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The scaffolding protein DLG5 promotes glioblastoma growth by controlling Sonic Hedgehog signaling in tumor stem cells

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Abstract

Background. Tumor invasion, a hallmark of malignant gliomas, involves reorganization of cell polarity and changes in the expression and distribution of scaffolding proteins associated with polarity complexes. The scaffolding proteins of the DLG family are usually downregulated in invasive tumors and regarded as tumor suppressors. Despite their important role in regulating neurodevelopmental signaling, the expression and functions of DLG proteins have remained almost entirely unexplored in malignant gliomas.

Methods. Western blot, immunohistochemistry, and analysis of gene expression were used to quantify DLG members in glioma specimens and cancer datasets. Over-expression and knockdown of DLG5, the highest-expressed DLG member in glioblastoma, were used to investigate its effects on tumor stem cells and tumor growth. qRT-PCR, Western blotting, and co-precipitation assays were used to investigate DLG5 signaling mechanisms.

Results. DLG5 was upregulated in malignant gliomas compared to other solid tumors, being the predominant DLG member in all glioblastoma molecular subtypes. DLG5 promoted glioblastoma stem cell invasion, viability, and self-renewal. Knockdown of this protein *in vivo* disrupted tumor formation and extended survival. At the molecular level, DLG5 regulated Sonic Hedgehog (Shh) signaling, making DLG5-deficient cells insensitive to Shh ligand. Loss of DLG5 increased the proteasomal degradation of Gli1, underlying the loss of Shh signaling and tumor stem cell sensitization.

Conclusions. The high expression and pro-tumoral functions of DLG5 in glioblastoma, including its dominant regulation of Shh signaling in tumor stem cells, reveal a novel role for this protein that is strikingly different from its proposed tumor-suppressor role in other solid tumors.

Key points

- DLG5 is a novel master regulator of Hedgehog signaling in glioblastoma.
- DLG5 has unique upregulation and tumor-promoting functions in malignant gliomas.
- DLG5 is necessary for the maintenance of the tumor stem cell population in GBM.

Glioblastoma (GBM) is the most common malignant tumor type originating in the adult Central Nervous System (CNS) and remains one of the deadliest forms of cancer.[1] Despite their considerable molecular heterogeneity[2] GBM tumors retain some common phenotypic features, such as diffuse invasion through neural tissue that makes complete resection

Importance of the study

Sonic Hedgehog (Shh) is a critical signaling pathway that promotes glioblastoma stem cell viability, invasion, and self-renewal. Our findings identify the scaffolding protein DLG5 as a necessary factor to maintain Shh activity in tumor stem cells, demonstrating for the first time a tumor-promoting role for a member of the DLG family that contrasts with the accepted tumor-suppressor role of DLG proteins in solid tumors. More importantly, the mechanism by which DLG5 regulates Shh signaling reveals a dependence that would be inescapable even for tumor cells with driver mutations in Shh members. This study highlights the importance of scaffold proteins involved in cell polarity as potential new targets to prevent growth, dispersion, and tumor stem cell persistence in malignant gliomas.

impossible.[3] GBM invasion is a complex process guided by the cytoarchitecture of the CNS and regulated by reactivation of developmental signaling pathways such as Notch, Wnt/β-catenin, and Sonic Hedgehog (Shh),[4] all of which allow tumor cells to respond to the same microenvironmental cues that promote the proliferation and migration of neural progenitors during development.

Tumor growth and invasion are also usually associated with the downregulation of proteins belonging to the *Crumbs*, *Par*, and *Scribble* scaffolding complexes. These protein complexes are required for cell and tissue polarity, i.e., the separation of spatially and functionally distinct domains within a cell or across a tissue layer.[5–7] Downregulation of scaffolding proteins is in many cases an early step towards the loss of epithelial integrity associated with tumor cell dispersion and these proteins are therefore regarded as tumor suppressors.[8] However, malignant gliomas originate from already-motile neural precursors[9] and can retain extensive multicellular networks during brain infiltration.[10] The expression of scaffolding proteins in GBM and their possible involvement in regulating major signaling mechanisms in these tumors has remained almost entirely unexplored.

The members of the *Disc Large Homolog* (DLG) family are scaffolding proteins found in *Scribble* and other macromolecular complexes required for stable cell-cell association and tissue organization.[11,12] These proteins are members of the Membrane-Associated Guanylate Kinase (MAGUK) superfamily and regulate the intracellular traffic of proteins or vesicles[13] thanks to specialized domains that bind cytoplasmic and cytoskeletal partners. DLG5 is unique in the MAGUK superfamily because of its much larger size and additional number of protein-protein binding domains (PDZ domains) compared to the other DLG members (Supplementary Figure S1). Indeed, due to its unusual structure, DLG5 has been proposed to be either a MAGUK or a member of the Caspase Activation and Recruitment Domain (CARD) family of adaptor proteins,[14] suggesting that it may have different functions from the other DLG members. Reports from bladder, prostate, liver, and breast cancers have shown that this protein is downregulated or lost in epithelial tumors and acts, expectedly, as a tumor suppressor.[15–17] DLG5 is also required for critical processes involving cell-cell adhesion during neural development, including the early closure of the neural tube and the formation of functional synapses[18,19]; however, its expression or functions have never been investigated in malignant gliomas.

Some of the known partners of DLG5 include cadherin/β-catenin complexes—required for Wnt signaling—[19] and Smo/Kif7 complexes—required for Shh signaling—,[20] suggesting that this protein could regulate major neurodevelopmental pathways. Of these, Shh signaling is of particular relevance because this pathway is highly upregulated in GBM[21] and correlated with tumor invasion,[22] maintenance of the tumor stem cell population,[23] and poor patient prognosis.[24] Strategies to inhibit Shh signaling as adjuvant therapy for GBM have been attempted in the clinical setting, with limited success to date (eg, clinical trials NCT00980343 and NCT01576666).

We report here that Shh signaling in GBM stem cells is critically dependent on DLG5 expression, which is highly upregulated in malignant gliomas compared to other solid tumors. Furthermore, we demonstrate multiple protumoral functions of DLG5 in GBM that have not been observed in other solid tumors, suggesting that this protein, and potentially other proteins involved in molecular scaffolding and cell polarity, can have unique functions in GBM that depart from their prior proposed roles in cancer.

Materials and Methods

Cells and Tissue Specimens

GBM stem cell lines (GSCs) established in our laboratory are described in the supplementary methods. Three lines (GBM146, GBM08, GBM30) were chosen for all the experiments in this study, together with the conventional cell line U251MG. Tissue microarrays containing normal human brain as well as low- and high-grade glioma were obtained commercially (Biomax arrays BS17017b, GL807, GL481, and GL803a). Stained tissue cores were diagnosed blindly by a neuropathologist (T.E.R.) to confirm agreement between observed histology and the pathology reported by the tissue provider. Frozen, age-matched tissue specimens were obtained from the University of Maryland Brain and Tissue Bank (normal brain) and from the Department of Neurosurgery at SUNY Upstate Medical University (GBM tissue). Institutional Review Board approval was obtained for tissue procurement and patients provided informed consent for the use of discarded tumor specimens for research (SUNY Upstate Medical University IRB 305315-22).

