



Published in final edited form as:

Cancer Res. 2011 December 1; 71(23): 7280–7290. doi:10.1158/0008-5472.CAN-11-1531.

Dishevelled 2 signaling promotes self-renewal and tumorigenicity in human gliomas

Teodoro Pulvirenti^{1,4}, Maartje Van Der Heijden², Leif A. Droms², Jason T. Huse³, Viviane Tabar², and Alan Hall¹

¹Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065.

²Department of Neurosurgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065.

³Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065.

SUMMARY

Glioblastoma multiforme is the most common glioma variant in adults and is highly malignant. Tumors are thought to harbor a subpopulation of stem-like cancer cells, with the bulk resembling neural progenitor-like cells that are unable to fully differentiate. Although multiple pathways are known to be involved in glioma tumorigenesis, the role of Wnt signaling has been poorly described. Here we show that Dishevelled 2 (Dvl2), a key component of the Wnt signaling pathway, is overexpressed in human gliomas. RNAi-mediated depletion of Dvl2 blocked proliferation and promoted the differentiation of cultured human glioma cell lines and primary, patient-derived glioma cells. In addition, Dvl2 depletion inhibited tumor formation after intracranial injection of glioblastoma cells in immunodeficient mice. Inhibition of canonical Wnt/ β -catenin signaling also blocked proliferation, but unlike Dvl2 depletion, did not induce differentiation. Finally, Wnt5a, a non-canonical Wnt ligand, was also required for glioma cell proliferation. The data therefore suggest that both canonical and non-canonical Wnt signaling pathways downstream of Dvl2 cooperate to maintain the proliferative capacity of human glioblastomas.

Keywords

glioma; cancer stem cell; Wnt signaling; Dishevelled; differentiation

INTRODUCTION

Glioblastoma multiforme (GBM) is a lethal brain tumor, with most patients dying within one year of diagnosis (1, 2). The last decade has witnessed very little advance in treatment, but the identification of stem-like cancer cells in brain tumors has provided new insights into the disease. Several groups have identified a sub-population of stem-like, cancer cells in brain tumors, using biomarker analysis and culturing techniques similar to those used to characterize normal neural stem cells. These cells demonstrate a significant tumor-initiating ability, are capable of self-renewal, and express neural stem cell markers, such as Nestin and

⁴Correspondence should be addressed to mailing address: 411 East 67th street, MSKCC, RRL537, New York, NY, 10021; phone: 212-639-3706, fax: 212-717-3604; pulviret@mskcc.org .

Conflict of Interest: The authors declare no financial conflict of interests.

CD133, but not markers of the differentiated neural lineage (3-7). Although these cells represent only a small fraction of the tumor bulk, their high self-renewal capacity is thought to sustain tumor growth. The identification of signaling pathways that maintain the proliferative capacity of these cells and/or regulate their decision to differentiate offers great potential for a better understanding of the disease at the molecular level.

For neural stem cells, the decision to divide or differentiate is strongly influenced by Wnt signaling, while other factors, such as EGF and FGF, stimulate their proliferation (8-10). Wnt ligands are capable of activating several distinct signal transduction pathways and promoting a large spectrum of cellular processes, such as proliferation, differentiation, polarity, adhesion and migration (11, 12). Wnt signaling pathways are usually categorized as canonical, or non-canonical. The former describes the classical β -catenin/gene transcription pathway, while the latter is most often linked with polarity establishment and cytoskeleton-mediated processes, but can also involve gene transcriptional effects (though not through β -catenin). The scaffold protein Dishevelled (Dvl) plays an essential role in all known Wnt signaling pathways. Activation of canonical Wnt signaling recruits Dvl to the plasma membrane, resulting in the disassembly of the β -catenin destruction complex and leading to the translocation of active β -catenin to the nucleus, with subsequent activation of gene expression. Activation of non-canonical Wnt pathways leads to Dvl-mediated activation of Rho GTPases, planar cell polarity proteins, and Ca^{2+} -dependent signals (13-17). In order to discriminate between canonical or non-canonical pathways, Wnt signaling utilizes different domains of Dvl to activate distinct downstream components. In particular, the DIX domain is responsible for binding to Axin and activation of the canonical/ β -catenin pathway, while the DEP domain functions only in the non-canonical Wnt pathway and is responsible for activation of the small GTPases, Rho and Rac (18).

Mutations in the genes encoding components of Wnt signaling pathways have been found in many human cancers, notably colorectal cancer (13, 19). Mutations in axin, β -catenin and APC are found in sporadic medulloblastomas along with nuclear localization of β -catenin, suggesting constitutive Wnt signaling (20-22). Wnt signaling through the canonical β -catenin pathway has also been reported to increase the stem-like behavior of astrocytes and glioma cell lines, while downregulation of canonical Wnt/ β -catenin pathway induces apoptosis in glioma cell lines (23, 24). High expression levels of Dvl2 have so far been observed in both lung and colon cancer. Dvl overexpression has been shown to be critical for Wnt signaling in non-small cell lung cancer (25, 26). Dvl has also been implicated, together with mTOR, in the progression of colorectal neoplasia (27).

In this study, we examine the role of Dvl in human gliomas. A tissue microarray analysis revealed Dvl2 overexpression in more than 70% of the analyzed GBM samples. Dvl2 depletion was found to block the proliferation of human gliomas and promote their differentiation both *in vitro* and *in vivo*. Finally, both canonical and non-canonical signaling pathways are required to maintain the proliferative capacity of glioma cells.

