Mesenchymal high-grade glioma is maintained by the ID-RAP1 axis

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High-grade gliomas (HGGs) are incurable brain tumors that are characterized by the presence of glioma-initiating cells (GICs). GICs are essential to tumor aggressiveness and retain the capacity for self-renewal and multilineage differentiation as long as they reside in the perivascular niche. ID proteins are master regulators of stemness and anchorage to the extracellular niche microenvironment, suggesting that they may play a role in maintaining GICs. Here, we modeled the probable therapeutic impact of ID inactivation in HGG by selective ablation of *Id* in tumor cells and after tumor initiation in a new mouse model of human mesenchymal HGG. Deletion of 3 *Id* genes induced rapid release of GICs from the perivascular niche, followed by tumor regression. GIC displacement was mediated by derepression of *Rap1gap* and subsequent inhibition of RAP1, a master regulator of cell adhesion. We identified a signature module of 5 genes in the ID pathway, including *RAP1GAP*, which segregated 2 subgroups of glioma patients with markedly different clinical outcomes. The model-informed survival analysis together with genetic and functional studies establish that ID activity is required for the maintenance of mesenchymal HGG and suggest that pharmacological inactivation of ID […]

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High-grade gliomas (HGGs) are incurable brain tumors that are characterized by the presence of glioma-initiating cells (GICs). GICs are essential to tumor aggressiveness and retain the capacity for self-renewal and multilineage differentiation as long as they reside in the perivascular niche. ID proteins are master regulators of stemness and anchorage to the extracellular niche microenvironment, suggesting that they may play a role in maintaining GICs. Here, we modeled the probable therapeutic impact of ID inactivation in HGG by selective ablation of Id in tumor cells and after tumor initiation in a new mouse model of human mesenchymal HGG. Deletion of 3 Id genes induced rapid release of GICs from the perivascular niche, followed by tumor regression. GIC displacement was mediated by derepression of Rap1gap and subsequent inhibition of RAP1, a master regulator of cell adhesion. We identified a signature module of 5 genes in the ID pathway, including Rap1gap, Id, and Id3 genes induced rapid release of GICs from the perivascular niche, followed by tumor regression. 

To determine whether ID proteins are essential for glioma maintenance and ascertain the potential therapeutic impact of target-
ing ID activity in this disease, we generated a new mouse model of malignant glioma that recapitulates mesenchymal tumors, the most aggressive subtype of HGG (20). This model has allowed us to address the significance of concurrent genetic inactivation of 3 Id genes (Id1, Id2, Id3) exclusively in brain tumor cells and after tumor initiation. The time-controlled deletion of Id genes in this mouse glioma model led to marked extension of survival, with rapid loss of GICs from the perivascular niche and tumor regression, an effect mediated by derepression of the RAP1-GT-Pase inhibitor, Rap1GAP. This finding is consistent with the strong prognostic value that emerged from the segregation of glioma patients into 2 subgroups, with divergent clinical outcome based on the activity of a 5-gene set, including Id genes and RAP1GAP.

