/3* are essential in mediating *NLGN3*-induced glioma cell progression *in vitro* and orthotopic primary glioma xenograft growth *in vivo*. *NLGN3*-stimulated glioma cell growth, proliferation and migration were largely inhibited by *Gai1/3* silencing, but enhanced by ectopic *Gai1/3* overexpression. Glioma xenografts are unable to grow in *NLGN3* KO brain suggesting an essential dependency on *NLGN3* for orthotopic glioma growth [28, 29]. Significantly, our results show that the growth of orthotopic primary glioma xenografts was largely inhibited with *Gai1/3* silencing. Conversely the *Gai1/3*-overexpressed primary glioma xenografts grew significantly faster than the control tumors. We concluded that possibly by mediating *NLGN3*-induced signaling transduction, *Gai1/3* are essential for glioma cell progression *in vitro* and *in vivo*.

One important finding of this study is that *Gai1* and *Gai3* are required for the orthotopic growth of bmLCs in mouse brain. One possibility is that neuron-secreted *NLGN3* is also important for orthotopic growth of bmLCs. Indeed, *ex vivo* cultured bmLCs were responsible to *NLGN3*. *NLGN3* activated Akt-mTOR and Erk1/2 signalings, and

promoted bmLCs proliferation and migration *in vitro*. Such actions were almost abolished with Gai1/3 DshRNA. Importantly, orthotopic growth of bmLCs in mouse brain was largely inhibited with Gai1/3 DshRNA, but augmented with ectopic Gai1/3 overexpression. In addition, Akt-mTOR and Erk1/2 activation in orthotopic bmLCs xenograft tissues was inhibited with Gai1/3 DshRNA, but enhanced with Gai1/3 overexpression. Other mitogens, including the BDNF and VEGF, could also be important contributors for the growth of brain-metastatic cancer cells [18]. Interestingly, we have previously shown that BDNF- and VEGF-induced mitogenic signalings rely on Gai1/3 [28, 29]. This could also demonstrate the essential role of Gai1/3 in mediating orthotopic growth of bmLCs in mouse brain.

NLGN3 is a crucial factor for neuron-glioma synapse formation and neuronal activity-dependent glioma progression [14, 15, 20]. Interestingly, Hanahan and his colleagues have shown that brain metastatic breast-cancer cells can form a specialized type of pseudo-tripartite synapse with glutamatergic neurons [58]. This will allow cancer cells to take up glutamate and activate GluN2B-mediated NMDAR (N-methyl-D-aspartate receptor) signaling, required for metastatic cancer cell growth [58]. Thus, another possibility is that bmLCs might form synapses with neurons to activate glutamate signaling, and neuron-secreted NLGN3 could be essential for the process. The underlying mechanisms warrant further characterizations.

NLGN3 expression levels negatively correlate with overall survival of glioblastoma patients [18, 19]. Furthermore high NLGN3 expression is associated with glioma recurrence [40]. Our current and previous results [26] have demonstrated that Gai1 and Gai3 levels are significantly upregulated in human glioma tissues, and their upregulation is correlated with poor patients survival, high tumor grade and NLGN3 upregulation. Gliomas, especially the high grade GBM, are composed of a pathologically heterogeneous mixture of cells exhibiting different cellular and nuclear polymorphism [59-61]. Therefore, Gai1/3 and NLGN3 upregulation in glioma tissues might be derived from both glioma cells and possible other cells in the tumor bulk (immune cells, endothelial cells and cancer stem cells, etc). Although the underlying mechanisms of Gai1/3-mediated NLGN3 signaling and glioma cell progression need further characterization, the results of the present and previous studies [26] strongly indicate that Gai1 and Gai3 could be valuable therapeutic targets for treating human glioma.

Conclusions

Gai1/3 mediation of NLGN3-induced signaling is essential for glioma growth *in vitro* and *in vivo*.

Acknowledgements

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Supplementary Material

Supplementary figures.

<http://www.thno.org/v11p8535s1.pdf>

Competing Interests

The authors have declared that no competing interest exists.

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