MATERIALS AND METHODS

Animal experiments

Cells were infected with viral vectors and selected in puromycin as described below. 6-8 weeks old NOD/SCID female mice were stereotactically injected with 2×10^5 cells into the striatum under a Memorial Sloan-Kettering Animal Care Committee-approved protocol. The animals were imaged using Magnetic Resonance Imaging (MRI) at MSKCC animal imaging core facility. Mice were maintained until development of neurologic signs and then sacrificed and perfusion-fixed with 4% para-formaldehyde (PFA). The brains were post-fixed for 24h in 4% PFA, incubated in 30% sucrose and then snap frozen in Optimal Cutting

Temperature (OCT) compound and cut on a cryostat (coronal sections, 25 μ M). Standard protocols were used for Hematoxylin-Eosin staining. Immunofluorescence staining was performed as described in the section “Immunofluorescence staining”.

Tissue microarray and tumor samples

For analysis of Dvl2 levels by immunohistochemistry, a tissue microarray (TMA) containing glioblastoma and normal brain samples was used (US Biomax, GL806). The TMA contains 35 cases of glioblastoma and 5 normal brain tissues, represented as duplicate cores per case. The immunohistochemical detection of Dvl2 was performed at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using Discovery XT processor (Ventana Medical Systems). Tissue sections were blocked for 30 minutes in 10% normal goat serum in 0.2% BSA/PBS, followed by incubation for 5h with 10 μ g/ml of the primary antibody (rabbit polyclonal anti-Dvl-2; Chemicon cat# ab5972 lot# j61682917) and incubation for 60 min with biotinylated goat anti-rabbit IgG (Vector labs, cat#:PK6101; 1:200 dilution). The detection was performed with DAB-MAP kit (Ventana Medical Systems). The glioblastoma samples for western blot analysis were collected from patients undergoing surgery at Memorial Sloan-Kettering Cancer Center, with consent. Normal tissue was obtained from a small cortical area removed during the surgical approach. The samples were snap frozen in liquid nitrogen, triturated using a plastic pestle and then collected in lysis buffer as described (see Protein analysis in Supplemental Material).

Cell lines and human GBMs

The human glioma cell lines U87, U138, U373 and U251 were cultured in MEM Gluta-Max (Gibco, 41090) supplemented with 10% FCS, 10mM Hepes, non-essential amino acids (Gibco, 11140) and antibiotics. LN229 were cultured in DME supplemented with 5% FCS and antibiotics. Primary glioblastoma samples were derived from patients undergoing surgery at Memorial Sloan-Kettering Cancer Center. Primary GBM samples were dissociated as described by Pollard and colleagues and grown as a monolayer on plastic cell culture dishes coated with 10ng/ml laminin (Sigma, L2020) (28). All patient-derived cells were cultured in NeuroCult NS-A basal medium, human (StemCell Technology, 05750), supplemented with NeuroCult NS-A proliferation supplements, human (StemCell Technology, 05754) and 10ng/ml human bFGF (Sigma F0291) and 20ng/ml human EGF (Preprotech AF-100-15) (In the main text we refer to this medium as neural stem cell medium for simplicity). For differentiation, primary GBM cells were plated on 10ng/ml laminin and the NeuroCult NS-A basal medium was replaced with MEM GlutaMax 5% FCS 48h before the infection.

Growth curve and neurosphere formation assay

Cells were infected with control or Dvl2 shRNAs and selected in 1 μ g/ml puromycin for 5d. 2×10^4 cells were plated per well in a 12-well plate and each sample was plated in triplicate. For glioma cell lines growing in 10% FCS, cell number was counted after 3d, 5d and 7d. Primary GBM cells were plated on laminin-coated dishes and grown as an adherent monolayer in serum-free neural stem cell medium. Cells were counted after 4d, 8d and 16d. For neurosphere formation, cells were grown and infected in an adherent monolayer in neural stem cell medium. After puromycin selection, cells were resuspended and grown in neural stem cell medium containing 0.7% methylcellulose (9). 5×10^3 cells were plated in each well of a 24-well plate in quadruplicate. The number of neurospheres with a diameter greater than 50 μ m was counted after 10d or 15d, for U87 or primary GBMs respectively.

Immunofluorescence analysis

Cells were plated on glass coverslips coated with laminin and infected with viral vectors as described above. After selection, cells were fixed in 4% formaldehyde, rinsed three times in PBS 1x and permeabilized in PBS containing 0.3% Triton and 10% goat serum. Samples were incubated overnight at 4°C with the primary antibody and 45min at room temperature with the secondary antibody and with Hoechst. Mouse brain cryo-sections were stained following the same procedure. For fluorescence imaging, images were taken using 10x or 20x objective lenses on a Zeiss Axio Imager.A1 microscope. The following antibodies were used: mouse anti-Nestin (Abcam ab22035, 1:100), mouse anti Tuj1 (Millipore MAB1637, 1:250), rabbit anti-GFAP (DAKO Z0334, 1:1000). For actin cytoskeleton detection Alexa 546-conjugated phalloidin was used (Invitrogen A22283, 1:100). Apoptosis was detected on fixed cells using the *In Situ* Cell Death detection kit (Roche, 11684795910), according to the manufacturer's instructions. Senescence was detected using the Senescence β -galactosidase kit (Cell Signaling, 9860S), following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2008. Student's t-test (2-tailed, unpaired) was used to determine the significance of results comparing cells infected with shControl and Dvl2 shRNA. Wilcoxon-Mann-Whitney Test was used to determine the significance of the *in vivo* tumor growth. *P<0.05, **P<0.01, ***P<0.001