Results

Suppression of ID function impacts tumor maintenance of HGG. To ask whether the consequences of Id deletion on tumor growth affect the cell-intrinsic properties of GICs, including the competence to adhere to the perivascular niche, we set out to develop a mouse model of malignant glioma in which Id deletion could be selectively targeted to glioma cells after tumor initiation without the confounding effects that might derive from Id deletion in other Id-expressing populations, such as endothelial cells, tumor stroma, or immune cells. Transduction of NSCs in the hippocampus, a neurogenic area of the adult brain, with a lentivirus that expresses oncopgenic ras (HrasV12) and shRNA against the tumor suppressor Tp53 (shp53) generates HGG (21). Although oncopgenic mutations affecting ras genes are uncommon in human GBM, Ras is frequently activated in HGG by aberrant signaling from multiple receptor tyrosine kinases (22). When we tested the same lentiviral vector expressing GFP instead of HrasV12 to identify infected cells, most of the GFP-positive cells costained with the radial glia and astrocytic marker GFAP and were negative for the neuronal marker NeuN (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI63811DS1). In order to temporally control deletion of Id selectively in tumor cells, we linked an IRES-Cre-ER cassette to HrasV12 cDNA and shp53, thus achieving Cre-recombinase activation by tamoxifen in vivo (Supplemental Figure 1B). To create a conditional Id-null mouse, we crossed mice harboring floxed alleles of Id1 (Id1<sup>floxed</sup>) and Id2 (Id2<sup>floxed</sup>) with constitutive Id3 knockout (Id3<sup>−/−</sup>) mice to generate Id1<sup>−/−</sup>Id2<sup>−/−</sup>Id3<sup>−/−</sup> (Id conditional triple-knockout; Id-cTKO) mice (13). HrasV12-Cre-ER-shp53 lentiviral particles were injected in the hippocampi of 4-week-old Id-cTKO mice, and tumor initiation/progression was examined. Definite tumor lesions were detected as early as 12 days after lentiviral transduction in 86% of the infected mice (6 out of 7; Figure 1A, top panels). At this stage, tumors consisted of highly proliferative Ki67<sup>+</sup> cells, showing reactivated expression of Id1 and Id2 (largely absent in the adult hippocampus) and robust positivity for nestin and oligodendrocyte transcription factor 2 (Olig2) (Figure 1A and Supplementary Figure 1C). Advanced tumors analyzed at the time of mouse euthanasia manifested features of HGG, such as multineuclated giant cells, necrosis and pseudo-palisades, mitotic figures, and propensity to invade the normal brain (Figure 1B). Tumors remained strongly positive for ID1, ID2, Olig2, and nestin; expressed glial fibrillary acidic protein (GFAP); and included individual entrapped BIII-tubulin-positive neurons. The high positivity for Ki67 and the endothelial marker CD31 were indicative of rapid growth and rampant tumor angiogenesis, respectively (Figure 1C). Together, these elements reflect those found in the human counterpart of the disease (glioma grade III–IV). Interestingly, double immunostaining experiments for ID1 and ID2 showed that the 2 ID proteins are frequently coexpressed in glioma cells (Figure 1D).

To ascertain the therapeutic potential of Id deletion in brain tumors, tumor-bearing Id-cTKO mice were treated with tamoxifen or vehicle (corn oil). First, we documented efficient loss of ID1 and ID2 expression and reduced positivity for Ki67 in advanced tumors analyzed by immunofluorescence 7 days after a single 4-day cycle of tamoxifen treatment (Supplemental Figure 2, A–C). The residual cells staining positive for ID1 (but not ID2) in tamoxifen-treated gliomas were, for the vast majority, tumor endothelial cells, as shown by the nuclear morphology and containing for CD31 (Supplemental Figure 2, A and B, and data not shown). Glioma cells that stained positive for the HrasV12 oncprotein also expressed ID proteins. However, the expression of HrasV12 was unaffected by Id deletion (Supplemental Figure 2D). This finding indicates that the tamoxifen-mediated ablation of Id genes in glioma cells does not impact retention of the tumor-initiating HrasV12 protein. Therefore, this mouse model allows selective and specific deletion of Id genes in glioma cells. Then, we evaluated survival in mice treated with tamoxifen or vehicle 12 days after lentiviral transduction and at biweekly intervals thereafter. Eighty-five percent of oil-treated mice (11 out of 13) succumbed because of intracranial tumors within an average of 10 weeks from lentiviral transduction. However, Id ablation resulted in significant extension of survival, with 64% of the mice (7 out of 11) alive after an average of 20 weeks (P = 0.002; Figure 2A). Tumor suppression was not merely an effect of tamoxifen, as tamoxifen treatment of wild-type animals infected with HrasV12-Cre-ER-shp53 lentivirus did not affect tumor growth (data not shown). The tumors that eventually developed and caused death of tamoxifen-treated mice accumulated high levels of ID1 and ID2, thus suggesting that Id expression by glioma cells is required for tumor growth in this model (Supplemental Figure 3A).