DLG5 Expression Datasets

Expression of DLG5 mRNA from a variety of cancers was extracted from datasets available from the repositories Oncomine Research (www.oncomine.org; 104 studies totaling 210 "tumor versus normal" comparisons) and The Cancer Genome Atlas (TCGA datasets, downloaded from the Broad GDAC Firehose, gdac.broadinstitute.org). Bulk RNAseq data to analyze DLG5 expression in GBM molecular subtypes (TCGA GBM 2016 dataset) was queried from the repository Gliovis (gliovis.bioinfo.cnio.es).

Immunohistochemistry

Tissue microarrays were processed for antigen recovery and immunohistochemistry following standard methods. Stained tissues were blindly scored by a neuropathologist (T.E.R.) for extent and intensity of DLG5 staining. Brain tissue sections from tumor-bearing mice were processed for hematoxylin-eosin histology or immunostaining with antibodies against bromodeoxyuridine (BrdU) and Gli1. Colocalization of BrdU or Gli1 with cell nuclei was analyzed using ad-hoc scripts in the software ImageJ.

Reagents for In Vitro Assays

All the procedures for cell transfection and DLG5 knockdown or overexpression are described in the supplementary methods. Transient, short-term experiments were performed with siRNAs whereas long-term incubations and *in vivo* studies were performed with the corresponding stably-expressed shRNAs. Antibodies used in this study are listed in Supplementary Table 1. Oligonucleotide sequences used for RNA interference or PCR are indicated in Supplementary Table 2. Shh signaling was induced by incubating GBM cells overnight with recombinant truncated human Sonic Hedgehog (1 μg/mL, Peprotech). Shh pathway inhibitors included vismodegib (Selleck Chemicals) and GANT-61 (Tocris Bioscience).

Biochemical Assays

Total RNA from cells or tissue was isolated using the PureLink RNA mini kit (Thermo Fisher); relative mRNA expression was quantified using standard qRT-PCR methods. For protein analysis, cells were lysed in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.6% w/v CHAPS, and protease/phosphatase inhibitors (Complete EDTA-Free and Phos-Stop, Roche Applied Science). Proteins were processed for Western blotting using standard methods. For immunoprecipitation, cell lysates were incubated with primary antibodies at 4°C overnight. Proteins were immuno-precipitated using protein G-agarose (Thermo Fisher) and further prepared for Western blotting. Some lysates were supplemented with the deubiquitinase inhibitor PR-619 (5 μM, LifeSensors) and the proteasomal inhibitor MG132 (10 μM, Selleck Chemicals), to prevent protein degradation and loss of ubiquitination.

In Vitro Studies

Twenty-four hours after any transfection procedures, GSCs were cultured in 96-well plates at an initial density of 5000 cells/well. Cell viability was quantified by the production of ATP using the Cell Titer-Glo reagent kit (Promega). GSC self-renewal was assessed by a limiting dilution assay[25]; the number and size of GSC-derived tumorspheres were quantified in each well 7 to 10 days after plating, using 12 to 16 replicates per dilution.

To measure cell invasion, transfected cells were allowed to form tumorspheres, and were subsequently embedded in Matrigel and cultured for 24–48 h. A migration index was calculated as the ratio of the area covered by the dispersed cells divided by the original area of the embedded spheroids. To measure invasion in a biologically-accurate model of the neural environment, GSC tumorspheres were labeled with calcein-AM (Sigma-Aldrich), seeded on brain slices freshly prepared from neonate mice, and cultured for up to 72 h following our established protocols.[26] The dispersion of the cells into brain tissue was imaged daily by fluorescence microscopy.

In Vivo Studies

All animal experiments were performed following institutional IACUC approval at SUNY Upstate Medical University. GSCs (2 μL containing 1.25 x 10^3 cells/μL) were implanted in the right-side striatum of athymic nude mice (FoxN1$^{nu/nu}$, 1:1 female:male ratio, Envigo). Animals were euthanized 15 days after tumor implantation and their brains were perfused and processed for cryosectioning. To quantify cell proliferation, BrdU (100 mg/kg in phosphate saline buffer) was injected intraperitoneally 6 hours before euthanasia. For overall survival assays, tumor-bearing mice were allowed to live until presenting neurological or physiological symptoms of excessive tumor burden, at which point they were euthanized.

Statistical Analyses

All experiments *in vitro* were repeated in triplicate with 3 independent replicates, except for cell invasion in brain slices (5–6 replicates) and self-renewal assays (12–16 replicates). Animal studies were performed in duplicate with $N = 5$/group for fixed-endpoint studies and $N = 12–14$/group for overall survival studies. Results are shown as means ± S.E.M. Multiple-comparison results were analyzed by one- or two-factor ANOVA with Tukey's multiple comparison test and correction for unequal variances if needed. Survival curves were compared by log-rank test. A value of $P < .05$ was taken to indicate statistically significant differences.

Results

DLG5 is Highly Expressed in High-Grade Glioma Compared to Other Solid Tumors

We first compared the expression of DLG5 in malignant gliomas –including GBM– against a spectrum of solid and liquid cancers by performing a meta-analysis of 104 studies that contained "tumor versus normal" comparisons across 21 different cancer types (Oncomine datasets, listed in Supplementary Table 3). Strikingly, our results showed that glioma was the tumor type with the highest median DLG5 expression over its corresponding normal

tissue (Figure 1A). These results were confirmed by comparing TCGA datasets collected from the Broad Firehose repository (Supplementary Figure S2). We further queried the TCGA datasets for GBM and confirmed that DLG5 had the highest mRNA expression when compared to the other members of the DLG family (Figure 1B). We validated this result by comparing the expression of DLG family members in a set of patient-derived GSC cultures collected in our laboratory (Figure 1C). Next, we carried out the immunohistochemical analysis of DLG5 in tissue cores from 27 normal brain specimens and 230 low- and high-grade gliomas, observing a positive correlation between tumor grade and DLG5 expression (Figure 1D–E). In agreement, Western blotting results confirmed the upregulation of DLG5 protein in GBM tissue compared to age-matched normal brain tissue (Figure 1F). Although the expression level of DLG5 was not sufficient as a prognostic marker of GBM patient survival (Supplementary Figure S3), our results demonstrate a significant upregulation of this protein that suggests a functional role for DLG5 in GBM cells.

DLG5 Promotes GBM Cell Viability and Invasion

To assess the effects of DLG5 on the phenotype of GBM cells, we chose three GSC lines representing different molecular subtypes (characterized in Supplementary Table 4) with a range of endogenous DLG5 expression. DLG5 knockdown and overexpression were validated in these cells by qRT-PCR, Western blotting, and rescue experiments to demonstrate the specificity of RNA-interference sequences (Supplementary Figure S4). DLG5 knockdown significantly decreased the invasion of GSCs in Matrigel plugs (Figure 2A–B) and brain slices (Figure 2C–D), in culture periods ranging from 24 to 72 hours. Cell viability was unaffected at these early time points and was not a confounding factor for loss of motility (Figure 2E). However, longer-lasting cultures of DLG5-deficient GSCs showed significant loss of cell viability after 72 hours, suggesting that loss of DLG5 eventually accumulates deleterious effects in the tumor cell population. Overexpression of DLG5 cDNA did not have a visible effect on cell migration, but increased cell viability significantly after 72 hours of culture (Figure 2F).