RESULTS

Endogenous Dishevelled is expressed at high levels in human glioblastomas

Overexpression of Dvl has been shown to potentiate the activation of Wnt signaling pathways (29, 30). To examine the potential role of Wnt signaling in high-grade brain tumors, we first compared the expression levels of Dvl2 (the most widely expressed isoform of Dishevelled) in normal and cancer brain tissues using the Oncomine database. Analysis based on a set of data including 80 glioblastoma samples showed that the levels of Dvl2 mRNA were increased in brain cancer tissue compared to normal tissue (Figure 1A) (31). The Cancer Genome Atlas (TCGA) data set was also analyzed for Dvl2 expression in brain cancer but the results appeared not significant (P=1.000; not shown). The level of Dvl2 protein was next analyzed in a group of 10, freshly-derived GBM samples. As shown in Figure 1B, Dvl2 is overexpressed, though to different extents, in all patient samples, when compared to normal brain tissue. EGF receptor (EGFR) overexpression and p53 loss are commonly found in human GBMs, but no significant correlation was found between Dvl2 and either EGFR or p53 expression (Figure 1B) (2, 32, 33). We also analyzed the status of isocitrate dehydrogenase-1 (IDH1) gene in these freshly-derived samples (34). As shown in supplementary Fig.1, only one sample showed a mutation in IDH1, the mutation being the less common R132G.

To further investigate the expression levels of Dvl2 in high-grade gliomas, a tissue microarray (TMA) containing 35 samples from patients diagnosed with grade IV glioma and 5 control brain samples was examined using a Dvl2-specific antibody. Tumors were scored as negative (0), medium positive (1), or very positive (2). Dvl2 is overexpressed in more than 70% of the GBM samples, with around 20% of these showing very high levels (Figure 1C). We conclude that Dvl2 is overexpressed in a significant number of human GBM samples, raising the possibility that Wnt signaling plays an important role in these tumors.

Dishevelled depletion blocks proliferation and induces differentiation of U87 glioma cells

To explore the role of Wnt signaling in human GBM, lentiviral shRNA vectors targeting Dvl2, which is essential for all known Wnt signaling pathways, were obtained (14). The

glioma cell line U87, originally derived from a human glioblastoma, harbors mutations in *PTEN* and p16^{ink4a}, and is highly proliferative and tumorigenic both *in vivo* and *in vitro*. Two different lentiviral constructs, which efficiently deplete Dvl2 in these cells significantly inhibited their proliferation (Figure 2A,B). This was associated with an increase in the cell cycle inhibitor p21^{WAF} (Figure 2B), a decrease in BrdU incorporation (Figure 2C) and inhibition of anchorage independent growth (Supplemental Figure 2A,B). The proliferation block associated with Dvl2 depletion was not accompanied by apoptotic death or senescence (Supplemental Figure 3A,B).

Time-lapse video microscopy (Figure 2D and Supplemental Movies 1 and 2), revealed a dramatic change in cell morphology induced around 72h after Dvl2 depletion. Control cells (infected with non-targeting shRNA lentivirus) remain highly motile and undergo cell divisions as expected (Supplemental Movie 1), but Dvl2-depleted cells become flat and non-motile, and elaborate elongated processes reminiscent of a differentiated state (Figure 2D, black arrows and Supplemental Movie 2). Western blot and immunofluorescence analysis revealed that Dvl2 depletion leads to a decrease in Nestin (a marker for neural stem/progenitor cells), an increase in Tuj1 (a neuronal marker) and a modest increase in GFAP (an astrocytic marker) (Figure 2E,F and Supplemental Fig.4) consistent with the induction of a differentiation-like program.

Since differentiation of stem/progenitor cells is associated with the loss of self-renewal, the impact of Dvl2 depletion on neurosphere formation was examined. The formation of non-adherent neurospheres, in serum-free media supplemented with EGF and FGF, is a widely recognized property of neural stem cells and is commonly used as an *in vitro* surrogate for self-renewal or stem cell-like properties (35). U87 cells efficiently form neurospheres after 10d of culture in neural stem cell medium, but this is dramatically reduced after Dvl2 depletion (Figure 2G-H). These data indicate that Dvl2 is necessary for the proliferation of U87 glioma cells and that Wnt signaling pathways have a role in regulating the neurosphere forming ability of these cells.

To confirm the specificity of RNAi depletion, rescue experiments were performed using a mouse Dvl2 (mDvl2) construct that is resistant to the sh2 lentivirus. A partial rescue of the proliferation defect and associated downregulation of p21^{WAF} was observed (Figure 3A,C). In addition, a partial rescue of differentiation was observed, with increased Nestin expression and decreased Tuj1 expression (Figure 3A,B). Consistent with these findings, overexpressing of Dvl2 in U87 cells resulted in an increase in Nestin expression levels together with an increase in proliferation and neurosphere formation (Supplemental Figure 6A-C).

To explore the wider significance of Dvl2 in human glioblastoma, four additional cell lines, U373, U138, LN229 and U251, each carrying different genetic mutations were infected with an shRNA lentiviral vector (36). Dvl2 depletion inhibited the proliferation of all four lines in serum cultures and in anchorage independent growth assays (Supplemental Figure 7A,B), while p21^{WAF} protein levels were increased in 3 of the 4 lines (Supplemental Figure 7C).

Dishevelled depletion induces the differentiation of primary, patient-derived glioblastoma cultures

To determine whether Dvl2 plays an important role in primary human glioblastoma (GBM), freshly derived tumor samples were obtained from three individual patients (GBM1/GBM2/GBM3) (Supplemental Table 1, Supplemental Fig.5). Tumors were dissociated and grown as adherent cultures on laminin coated dishes (28). GBM cells were then infected with lentiviral shRNA vectors targeting Dvl2 or a control shRNA. Dvl2 depletion inhibited the proliferation of all three GBMs in serum cultures (Figure 4), as well as their ability to grow

as neurospheres in neural stem cell medium (Figure 5H). All three GBMs underwent significant morphological changes reminiscent of differentiation, together with down-regulation of Nestin and up-regulation of the glial marker GFAP (Figure 5A-G). A significant increase in p21^{WAF} and Tuj1 was also seen by western blot in 2 out of 3 samples (Figure 5G). We conclude that Dvl2-mediated signaling is required to maintain the self-renewal ability of both glioma cell lines and patient-derived GBM samples.