ID proteins preserve GICs and are required for association of GICs with endothelial cells. To identify the defining features precipitated by deletion of Id genes in glioma, we sacrificed controls and tamoxifen-treated mice 6 weeks after tumor initiation. The majority of tumors (80%) in the tamoxifen-treated cohort displayed dramatic reduction of tumor volume compared with that of carrier-treated tumors and consisted of clusters of tumor cells confined to the hippocampus that had not progressed relative to tumors detected 12 days after viral infection (Figure 2, B and C, also compare Figure 2B with Figure 1A). As expected, these tumors either lacked or expressed negligible levels of ID proteins. Id-deleted glioma lesions showed a marked reduction in the proliferation rate (4 fold, as measured by Ki67 immunoreactivity) and a striking decrease of the stem cell markers nestin and SSEA1 compared with that of control tumors (Figure 2, B–E, and Supplemental Figure 3B). It is important to emphasize that expression of nestin in tamoxifen-treated tumors was lower at this stage than in the controls at the earliest time point (compare Figure 2B with Figure 1A), suggesting that nestin downregulation was independent of tumor size and specifically implemented by Id loss. The decrease of nestin expression was recapitulated in vitro when tumor explants established from HrasV12-Cre-ER-shp53 glioma-bearing Id-cTKO mice were cultured in the presence of tamoxifen (Supplemental Figure 3C). In contrast, expression of GFAP was higher in tamoxifen-treated tumors compared with that in controls (Supplemental Figure 3D).
To analyze the consequences of Id ablation on GIC-enriched populations, we isolated cells from HrasV12-Cre-ER-shp53 glioma. We selected GICs based upon their ability to self-renew as tumor spheres in stem cell–permissive culture conditions (23); retain expression of the NSC and GIC markers SSEA1, integrin \( \alpha_6 \), and nestin; and display robust glioma-initiating capacity when orthotopically transplanted into the brains of immunodeficient mice (Figure 3). Treatment of GICs isolated from Id-cTKO mice with tamoxifen abolished the tumor sphere-forming capacity, but the same treatment had no effect on GICs derived from...
Id1+/+Id2+/+Id3–/– mice (Figure 3, B and C). To accurately determine the frequency of glioma cells that self-renew and generate spheres within the glioma cell population, we performed limiting dilution analysis using cells from freshly dissociated tumors (24). Based on the Poisson distribution and the intersection at the 37% level, we estimated the minimal frequency of glioma cells endowed with stem cell capacity to be 0.29% ± 0.02% and 3.84% ± 0.51% in tumors derived from tamoxifen-treated animals and controls, respectively (P = 0.006) (Figure 3D). Finally, following orthotopic implantation of HrasV12-Cre-ER-shp53-Id-cTKO GICs into the brains of immunodeficient mice, tamoxifen efficiently blocked tumor formation (0 out of 5 mice developed tumors), whereas highly aggressive gliomas developed in the control group (5 out of 5 mice developed tumors; Figure 3E). The concordant phenotypes in vivo and in vitro, as well as across multiple experimental systems, support the view that ID proteins play a key role in maintaining self-renewal and the tumorigenic capacity of GICs.