DLG5 is Essential to Maintain the Self-Renewal Properties of GSCs

We next assessed the impact of DLG5 on self-renewal of GSCs and expression of stem cell-related genes. Knockdown of DLG5 reduced the tumorsphere-forming ability of GSCs, resulting in fewer (Figure 3A–B, Supplementary Figure S5A) and smaller (Figure 3C, Supplementary Figure S5B) tumorspheres. This effect was observed in both mesenchymal (GBM30) and proneural (GBM146, GBM08) GSCs, suggesting that DLG5 is required for subtype-independent mechanisms of self-renewal. When we combined control and DLG5-deficient GSCs labeled with different fluorescent markers we observed that DLG5-deficient cells were eventually lost from the tumorspheres, suggesting that they were unable to "compete" with the self-renewal of control tumor stem cells (Supplementary Figure S5C–G). In agreement with this loss of self-renewal ability, DLG5 knockdown resulted in decreased mRNA and protein expression for several major genes that regulate stemness status in GSCs, including *SOX2*, *OLIG2*, *NANOG*, and *OCT4/POU5F1* (Figure 3D–E, Supplementary Figure S5H). Overexpression of DLG5 resulted in the opposite effects, with a four-fold increase in the proportion of self-renewing tumor stem cells and significant upregulation of stemness-related genes (Figure 3F–G).

Downregulation of DLG5 Reduces GBM Progression

To confirm the role of DLG5 in GBM *in vivo*, we evaluated the progression of control or DLG5-deficient, GSC-derived, orthotopic tumors in nude mice. Experiments terminated at a fixed endpoint revealed significantly reduced tumor cell proliferation (Figure 4A–B) and smaller tumor size (Figure 4C–D) in animals implanted with DLG5-deficient compared to control GSCs. Accordingly, animals carrying DLG5-deficient tumors showed a modest but significantly extended survival over their controls (Figure 4E).

Downregulation of DLG5 Inhibits Shh Signaling in GBM

GSCs repurpose early developmental signaling pathways to maintain their continuous self-renewal and propagation.[27] Shh signaling is a key mechanism of cell determination during early neural development, which is reactivated in GBMs and promotes GSC self-renewal and tumor invasion. Indeed, inhibition of Shh in culture, using the Smo inhibitor vismodegib or the Gli antagonist GANT-61, was sufficient to decrease the expression of stemness-related genes as well as GSC viability and self-renewal (Supplementary Figure S6). Because DLG5 has been proposed to regulate the traffic of the Shh receptor Smo, we hypothesized that alteration of DLG5 would have an inordinate effect on this signaling pathway in GSCs.

Knockdown of DLG5 decreased the expression of the key Shh components, Smo and Gli1 (Figure 5A–B, Supplementary Figure S7A), as well as Gli1-regulated downstream genes such as *PTCH1*, *cMYC*, and *CD44* (Figure 5A). In agreement, DLG5-deficient intracranial tumor xenografts had reduced expression of human *GLI1* (Shh), *OLIG2* (stemness), and *CD44* (mesenchymal marker) (Figure 4F–H). In addition, Gli1 immunostaining in those tumors showed qualitatively lesser intensity and co-localization with the cell nuclei—the latter being indicative of Gli activity,[28]—suggesting not only a decrease but also inactivation of Gli1 after DLG5 knockdown. DLG5 overexpression in cultured GSCs had the opposite effect, resulting in upregulation of Smo and Gli1 (Figure 5B). Indeed, analysis of gene expression in a collection of GSC cultures confirmed a positive correlation between DLG5 expression and expression of Shh components in GBM cells (Supplementary Figure 7B).