Dishevelled depletion inhibits glioblastoma tumorigenicity *in vivo*

To determine whether Dvl2 depletion is effective at suppressing *in vivo* tumorigenicity, intra-cranial injections of controls, or tumor cells originating from a patient tumor (GBM1), or cell lines (including a GFP-U87 line) expressing Dvl2 shRNA were performed in NOD/SCID mice (for a total of 8 control mice and 12 mice injected with Dvl2-depleted cells). Magnetic resonance imaging (MRI) performed 13 weeks after injection of cells derived from patient GBM1 revealed an extensive and invasive tumor mass (Figure 6A). However, GBM1 cells that had been infected with Dvl2 shRNA lentivirus 7d prior to intra-cranial injection did not produce tumors detectable by MRI or histology at this time (Figure 6A). In one out of 3 mice injected with Dvl2 depleted GBM1, a small lesion was detectable by MRI at 21 weeks after injection (not shown; the animal was left alive for 25 weeks). Overall, the absence of tumor mass was reflected in the survival curve, indicating that Dishevelled signaling is critical for glioma development *in vivo* (Figure 6F). To investigate the fate of Dvl2-depleted GBM1 cells *in vivo*, brain cryo-sections were obtained. Immunohistological studies showed a highly proliferative tumor mass in mice injected with control, but not Dvl2-depleted cells (Figure 6B). Similarly, using an antibody specific for human Nestin, rare human GBM1 cells were found at the Dvl2 depleted injection sites (Figure 6C). The small lesion found in one out of 12 mice injected with Dvl2-depleted cells, was positive for human Nestin (not shown).

To determine whether Dvl2-depleted cells die after injection, or adopt a differentiated phenotype and integrate into the mouse brain, a GFP-expressing U87 cell line (U87-GFP) was generated. Control cells (U87-GFP) generated a tumor 5 weeks after intra-cranial injection, while Dvl2-depleted U87-GFP cells induced no lesions detectable by MRI up to 20 weeks after injection (Figure 6G, Supplemental Figure 8A,B). Subsequent analysis of brain cryo-sections for GFAP (an astrocytic differentiation marker) and GFP, revealed cells that had infiltrated the mouse brain and were positive for both (Figure 6D,E, arrows). This result indicates that the injected Dvl2 depleted cells are still present in the mouse brain, and some adopt a differentiate-like state (Figure 6D,E).

Canonical and non-canonical Wnt signaling pathways cooperate in the regulation of glioblastoma cell proliferation

Although different Wnt ligands can activate distinct signal transduction pathways, Dishevelled is thought to be essential in all cases (14). To begin to explore the nature of Dvl2 signaling in glioblastoma, we used U87 cells. siRNA oligonucleotides were used to deplete β -catenin, an essential component of the canonical Wnt signaling pathway. Despite a relatively modest RNAi knockdown (Figure 7B), β -catenin depletion strongly inhibited the proliferation of U87 cells in culture (Figure 7A). However, it did not induce differentiation, as judged by morphology or protein markers (p21^{WAF}/Nestin/Tuj1) (Figure 7B; Supplemental Figure 9B). As an alternative approach to inhibit canonical Wnt signaling, a truncated (dominant negative) form of the transcription factor TCF-4 (DN-TCF), previously shown to block β -catenin mediated transcription, was used (37, 38). This also inhibited the proliferation of U87 cells (Figure 7A), but did not induce any differentiation markers (Figure 7B; Supplemental Figure 9C). To further investigate the role of canonical Wnt/ β -catenin signaling in the Dvl-dependent differentiation event, we used a non-

phosphorylatable/active form of β -catenin. If the block of canonical Wnt/ β -catenin signaling is responsible for the differentiation-like phenotype caused by Dvl2 depletion, an active form of β -catenin should be able to rescue this effect. As shown in Supplemental Figure 10, U87 cells stably expressing active β -catenin (β -catenin with serine to alanine substitutions at position 35, 37, 41 and 45, or β -catenin-S4A) were not able to rescue the proliferation block or the differentiation phenotype caused by Dvl2 depletion. We conclude that β -catenin-mediated, canonical Wnt signaling is required to maintain the proliferative capacity of human glioblastoma cells, as suggested by others (23). However, since loss of β -catenin/TCF-4 does not phenocopy the loss of Dvl2, and active β -catenin does not restore the normal proliferative behavior of these cells, we conclude that additional signals downstream of Dvl2 are also involved.

To investigate the possible involvement of non-canonical Wnt signaling, lentiviral constructs encoding shRNAs targeting the non-canonical Wnt ligand Wnt5a were used. Depletion of Wnt5a with two different shRNAs inhibited the proliferation of both U87 cells and primary cells derived from patient GBM1 (Figure 7C-F), but was not able to induce differentiation (Figure 7G). Furthermore, Wnt5a depleted cells become flat and show high levels of β -galactosidase, a known marker of cellular senescence (Figure 7H). Finally, depletion of Wnt5a dramatically inhibited the ability of primary GBM1 cells to form neurospheres in neural stem cell medium (Figure 7F). These results suggest a role for a non-canonical Wnt pathway in regulating glioma cell proliferation.