Next, we tested whether loss of Id impacts the interaction between GICs and endothelial cells in the tumor perivascular niche. First, we determined that a substantial fraction of SSEA1-positive tumor cells were also positive for ID immunostaining, thus indicating that GICs express ID proteins in vivo (Supplemental Figure 4). Then, we asked whether deletion of Id genes in GICs from established tumors influenced the residency of GICs in the perivas-
The effect of Id ablation in Ras-V12-IRES-Cre-ER-shp53 GICs in vitro and after orthotopic transplantation in vivo. (A) Cells isolated from Ras-V12-IRES-Cre-ER-shp53 gliomas generated in Id-cTKO mice were cultured in medium containing EGF and FGF-2 for 3 passages and immunostained using antibodies against Nestin, SSEA1, and integrin α6 (ITGα6). Scale bars: 20 μm. (B) Microphotographs of tumor spheres from GICs 14 days after plating at <1 cell per μl in semiadherent conditions. GICs isolated from advanced Ras-V12-IRES-Cre-ER-shp53 gliomas in Id-cTKO or wild-type control mice were cultured in medium containing EGF and FGF-2 for 2 passages and then subjected to treatment with vehicle or 4-OHT for 5 days. Dissociated cells were assayed by microscopic analysis for sphere formation. Scale bars: 500 μm (top panels); 250 μm (bottom panels). (C) Quantification of gliospheres as a percentage of plated cells from cultures with the indicated genotypes and treatments. Data represent the mean ± SD of triplicate samples; *P = 0.003. (D) Regression plot for cells isolated from controls or tamoxifen-treated tumors. Cells from 2 independent tumors per treatment were plated at limiting dilution (1–400 cells per well) in triplicate plates. Data represent the mean ± SD; P = 0.006. Experiments were repeated twice. (E) H&E staining of representative brain sections of immunodeficient mice subjected to stereotaxic injection with Ras-V12-IRES-Cre-ER-shp53 Id-cTKO GICs and treated with oil or tamoxifen. Scale bars: 250 μm.
the proneural class (overall probability for mesenchymal classification: 0.895 ± 0.11) (Table 1). This finding was corroborated by the robust positivity of the tumor cells for fibronectin and connective tissue growth factor, 2 markers of mesenchymal GBM that did not show significant changes after Id deletion (refs. 26, 28, and Figure 5A). However, knowledge-based pathway analysis applied to the genes differentially expressed between control and Id-depleted tumors revealed regulation by ID proteins of functional gene categories linked to plasma membrane, extracellular matrix, cell-cell signaling, cell adhesion, etc. (Supplemental Table 1). These categories are very similar to those acutely perturbed by Id deletion in NSCs through direct regulation of the bHLH target gene Rap1gap, the inhibitor of RAP1-GTPase (13). Interestingly, Rap1gap mRNA was markedly elevated in RasV12-IRES-Cre-ER-shp53 glioma following tamoxifen-mediated deletion of Id genes (Figure SB).

Depending on the cellular contexts, RAP1 can act as a key regulator of cell-cell or cell-ECM adhesion or both. The function of RAP1 for cell-ECM adhesion is implemented through its ability to operate as intracellular mediator of integrin signaling (29–32). Recently, integrin signaling emerged as an essential determinant for the anchorage of GICs to the perivascular brain tumor niche (10). First, we established that Rap1GAP was expressed in the glioma model (Supplemental Figure 6 and ref. 18). Having determined that Id deletion restored the normal expression of Rap1GAP in glioma, we examined the role of Rap1GAP in GICs and determined whether RAP1 is the significant target of Rap1GAP activity. To do this, we took advantage of a widely used cellular system developed to study the mechanistic events determining the glioma stem cell state. In this system, primary astrocytes are reprogrammed to a transformed stem cell phenotype (induced glioma-initiating cells [iGICs]) by the expression of a mutant form of the EGFR, EGFRvIII, concurrently with silencing of key tumor suppressor genes frequently deleted in human glioma.

**Table 1**

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LDA scores range between 0 (not belonging) and 1 (belonging) for each mouse glioma in relation to its allocation within proneural and mesenchymal subgroups. The probability for mesenchymal classification of controls is 0.995 ± 0.005. The probability for mesenchymal classification of tamoxifen-treated tumors (without ID expression) is 0.895 ± 0.11.