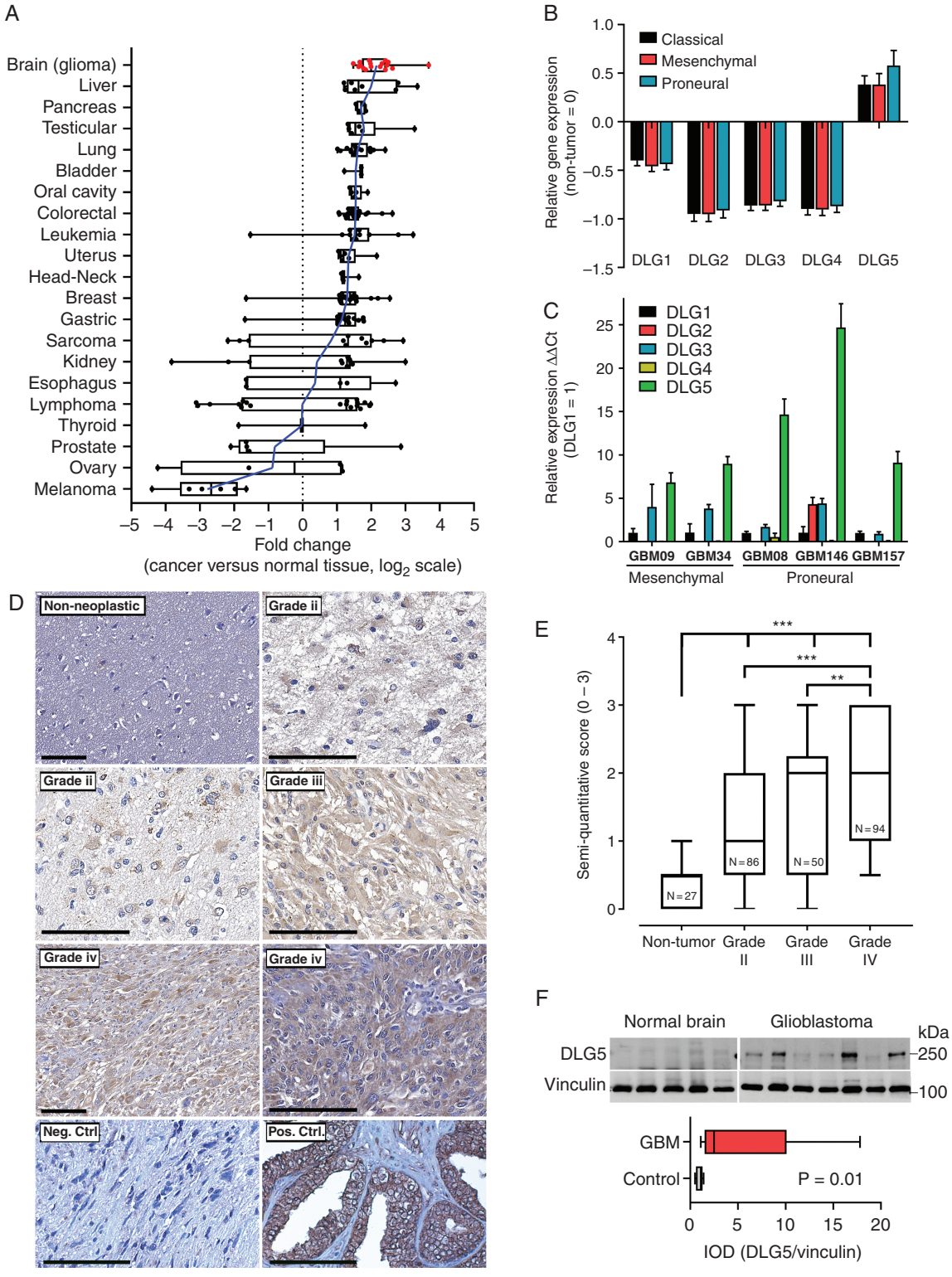

Fig. 1 Expression of DLG5 in malignant gliomas and correlation with glioma grade. (A) Relative expression (fold change) of DLG5 mRNA in solid and liquid cancers compared to their normal tissues. Each dot represents an independent "tumor versus normal" comparative study in which DLG5 expression was significantly different in the tumor samples compared to their controls (Oncomine Research database). (B) RNAseq data queried from the TCGA GBM 2016 dataset, showing comparative expression of DLG family members in GBM subtypes versus normal brain tissue. (C) mRNA expression of DLG family members quantified by qRT-PCR in primary cultures of GSCs (ΔΔCt for DLG1 = 1 for each GSC). *DLG2*

More importantly, DLG5 knockdown completely prevented the upregulation of Gli1 in response to the cognate Shh ligand added to the cultures (Figure 5C). Accordingly, the enhancing effect of recombinant Shh on tumor cell proliferation and invasion was abolished in DLG5-deficient cells (Figure 5D–E). Together, these results suggest that not only the loss of DLG5 diminishes the endogenous activation of the Shh pathway but also makes GSCs insensitive to exogenous Shh, revealing DLG5 as a critical factor sustaining Shh signaling that supports GBM progression.

Downregulation of DLG5 Increases Gli1 Degradation in GSCs

We finally investigated the mechanism by which DLG5 could regulate Shh signaling in GBM cells. DLG5 knockdown decreased the expression of several proteins involved in molecular scaffolding and cell polarity (Supplementary Figure S8), such as DLG1, the PARD3/PARD6 components of the *Par* complex, and atypical PKC that is known to activate Gli.[29] However, in our cultured cells we were unable to detect a direct association of DLG5 with these polarity proteins, or with the components of the *Scribble* complex (SCRIB and LLGL1), which could have suggested a direct involvement of DLG5 on cell polarity functions. We were also unable to detect a direct association of DLG5 with Smo (reported in embryonic fibroblasts[20]) or Gli1, all of which suggested an indirect regulation of DLG5 over Shh signaling.

DLG5 has been reported to carry ubiquitin-ligases that mark proteins for proteasomal degradation, such as β-TRCP or cullins,[30,31] which are also known to interact with Gli family members.[32,33] We, therefore, hypothesized that loss of DLG5 affected the expression and/or localization of these ubiquitin-ligases, leading to increased Gli1 degradation. In agreement with this hypothesis, DLG5 downregulation increased the expression of the ubiquitin ligase cullin-3 (Cul3) and total protein ubiquitination in cell lysates (Figure 6A–B). More generally, DLG5 correlated negatively with Cul3 and other ubiquitin ligases in GSCs (Supplementary Figure S7). Moreover, the downregulation of Gli1 seen after DLG5 knockdown was prevented by the proteasomal inhibitor, MG132 (Figure 6C), suggesting that loss of DLG5 promotes the degradation of Gli1 in the tumor cells. To further test this hypothesis we co-transfected FLAG-tagged Gli1, HA-tagged ubiquitin, and control or DLG5 siRNAs in U251MG cells, which allowed us to monitor ubiquitination after DLG5 knockdown (by detection of HA) and to recover newly-expressed Gli1 by immunoprecipitation with anti-FLAG antibody (Figure 6D). As seen in GSCs, DLG5 knockdown also increased the total expression of Cul3 in U251MGs. Moreover, a Gli1 complex co-precipitated with Cul3 and had increased ubiquitination in DLG5-deficient cells. Taken together, these results strongly suggest that loss of DLG5 results in loss of regulatory control over certain ubiquitin ligases, resulting in increased ubiquitination of a Gli1-containing complex. This leads to increased Gli1 degradation and therefore dominant downregulation of Shh signaling in GBM cells.

Discussion

One of the hallmarks of GBM is its highly invasive behavior, which contributes to its poor prognosis and lethality. Tumor invasion has been typically associated with the loss or redistribution of scaffolding proteins that separate subcellular domains and regulate cell polarity, leading to cell reorganization in the direction of motility.[34] However, several studies have shown that progression of solid tumors, including GBM, can also proceed with the collective invasion of cells that retain their cell-cell junctions and multicellular organization,[10,35] suggesting that scaffolding proteins may alternatively gain functions in cancer cells that are not related to the maintenance of cell polarity. In the present study, we have described the unexpected upregulation of the scaffolding protein DLG5 in gliomas and demonstrated that this protein has novel functions that depart from its reported role in normal tissues, becoming a regulator of tumor stem cell properties and a necessary factor for GBM progression.

The Shh signaling pathway is a key mechanism of neural patterning during development, which persists in the adult CNS to regulate the identity and fate of adult neural stem cells.[36] This pathway is also essential for the maintenance of the tumor stem cell population in cancers, including GBM.[37,38] Indeed, Shh prevents GSC differentiation,[39] promotes GSC migration,[23] and contributes to chemo- and radio-resistance of GBM.[40,41] Accordingly, Shh signaling has been highlighted as a high-relevance target in GBM patients,[42] ripe for combination therapies. Our discovery of DLG5 as a higher-order regulator of Shh in GSCs provides unique insight into the mechanisms that control this pathway in GBM and new opportunities to disrupt it.

The proteins of the DLG family are downregulated in multiple types of cancer and therefore regarded as tumor suppressors.[15,16] In agreement, a short report described the anti-mitotic effect of DLG3 overexpressed in GBM cells,[43] supporting the anti-tumor role of DLG family members even in brain cancer. Moreover, a recent report detected the expression of DLG5 in two GBM cell lines and proposed that this protein could reduce GBM progression by upregulating Hippo signaling,[44] even though DLG5 suppresses this pathway in normal cells.[45] It is worth noting

and *DLG4* mRNAs were undetectable in most cultures. (D) Immunohistochemistry of tissue microarrays; weak cytoplasmic staining of DLG5 in normal brain was only found in neurons. *pos. ctrl:* positive control (prostate tissue); *neg. ctrl.*: negative control (GBM tissue without primary antibody). Scale bars in all images: 100 μm. (E) DLG5 expression versus glioma grade (** $P < .01$, *** $P < .001$ by Kruskal-Wallis test). (F) Western blotting of DLG5 in total homogenates from human normal brain and GBM tissues (median age: 58–62 years) Equal protein load was controlled by total protein measurement and optical density of the cytoskeletal protein vinculin (*IOD*: integrated optical density for blot bands); $P = .01$ by Student's *t*-test.