DISCUSSION

To explore the role of Wnt signaling in human glioblastoma, Dvl2, a key downstream component of all known Wnt signaling pathways, was depleted using RNAi. Dvl2 depletion not only inhibited the proliferation, but also promoted the differentiation of human glioma cell lines as well as freshly-derived patient glioblastoma samples. Dvl2 depletion inhibited neurosphere formation, an assay for the cancer stem-like subpopulation, as well as tumorigenicity, after intracranial injections in the mouse. Surprisingly, however, inhibition of the canonical Wnt signaling pathway, by β -catenin depletion, or expression of a dominant negative version of the TCF transcription factor, blocked proliferation, but did not induce differentiation. We have gone on to show that a non-canonical Wnt signaling pathway, involving the ligand Wnt5a, is also required to maintain the proliferative capacity of human glioblastoma cells. We conclude that a combination of canonical and non-canonical Wnt signaling is required to maintain glioblastoma proliferation.

Human glioblastomas are associated with a variety of genetic changes, including mutations in PTEN, EGFR, PDGFR, p53 and p16^{INK4a}, and their contribution to disease development has been explored experimentally using mouse models (2, 39). Overexpression of PDGFR in neural progenitors, for example, induces the formation of oligodendrogliomas (40), while inactivation of both p53 and PTEN prevents neural stem/progenitor cell differentiation, in part at least through upregulation of c-myc, and leads to the generation of malignant glioma (41). Other major signaling pathways involved in the regulation of neural stem cells have been reported to be altered in gliomas, including Notch and Hedgehog (42). In fact, mutations in the Hedgehog signaling pathway have been found in another brain cancer, medulloblastoma, and inhibition of this pathway blocks the proliferation and self-renewal of stem-like glioma cells (43). Canonical Wnt signaling is a major player in maintaining the self-renewal capacity of neural stem cells and has been reported to be upregulated in brain cancers, including glioma (10, 44, 45). A recent report identified PLAGL2 as a glioma oncogene and showed that it exerts its effects by promoting canonical Wnt signaling to suppress differentiation and promote self-renewal of neural stem/progenitor cells (23).

Here we show for the first time that Dvl2 is overexpressed in high-grade gliomas, suggesting a role for active Wnt signaling in regulating the biology of these tumors. Despite the different amplification levels of EGFR, or p53 status, in the various cell lines and GBM cells used in this study, Dvl2 depletion induces a block of proliferation and a morphological change that has the characteristics of a differentiation-like phenotype. The fact that this phenotype was not observed upon a block of Wnt/ β -catenin pathway, suggests that more than just the well-characterized canonical Wnt signaling is involved downstream to Dvl in this process.

Mutations in components of non-canonical Wnt signaling have not been reported so far in human gliomas, though expression of the non-canonical Wnt ligand, Wnt5a, has been reported to increase with glioma grade (24, 46). We report here that depletion of Wnt5a blocks proliferation and induces senescence of glioma cells, showing that a non-canonical Wnt signaling pathway is required to maintain the proliferative capacity of glioma cells. Therefore, we demonstrate that both canonical and non-canonical Wnt signaling pathways maintain the proliferative capacity of human glioblastoma cells. Since loss of Dvl2 not only inhibits proliferation, but also promotes a differentiation-like program, we conclude that inhibition of both Wnt signaling pathways might be required to initiate differentiation, though it is possible that other signaling pathways downstream to Dvl2 are also involved. The identification of Wnt5a as an essential player in maintaining the proliferative capacity of glioma cells may provide new therapeutic opportunities for treating this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jeremy Rich, Dr. Angelo Vescovi, Dr. Tania Maffucci and members of the Hall laboratory for many constructive discussions. We thank Dr. E. R. Fearon for the DN-TCF4 construct. We thank the High-Throughput Screening core facility at MSKCC for providing the shRNAs clones. We thank Dr. K. Manova-Todorova and Dr. A. Baldys at the Molecular Cytology Core Facility at MSKCC (Support Grant NCI P30-CA008748) for the TMA staining. We thank the Animal Imaging core facility at MSKCC for the Magnetic Resonance Imaging. This study is dedicated to Agata Annino, a dear colleague who died of brain cancer at the age of 32.

GRANT SUPPORT The work was supported by a National Institutes of Health (NIH) grant GM081435 to A. H. and a National Cancer Institute core center grant (P30-CA 08748). T.P. was supported in part by an American Italian Cancer Foundation Fellowship and an MSKCC Brain Tumor Center award.

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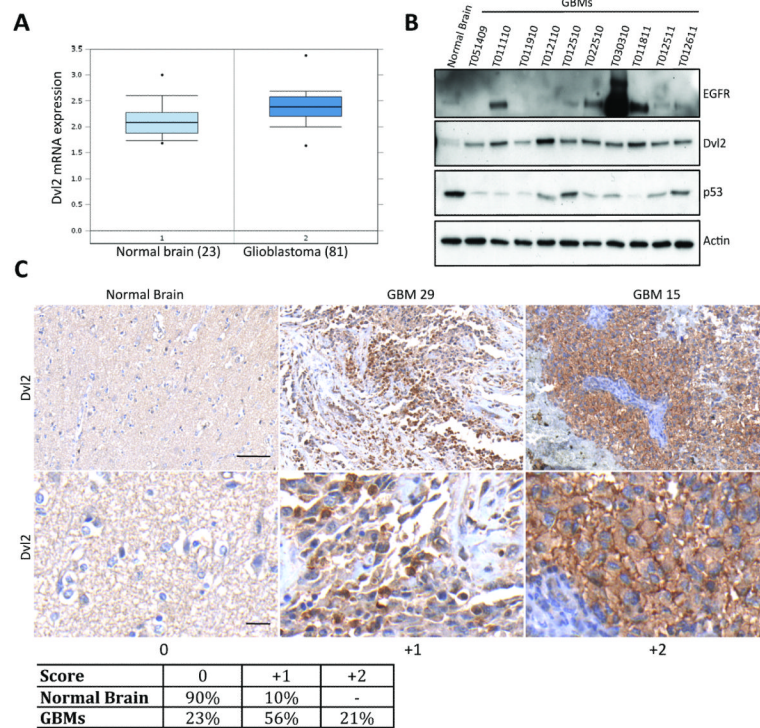