**Figure 4**

Ablation of Id disrupts adhesion of GICs to endothelial cells in the perivascular niche. (A) Immunostaining for SSEA1 (red) and CD31 (green) on a representative Ras-V12-IRES-Cre-ER-shp53 HGG in Id-cTKO mice 6 weeks after treatment with tamoxifen or oil. Nuclei were counterstained with DAPI (blue). (B) Quantification of the percentage of SSEA1+ cells from A within 10 μm from CD31+ cells (n = 4 per each treatment group). *P = 0.0005. (C) Immunostaining for SSEA1 (red) and CD31 (green) on representative Ras-V12-IRES-Cre-ER-shp53 HGG tumors collected from Id-cTKO mice 7 days after treatment with tamoxifen or oil. Nuclei were counterstained with DAPI (blue). (D) Quantification of the percentage of SSEA1+ cells from C within 10 μm from CD31+ cells (n = 4 per each treatment group). *P = 0.0005. (E) Immunostaining for SSEA1 (red) and cleaved caspase-3 (green) on representative Ras-V12-IRES-Cre-ER-shp53 HGG from Id-cTKO mice 7 days after treatment with tamoxifen or oil. Nuclei were counterstained with DAPI (blue). Data represent the mean ± SD. Asterisks in images indicate the lumen of blood vessels; arrows indicate SSEA1 + cells within 10 μm from CD31+ cells; arrowheads indicate SSEA1 + cells >10 μm from CD31+ cells. Scale bars: 20 μm.
We engineered astrocytes derived from \( Id1^{L/L}Id2^{L/L}Id3^{L/L} \) mice to express \( EGFRvIII-IRES-CreER \) and \( shp53 \) and treated the resulting neurospheres with vehicle or tamoxifen to delete \( Id \) genes. Loss of \( Id \) in iGICs induced the bHLH-ID targets \( Rap1gap \) and \( Cdkn1c \) and led to morphological and molecular changes indicative of multilineage neural differentiation (Supplemental Figure 7, A–C). These effects occurred in the absence of signs of apoptosis (Supplemental Figure 7D). Therefore, we used this system to ask whether the ID/RAP1 pathway controls adhesion of GICs to endothelial cells, a property that mirrors adhesion of GICs to the perivascular niche in vivo (4). Loss of \( Id \) genes induced by tamoxifen inhibited the ability of iGICs to adhere to brain endothelial cells and compromised their self-renewal capacity as gliomaspheres (Figure 6, B–D, and Supplemental Figure 7F). However, when \( Id \) genes were deleted but RAP1 activity was preserved by expressing a constitutively active RAP1 mutant resistant to Rap1GAP inhibition (RAP1-G12V;Q63E, Supplemental Figure 7E and refs. 36, 37), adhesion to endothelial cells was rescued (Figure 6,
B and C, and Supplemental Figure 7F). Expression of RAP1-G12V; Q63E rescued also the gliomasphere-forming capacity of iGICs depleted of Id genes, albeit less efficiently than their adherence (Figure 6D). Thus, inhibition of RAP1 is the crucial event precipitated by recombination of Id genes in tumor cells, which leads to the disruption of the physical contact between GICs and endothelial cells.

Next, we asked whether upregulation of Rap1GAP is sufficient to alter adhesion of GICs to endothelial cells. To this aim, we used early-passage, patient-derived GICs that, under serum-free culture conditions, retain phenotypes and genotypes closely mirroring primary tumor profiles as compared to serum-cultured glioma cell lines that have largely lost their developmental identities (23). Enforced Rap1GAP expression in human GICs inhibited RAP1 activity and severely compromised the adhesion of human GICs to endothelial cells (Figure 7, A–C). The frequency of glioma cells that self-renew as gliomaspheres was drastically decreased by enforced expression of Rap1GAP (0.275% ± 0.035% in Rap1GAP-expressing GICs versus 8.417% ± 0.752% in controls; \( P = 0.003 \)) (Figure 7D). Rap1GAP also decreased the size of tumor spheres, thus underscoring the glioma suppressor function of Rap1GAP (Figure 7, E and F). To ask whether the effects of Rap1GAP in GICs are indirect consequences of changes of cell cycle and/or cell viability, we determined the fraction of BrdU-positive cells (to measure entry into S phase) and analyzed cleaved caspase-3 (to assay cell viability) of human GICs expressing Rap1GAP. These experiments showed that Rap1GAP prevented the adhesion of GICs to brain endothelial cells and compromised their self-renewing capacity as tumor spheres, without changing their ability to progress through S phase and in the absence of marks of apoptosis (Figure 8 and Supplemental Figure 8). Conversely, expression of the cell cycle inhibitor p27Kip1 in GICs inhibited S phase progression (and consequently the growth of GICs as spheres) but did not affect the competence of GICs to establish interactions with brain endothelial cells in the same assay (Figure 8 and Supplemental Figure 8). Taken together, these find-
The number of adherent GFP+ cells was determined by high-power microscopy review. Data represent the mean ± SD of triplicate samples at <1 cell per μl was determined by microscopy review. Data represent the mean ± SD of triplicate samples. *P = 2.4069 × 10−5.