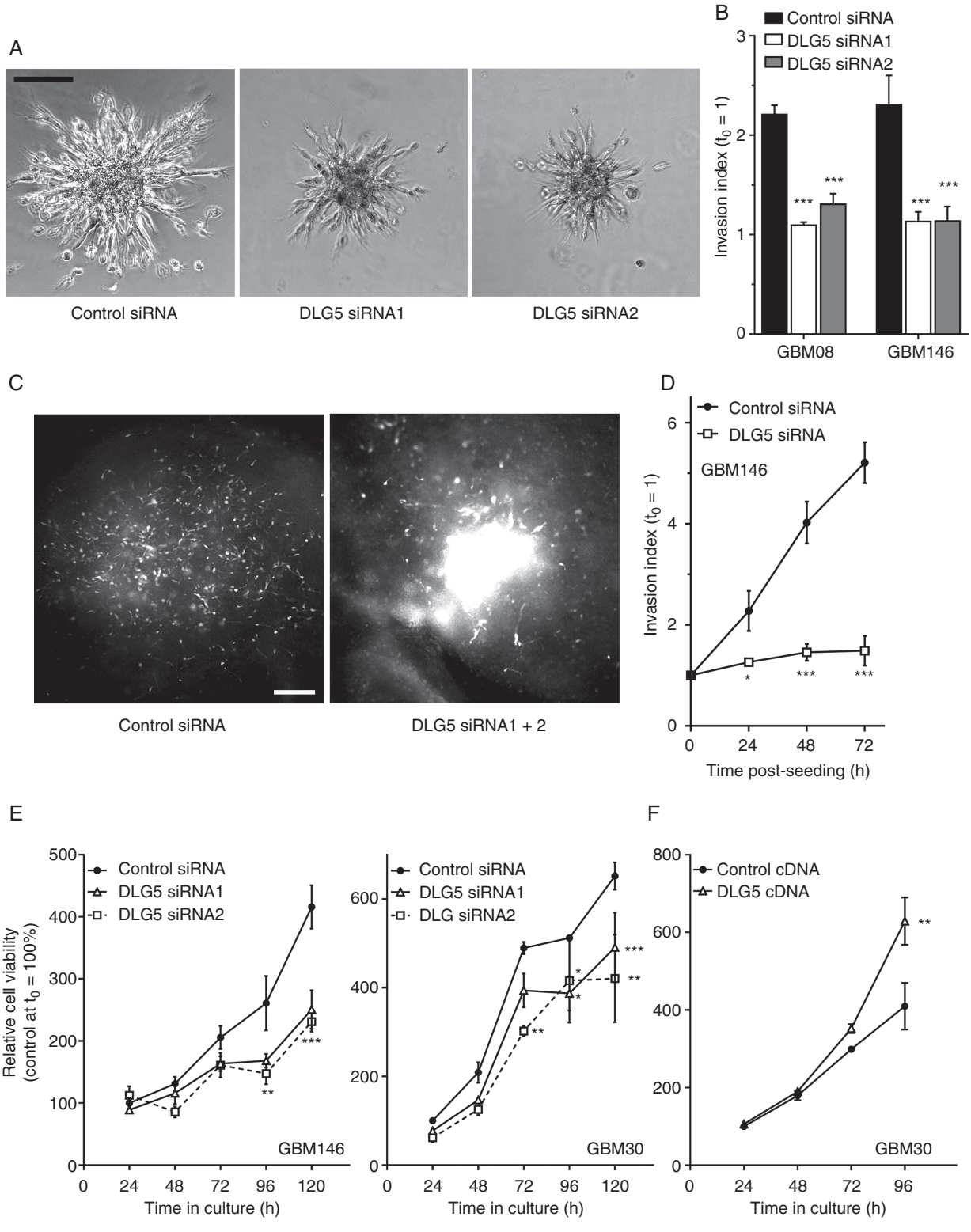

Fig. 2 DLG5 regulates GBM cell invasiveness and viability. (A–B) Representative images (A, GBM146 cells) and quantification (B) of GSC invasion through Matrigel after 48 h. (***$P < .01$ by one-way ANOVA for each cell line). (C–D) Representative image and quantification of GSC dispersion in an organotypic brain slice culture. Cells (GBM146) were transfected with control or combined DLG5 siRNAs (*$P < .05$, ***$P < .001$ by two-way ANOVA for repeated measures). E-F) Cell viability curves of GSCs after DLG5 knockdown or overexpression. (*$P < .05$, **$P < .01$, ***$P < .001$ by two-way ANOVA for repeated measures).