Figure 1. Dvl2 is overexpressed in human glioblastomas

(A) Oncomine microarray data analysis for Dvl2 expression in glioblastoma (GBM) vs. normal brain tissue is shown. The Student t-test was conducted using the Oncomine software; $P=0.001$. The boxes represent the 25th through 75th percentiles; the horizontal lines represent the medians; the points represent the end of the ranges. (B) Expression of Dvl2 in normal brain and 11 fresh derived GBM samples, analyzed by western blot. The levels of expression of EGFR and p53 were also analyzed. Normal tissue was obtained from a small cortical area removed during the surgical approach. (C) Immunohistochemistry of Dvl2 on tissue microarray containing 35 GBM samples and 5 normal brain samples. Scores (0), (1) and (2) represent negative, medium positive and highly positive staining respectively. Top panel shows representative staining of normal brain and two GBM cases, scale bar 100 μ m; bottom panel is a magnification of the top panel, scale bar 20 μ m.

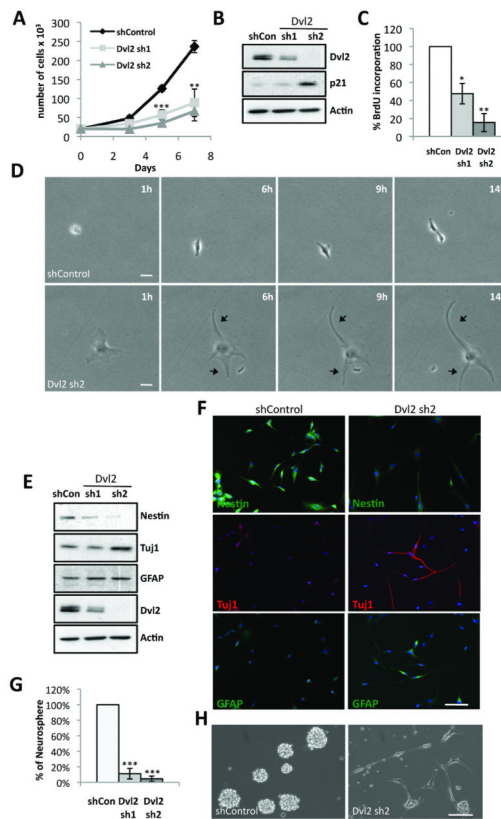


Figure 2. Dvl2 depletion blocks proliferation and induces differentiation of U87 glioma cells
 U87 cells were infected with a lentivirus containing a non-targeting shRNA (shControl), or two different shRNAs against Dvl2 (sh1 and sh2). The cells were selected in puromycin for 5d. (A) U87 growth following Dvl2 depletion 3d, 5d and 7d after plating in medium containing 10% fetal calf serum (FCS). (B) Protein levels of Dvl2 and the cyclin/Cdk inhibitor p21^{WAF} determined by western blot 7d after lentiviral infection. (C) BrdU incorporation in control and Dvl2-depleted cells in medium containing 10% FCS. (D) Individual frames from a time-lapse sequence (Supplementary Movies 1 and 2) showing the morphological changes, indicated by the arrows, in U87 cells after Dvl2 depletion; control cell (upper panel), Dvl2 depleted cell (lower panel). Scale bar is 20 μ m. (E and F) Decrease in the neural stem/progenitor cell marker Nestin and increase in the differentiation markers Tuj1 and GFAP visualized by western blot and fluorescence microscopy 7d after infection with Dvl2 shRNA lentivirus. (G) Inhibition of neurosphere formation after Dvl2 depletion. 7d after lentiviral infection, cells were plated and grown for 10d in serum-free neural stem cell medium containing 0.7% methylcellulose. The number of neurospheres is expressed as % of control. Data represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control cells (Student's t test). (H) Loss of neurosphere forming ability and morphological changes following Dvl2 depletion as shown by phase-contrast microscopy. Scale bar in F and H is 100 μ m.

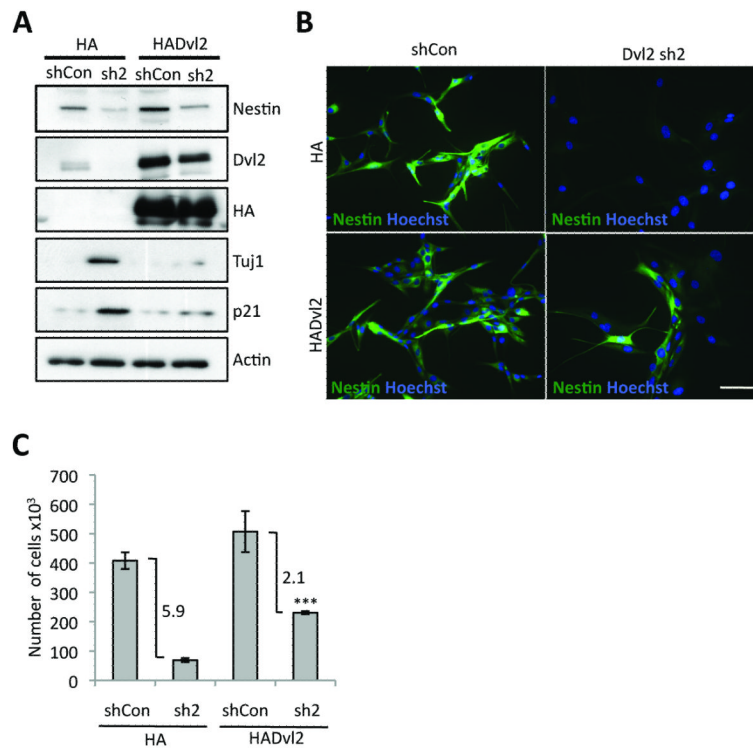


Figure 3. Expression of mouse Dvl2 rescues the differentiation-like phenotype in U87 glioma cells U87 cells were infected with a retrovirus empty vector (HA), or expressing mouse Dvl2 (HADvl2). The cells were infected with a lentivirus containing an shControl or Dvl2 sh2 as previously described. (A) Rescue of the effect on Nestin, Tuj1 and p21 levels visualized by western blot. (B) Rescue of Nestin expression analyzed by fluorescence microscopy. (C) After lentiviral infection, cells were plated and grown in medium containing 10%FCS and counted after 7d. The numbers next to the bars indicate the fold decrease in proliferation compared to control. The stars indicate the significance of the rescue. Data represent mean \pm SEM. ***P<0.001 relative to control cells (Student's t test). Scale bar in B is 100 μ m.