The expression of RAP1GAP is significantly downregulated in 81 samples from human glioblastoma (class 2, dark blue) compared with 23 samples from nontumor human brain (class 1, light blue). P = 3.83 × 10−22. In each possible combination of bHLH genes, bHLH target genes, and ID genes was recursively analyzed, the proportional hazards regression analysis revealed that a 5-gene set, including TCF12, ID2, ID3, RAP1GAP, and CDKN1C, was strongly predictive of survival and, using hazard ratios to determine effect size, outperformed any

**Figure 8**
Loss of cell adhesion by Rap1GAP in GICs is independent of proliferation. (A) Western blot analysis for Rap1GAP and p27Kip1 expression in GICs transduced with pLOC-Rap1GAP-GFP, pLOC-p27Kip1-GFP, or pLOC-GFP. β-Actin is shown as loading control. (B) Microphotographs of human GICs transduced with pLOC-Rap1GAP-GFP, pLOC-p27Kip1-GFP, or pLOC-GFP and assayed for adhesion to endothelial cells. Scale bars: 100 μm. (C) The number of adherent GFP+ cells was determined by high-power microscopy review. Data represent the mean ± SD of triplicate samples from 2 independent experiments. *P = 2.4069 × 10−5. (D) The number of tumor spheres obtained from human GICs treated as in B and seeded at <1 cell per μl was determined by microscopy review. Data represent the mean ± SD of triplicate samples. *P = 5.05863 × 10−5.

**Figure 9**
The ID/RAP1 pathway carries prognostic value in human malignant glioma. (A) The expression of RAP1GAP is significantly downregulated in 81 samples from human glioblastoma (class 2, dark blue) compared with 23 samples from nontumor human brain (class 1, light blue). P = 3.83 × 10−22. In box-and-whisker plots, horizontal bars indicate the medians, boxes indicate 25th to 75th percentiles, and whiskers indicate 10th and 90th percentiles. (B) Kaplan-Meier analysis comparing survival of patients carrying HGG expressing high TCF12, RAP1GAP, and CDKN1C and low ID2 and ID3 (green line) or low TCF12, RAP1GAP, and CDKN1C and high ID2 and ID3 (blue line) and the overall patient population (red line). P < 0.001.
other gene combination from the defined pathway (hazard ratio, 0.318; \( P < 0.0001 \)) (Supplemental Table 2). Comparing the samples at both extremes of this 5-gene signature categorizes patients in 2 subgroups, with either high or low activity of the \( \text{Id/bHLH/RAP1GAP/CDKN1C} \) pathway and opposite clinical outcomes (\( P = 0.00483 \), log-rank test; Figure 9B). High levels of \( \text{Id1} \) could not contribute to the poor-prognosis \( \text{Id1}- \) based pathway, because high \( \text{Id1} \) expression was individually associated with better clinical outcome in the proneural subgroup and the overall population of patients with HGG (Supplemental Table 2 and Supplemental Figure 10). This notion is consistent with results from a recent study that reported a significant survival benefit of high expression of \( \text{Id1} \) in mouse and human glioma of the proneural subclass (19). Conversely, the HGG subgroup that displays high activity of the \( \text{Id} \) pathway is markedly enriched in mouse and human glioma of the proneural subclass (19).

The ID/Rap1GAP/RAP1 pathway controls residency of GICs in the perivascular niche of malignant glioma. High levels of ID proteins inhibit transcriptional activation of the \( \text{RAP1GAP} \) gene by bHLH transcription factors in GICs. The balance of the RAP1-GTPase is shifted toward active RAP1-GTP that activates integrin signaling and anchorage of glioma cells to the tumor endothelium. Loss of ID activity derepresses bHLH-mediated activation of Rap1GAP expression, with inhibition of RAP1-GTPase and relative accumulation of RAP1 as RAP1-GDP. Loss of RAP1-GTP results in the release of GICs from the perivascular space, with ensuing loss of stem and tumor-initiating capacity.