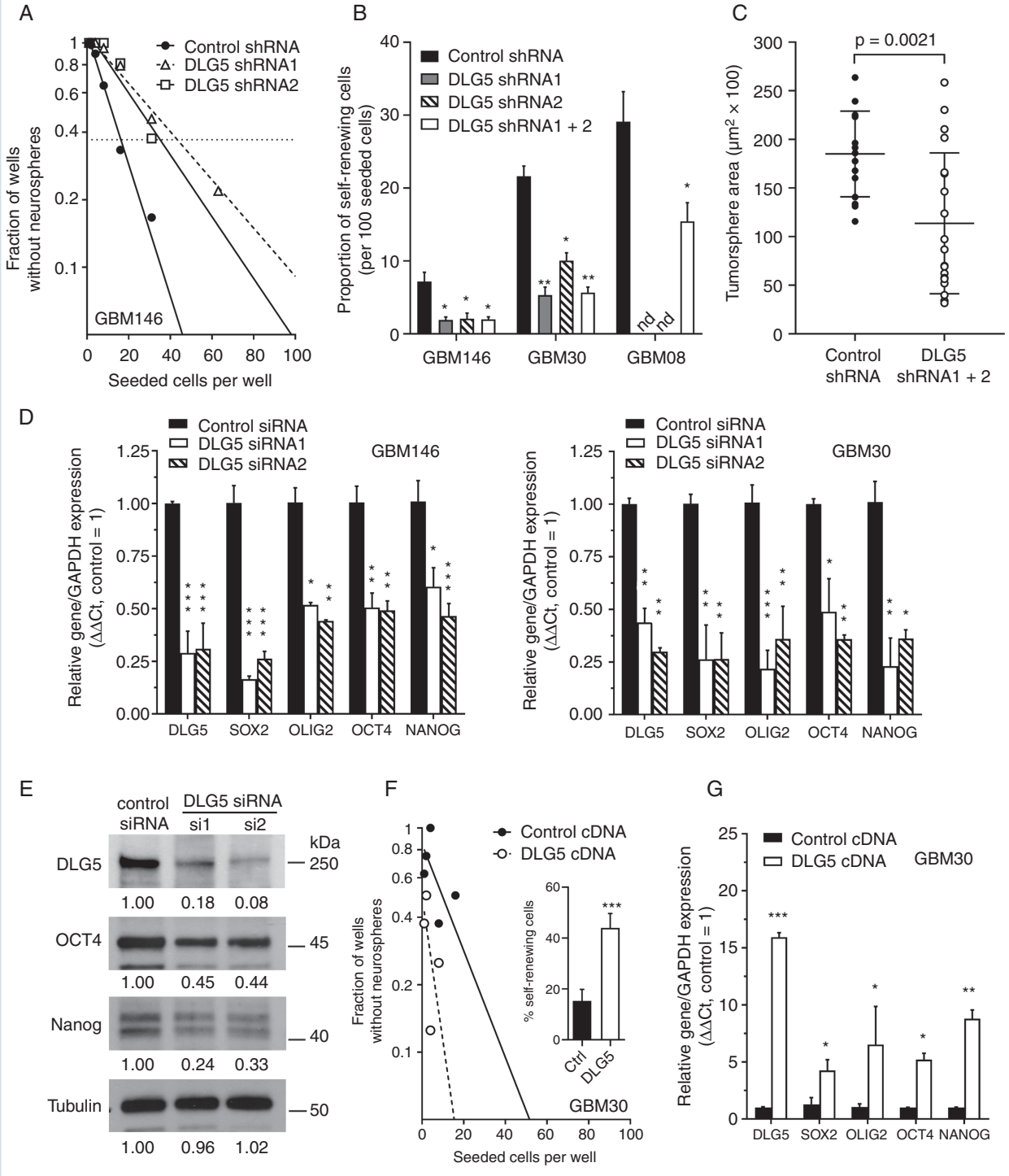

Fig. 3 DLG5 regulates GSC self-renewal and stemness-related gene expression. (A) Limiting-dilution curves of GBM146 cells transduced with control or two independent DLG5 shRNAs. (B) Proportion of self-renewing cells in three GSC lines transfected with independent or combined DLG5 shRNAs (*$P < .05$; **$P < .01$ by one-way ANOVA for each cell line); *nd:* not determined. (C) Quantification of transversal area for tumorspheres (GBM30 cells) formed after one-week incubation in the limiting-dilution assay (seeding: 30 cells/well; $N = 8$/group); $P = .0021$ by Student's *t*-test. (D) mRNA expression of stemness-related genes (*SOX2, OLIG2, NANOG*, and *OCT4*) after transient DLG5 knockdown in GSCs; * $P < .05$, ** $P < .01$, *** $P < .001$ by two-way ANOVA for repeated measures. (E) Western blot of stemness-related proteins after DLG5 knockdown; the numbers indicate relative optical density for all the protein bands, normalized to tubulin control. (F) Limiting-dilution curves and proportion of self-renewing GBM30 cells (*inset*) stably transduced with a control vector or DLG5 cDNA. G) mRNA expression of stemness-related genes in the same cells from panel (F); * $P < .05$, ** $P < .01$, *** $P < .001$ by two-way ANOVA.

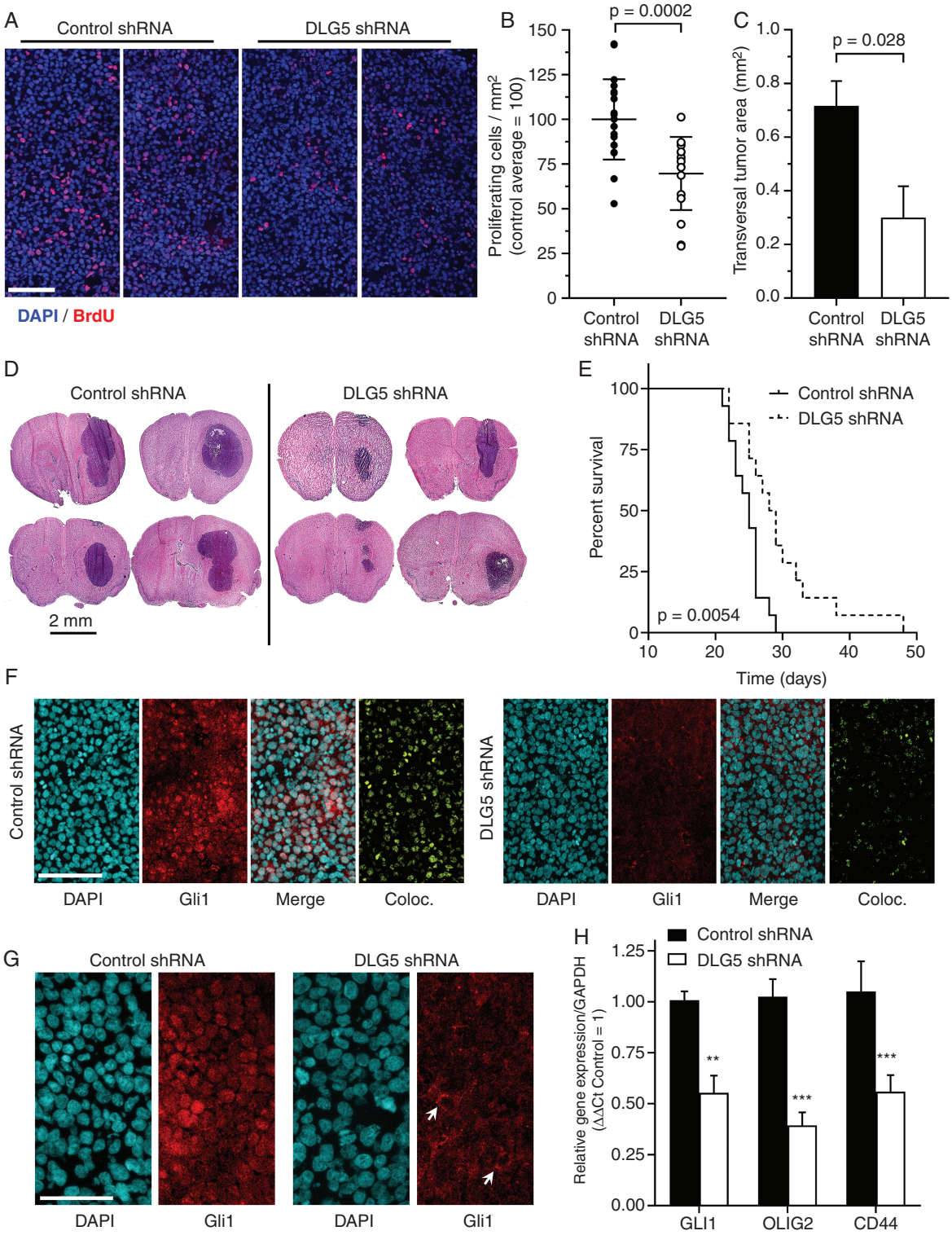

Fig. 4 DLG5 deficiency reduces GBM progression in vivo. (A–B) Representative immunohistochemistry (A) and quantification (B) of BrdU-positive cells in control and DLG5-deficient intracranial tumors (GBM30 cells) processed two weeks after implantation. Tissue sections were counterstained with the nuclear dye DAPI ($N = 5$ independent tumors/group, 3–5 tissue sections/tumor; $P = .0002$ by Student's t-test). (C–D) Cross-sectional area (C) and representative images (D) of H&E stained intracranial tumors ($N = 5$ independent tumors/group). E) Overall survival curves for mice carrying control and DLG5-deficient intracranial tumors ($N = 14$/group; $P = .0054$ by log-rank test). (F) Immunohistochemistry for Gli1 in control and DLG5-deficient tumors; tumors were counterstained with DAPI. *coloc:* feature-extraction processing from Gli1/DAPI merged images, showing nuclear co-localization of Gli1. G) Higher magnification detail of images in (F), showing nuclear localization of Gli1 (control) and cells devoid of Gli1 in the cell nuclei (DLG5 shRNA, arrows). H) mRNA expression of human genes (*GLI1*, *OLIG2*, *CD44*) in control and DLG5-deficient xenografts (**$P < .01$, ***$P < .001$ by two-way ANOVA). Scale bars: (A) and (F), 100 μm; (G): 50 μm.