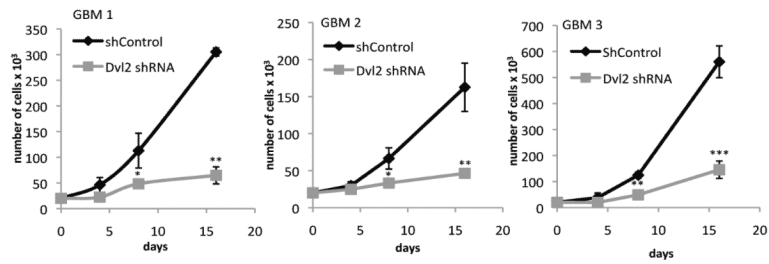


Figure 4. Dvl2 depletion blocks proliferation of patient-derived primary GBMs

Dvl2 depletion blocks proliferation of patient-derived primary GBMs. Primary cells were obtained after dissociation of three different patient-derived GBM tumor samples (GBM1, 2 and 3). Cells were plated on laminin-coated plastic dishes to form an adherent monolayer. Cells were infected with non-targeting (shControl) or Dvl2 targeting shRNA (sh2) lentivirus and selected in puromycin as previously described for 5d. Cells were plated for a growth curve in neural stem cell medium. Cell number was determined 4d, 8,d and 16d later. Data represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control cells (Student's t test).

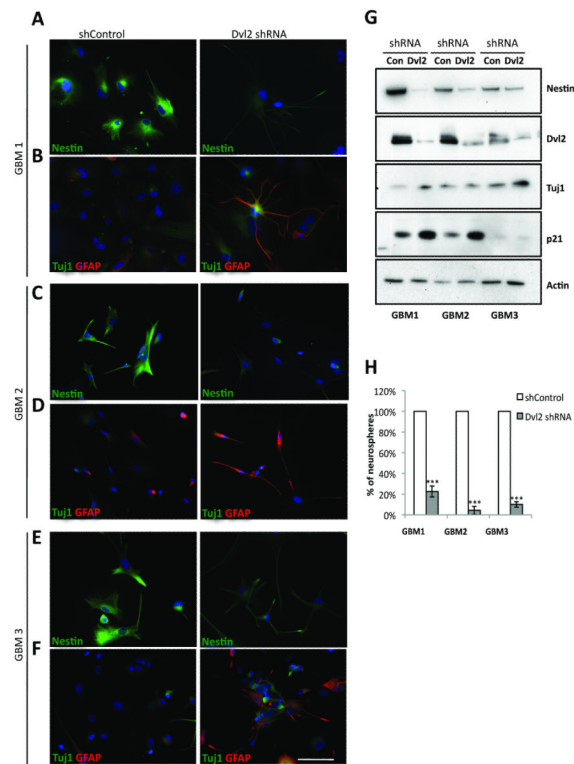


Figure 5. Dvl2 depletion blocks neurosphere formation and promotes differentiation of patient-derived primary GBMs

Primary cells were infected and selected as previously described. (A-F) Immunofluorescence analysis of cells isolated from patients GBM1, 2 and 3 after Dvl2 depletion. Cells were stained for the neural/progenitor stem cell marker Nestin (A,C,E), and two differentiation markers, Tuj1 and GFAP (B,D,F). Scale bar is 100 μ m. (G) Western blot analysis of cells isolated from patients GBM1, 2 and 3 after Dvl2 depletion. A decrease in Nestin can be seen in all three samples, an increase on Tuj1 is clearly seen in GBMs 1 and 3, and an increase in p21WAF is clearly seen in GBMs 1 and 2. (H) Neurosphere formation by GBM1, GBM2 and GBM3 patient-derived cells before and after Dvl2 depletion. The number of neurospheres is expressed as % of the control. Data represent means \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 relative to control cells (Student's t test). Scale bar is 100 μ m.