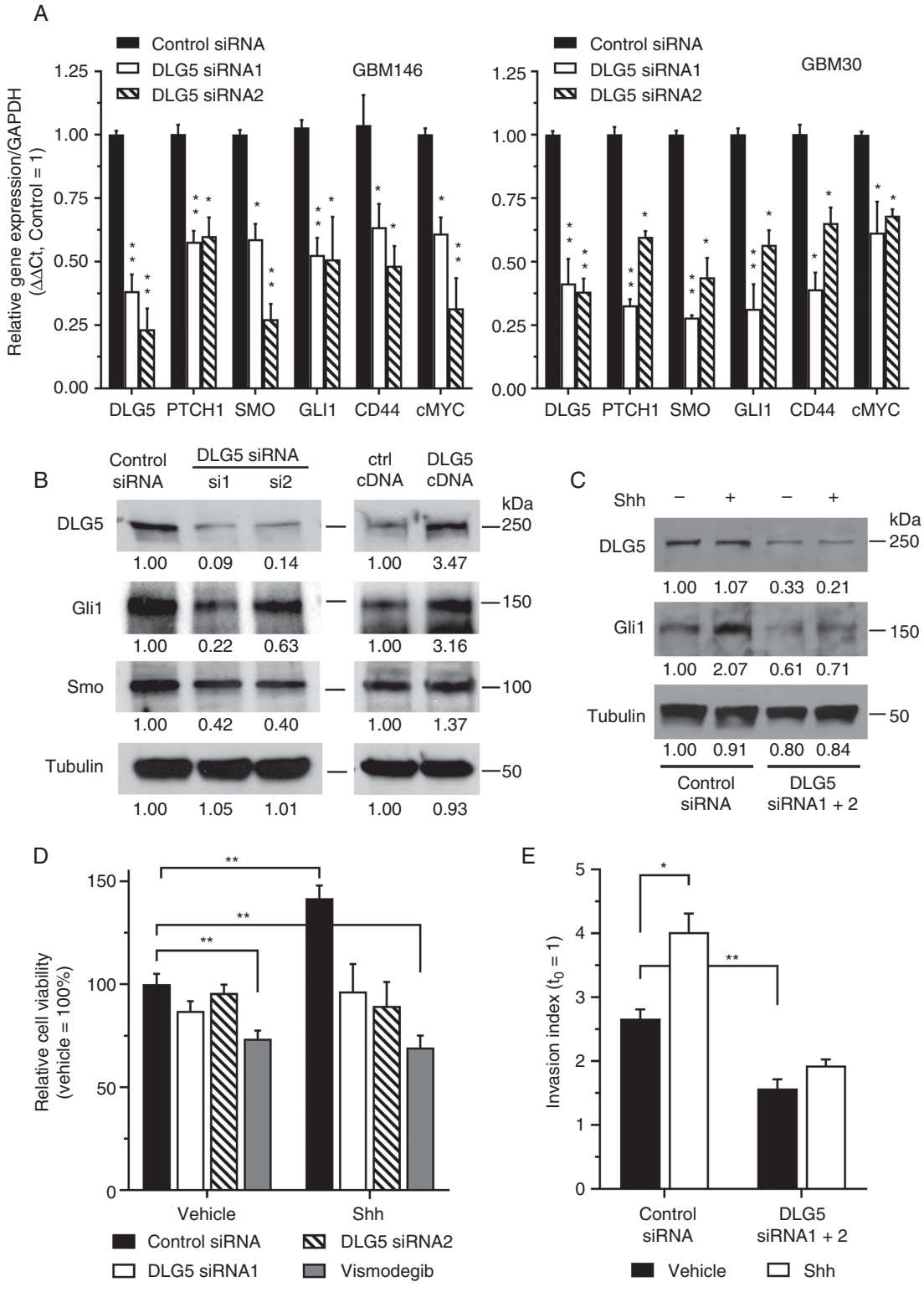

Fig. 5 DLG5 regulates Shh signaling. (A) mRNA expression of Shh-pathway components and Shh-downstream genes in control and DLG5-deficient GSCs (*$P < .05$, **$P < .01$, ***$P < .001$ by two-way ANOVA for repeated measures). (B) Western blot of Shh components after DLG5 knockdown or overexpression. (C) Effect of purified recombinant Shh (1 μg/mL, overnight) on Gli1 expression in GBM146 cells transfected with control or DLG5 siRNAs. The numbers in (B) and (C) indicate relative optical density for all the protein bands, normalized to tubulin control. (D–E) Effect of added Shh (1 μg/mL) on GSC viability (D) and Matrigel invasion (E); experiments were performed with GBM146 cells transfected with control or DLG5 siRNAs and cells were analyzed after 48h. Vismodegib (25 μM) was used to control for Shh specificity. * $P < .05$, ** $P < .01$ by two-way ANOVA.

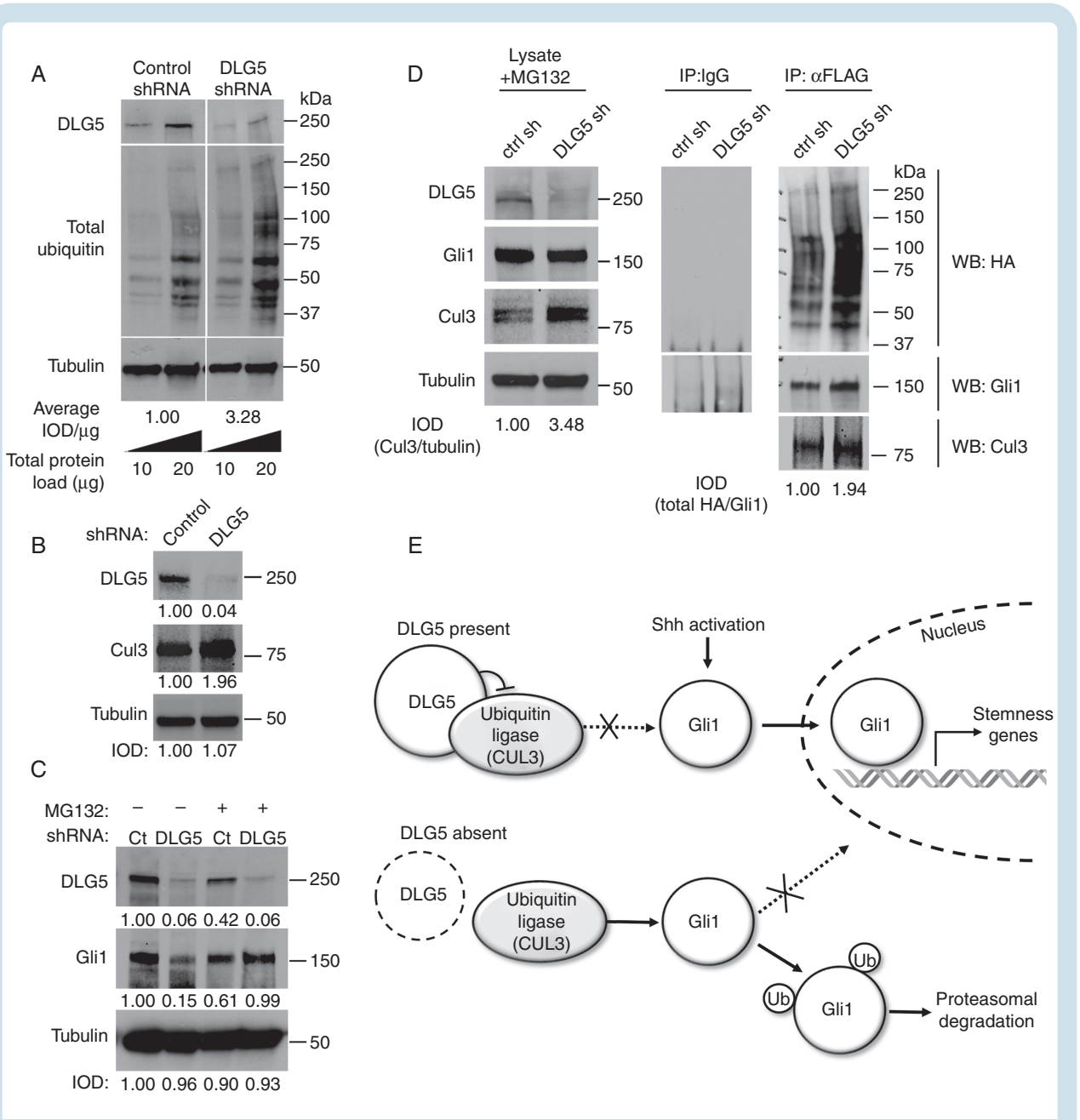

Fig. 6 DLG5 regulates protein ubiquitination and proteasomal degradation of Gli1. (A) Total protein ubiquitination, detected with anti-ubiquitin antibody, in control and DLG5-deficient GSCs (GBM08). *IOD:* integrated optical density for total ubiquitin/tubulin; results show representative images for two protein loads. (B) Expression of the E3 ubiquitin ligase cullin-3 (Cul3) in control and DLG5-deficient GSCs (GBM08). (C) Expression of Gli1 in GBM08 cells carrying control (*Ct*) or DLG5 shRNAs and treated for 24 hours with the proteasomal inhibitor MG132 (10 μM). Numbers in (B) and (C) indicate relative optical density for all the protein bands, normalized to tubulin control. (D) U251MG cells co-transfected with FLAG-Gli1, HA-ubiquitin, and DLG5 or control shRNAs were lysed 48 hours after transfection in the presence of PR-619 and MG132. Lysates were processed for immunoprecipitation (*IP*) with a control IgG (*IgG*) or anti-FLAG (*αFLAG*) antibody. Blots were probed to detect ubiquitin (HA-positive) co-precipitated with Gli1. *IOD:* optical density in cell lysates or IP samples, at equal protein loading per lane. (E) Working model of DLG5 as a regulator of Gli1 activity and turnover in GBM cells.

that those results were obtained with conventional GBM cell lines that have lost the properties of tumor stem cells and lack functional pathways for the maintenance of tumor stemness. Any effects of DLG5 in those cells should therefore be interpreted with caution because the maintenance of stemness seems to be the function most affected by DLG5, both in developmental models and, as we have shown here, in GBM.

Our study of DLG5 is the first to depart from the conventional view on DLG members as tumor suppressors, supporting a reinterpretation of the role of these proteins in nonepithelial cancers such as GBM.[46] For DLG5,

this reinterpretation is helped by the fact that the protein is an atypical member of the MAGUK family of polarity proteins and has been proposed as a member of an alternative family that has no relationship with cell polarity.[14] Moreover, our own results suggest that loss of DLG5 does not result in upregulation of other polarity complexes (Supplementary Figure S8) and this protein may not be a standard component of the *Scribble* complex, where it has yet to be detected. Interestingly, downregulation of DLG5 increased the expression of the *Scribble* component LLGL1 (Supplementary Figure S8), which promotes oligodendrocyte precursor differentiation,[47] thus supporting the role of DLG5 as necessary to maintain cell stemness and prevent differentiation. It should be noted that DLG5, and even Gli/Shh signaling, are necessary but likely not sufficient to maintain the GSC population, as suggested by the lack of prognostic association between DLG5 or Gli1 expression and overall patient survival (Supplementary Figure S3).

The scaffolding function of DLG5 in glioma cells could serve not only to direct the subcellular localization of protein complexes but also, depending on the cargo, to keep certain pathways active or restricted to specific subcellular domains. Based on our data showing that DLG5 associates with ubiquitin-ligases that can tag Gli1 for degradation we propose a working model (Figure 6E) in which DLG5 keeps active Shh/Gli signaling by "sequestering" ubiquitin-ligases such as Cul3 and restricting their distribution in the cell. Downregulation of DLG5 would disrupt this control and initiate a proteasome-dependent cascade that would turn off Shh signaling, ultimately damaging GSC self-renewal and survival. This reveals a dependence that could be inescapable even for tumor cells with activating mutations in Shh members. While these conclusions are preliminary, they are reinforced by the fact that proteasomal inhibition after DLG5 knockdown was sufficient to restore Gli1 expression even in absence of added Shh ligand (Figure 6C).

While DLG5 appears at first sight as an unlikely druggable target, it is worth noting that this scaffolding protein contains a number of PDZ domains that have high specificity to bind and carry different molecular partners, allowing the fine-tuning of DLG5's functions.[20] Accordingly, novel therapeutic approaches have been proposed for highly-selective targeting of PDZ domains,[48] which could be a viable approach towards disrupting DLG5 functions and targeting the persistence of tumor stem cells in GBM.

In sum, we report here for the first time a robust protumoral role for the scaffolding protein DLG5 in GBM, which is mediated by regulation of Shh activity to maintain a self-renewing, migratory, tumor stem cell population. Disruption of DLG5 and related cell polarity proteins may offer new avenues to prevent dispersion and recurrence of these malignant brain tumors.

Supplementary material

Supplemental material is available at *Neuro-Oncology* online.

Keywords

GLI signaling | glioma invasion | scaffold proteins | Shh signaling | tumor stem cells

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Authorship statement. SK and MSV designed the overall study and experimental design; SK, MSN, TER, and MSV designed and developed experimental methods; SK, MSN, SLL, JAL, SR, and TER performed experiments, data collection, and analysis; LSC, TER, and MSV provided resources and performed data curation; SK and MSV wrote the manuscript with input from all co-authors.

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