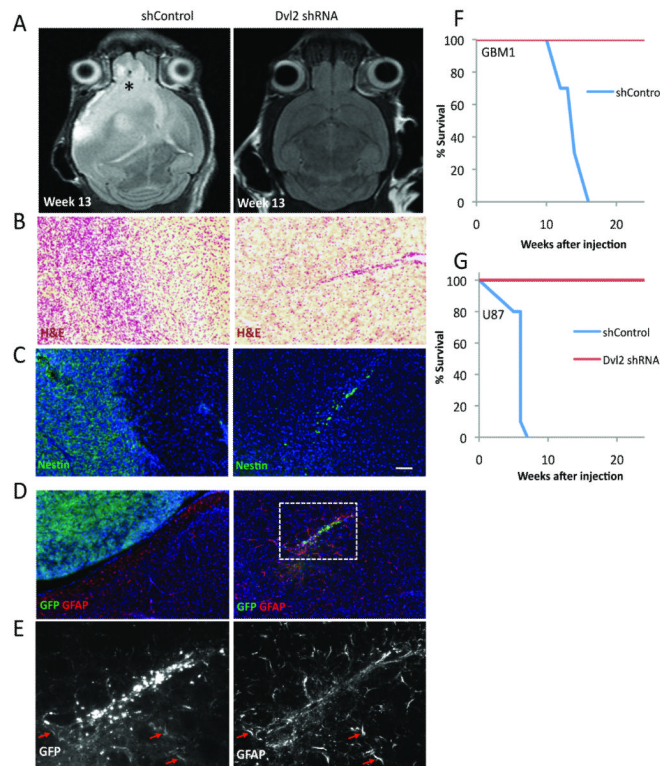


Figure 6. Dvl2 depletion suppresses tumorigenicity of primary GBM cells

GBM1 cells were infected with control and Dvl2 shRNA lentivirus as previously described and selected in puromycin for 5 days. 2×10^5 cells were stereotactically injected into the striatum of 3 NOD/SCID mice per group. (A) Tumor growth as determined by Magnetic Resonance Imaging (MRI) thirteen weeks after injection. Left panel - control cells; right panel - Dvl2 -depleted cells. The asterisk in the left panel indicates the tumor. (B) Intracranial tumor characterization by H&E staining showing a high proliferative mass in mice injected with control cells compared to Dvl2 depleted cells. (C) Immunofluorescence microscopy of brain cryo-sections. The cells were stained with an antibody specific for human Nestin, to identify human cells in the mouse brain, and Hoechst to visualize cell nuclei. Scale bar is $50 \mu\text{m}$. (D) U87 cells stably expressing GFP (U87-GFP) infected with control (left panel) and Dvl2 shRNA lentivirus (right panel) and injected into the striatum of NOD/SCID mice. 2×10^5 cells were injected per animal and 5 mice were used for control and 9 for Dvl2-depletion. GFP was used to identify injected U87 cells. Cryo-sections were stained for the differentiation marker GFAP to determine cell fate in vivo. Scale bar $100 \mu\text{m}$. (E) Enlargement of the area indicated by a dotted line in the right panel in (D) showing colocalization of GFP and GFAP markers (arrows). (F) Survival curve of NOD/SCID mice injected with 2×10^5 control or Dvl2-depleted GBM1 cells (Wilcoxon-Mann-Withney Test, rank sum test: $p=0.017$). (G) Survival curve for mice injected with U87 cells infected with shControl or Dvl2shRNA lentivirus (Wilcoxon-Mann-Withney Test, rank sum test: $p=0.021$). Data represent means \pm SEM. Scale bar is $100 \mu\text{m}$.

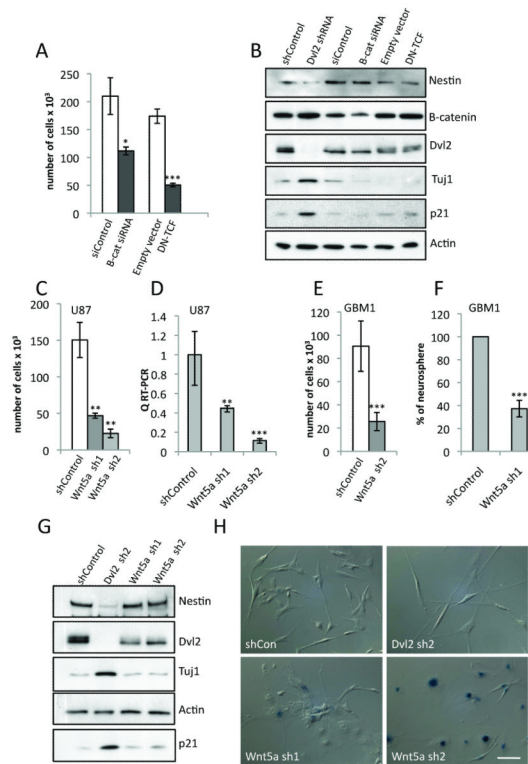


Figure 7. Canonical and non-canonical Wnt signaling maintain glioma cell proliferation
 (A) U87 cells were: (i) transfected with a non-targeting siRNA oligonucleotide (siControl), or a siRNA oligonucleotide targeting β -catenin, or (ii) infected with a control, or a dominant-negative (Delta-N) TCF expressing retrovirus. Cells were plated in medium containing 10% FCS and cell numbers determined after 5d. (B) Cells were treated as in (A) and Nestin, Tuj1 and p21^{WAF} protein levels determined by western blot analysis. Dvl2-depleted U87 cells were used as a control. The number of neurospheres was expressed as % of control. (C) U87 cells were infected with non-targeting shRNA lentivirus (shControl) or lentivirus expressing two distinct shRNAs targeting Wnt5a (Wnt5a sh1 and sh2). Cells were selected in purmoycin for 5d, plated in medium containing 10% FCS and cell numbers determined after 5d. (D) Wnt5a mRNA depletion efficiency by the two shRNAs in U87 was determined by QRT-PCR and normalized to GAPDH expression. (E) Cells from patient GBM1 were infected with lentivirus containing non-targeting shRNA (shControl), or shRNAs directed against Wnt5a. 7d after infection, cells were plated in neural stem cell medium as an adherent monolayer on laminin-coated dishes. Cell numbers were determined 8d later. (F) Cells from patient GBM1 were treated as in (E) and the neurosphere formation ability determined 15d after plating in neural stem cell medium containing 0.7% methylcellulose. The number of neurospheres was expressed as % of control. (G) U87 cells were treated as in (C) and Nestin, Tuj1 and p21^{WAF} protein levels determined by western blot analysis. Dvl2-depleted U87 cells were used as a control. (H) Cells were treated as in (C) and then fixed and stained for β -galactosidase (blue) to detect senescence. Data represent means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control cells (Student's t test). Scale bar is 100 μ m.