### Table 2

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</tr>
<tr>
<td>Yes</td>
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\( ^a \)The unfavorable score is defined by the high expression of \( \text{Id2} \) and \( \text{Id3} \) in association with the low expression of TCF12, RAP1GAP, and CDKN1C.

The mouse model described here will be of general use for the in vivo identification of other oncogenic pathways to which specific tumor types may be addicted. We have shown that ID proteins function in a cell-intrinsic manner in GICs to preserve the intimate interactions of GICs with endothelial cells in the perivascular niche and maintain self-renewal, two properties that have been consistently associated with the most aggressive features of glioma cells (2–6). The selective ablation of \( \text{Id} \) genes in glioma cells has allowed us to define a GIC-intrinsic role of ID proteins for the communication with the perivascular tumor niche. Loss of \( \text{Id} \) genes in glioma cells leads to rapid and concurrent loss of perivascular anchorage and self-renewal of GICs, without appreciable changes of cellular viability. The effect of \( \text{Id} \) loss on the adhesion properties of GICs is mediated by derepression of the bHLH target gene \( \text{Rap1gap} \), resulting in RAP1-GTPase inactivation. Consistent with the role of Rap1GAP as mediator of the effects triggered by \( \text{Id} \) loss in GICs, the interactions of GICs with endothelial cells are impaired when \( \text{Rap1gap} \) is ectopically introduced into GICs (Figure 10). The consequent loss of self-renewal matches impaired self-renewal and loss of tumor-forming capacity of GICs after targeted inhibition of integrin signaling (6). Conversely, preventing inactivation of RAP1-GTPase through a constitutively active RAP1 protein rescues \( \text{Id} \)-deleted GICs from the inhibitory effects of \( \text{Rap1gap} \). Interestingly, \( \text{Rap1GAP} \) has recently emerged as a candidate tumor suppressor gene in other tumor types (41–44). Besides repressing Rap1GAP, the expression of ID proteins in malignant glioma is essential to prevent expression of cyclin-dependent kinase inhibitors (e.g., p57Kip2\(^\text{\textsuperscript{3}}\)), thus explaining the proliferative arrest precipitated by acute \( \text{Id} \) ablation in GICs. We suggest that loss of GICs at the glioma perivascular niche results from the suppression of the dual function of ID proteins as enhancers of proliferation and essential factors for the adhesion of GICs to the glioma perivascular niche. Recruitment of ID proteins as drivers of glioma malignancy is critical to enforce repression of \( \text{Rap1GAP} \) and \( \text{CDKN1C} \) in GICs and constitutes the molecular basis for the strong association between activity of the \( \text{Id/bHLH/RAP1GAP/CDKN1C} \) pathway and survival in human glioma.
Combined with the findings recently reported on the role of ID1 in HGG (19), our study provides a clearer picture of how ID proteins operate in malignant glioma. In proneural HGG and those initiated with a Kras mutation, high expression of ID1 marks a highly self-renewing population of tumor cells that does not coexpress ID2 and ID4, is associated with poorer tumor-initiating capacity relative to that of the population with low ID1 expression, and correlates with better survival of patients with proneural glioma (19). It will be interesting to test the effects of combined loss of 3 Id genes in these mouse models, therefore addressing the consequences of inactivating multiple Id genes when they are expressed by different types of tumor cells. Conversely, the mesenchymal gliomas generated here coexpress multiple ID proteins, and the combined ablation of 3 Id genes in tumor cells has dramatic negative impact on self-renewal, tumor-initiating capacity, and residency in the perivascular niche of glioma cells. In future work, it will be important to address whether deletion of 1 or 2 Id genes in the mesenchymal model mouse of malignant glioma reported here will also lead to a similar dissociation of self-renewal and tumor growth, as observed in the other tumor models (19).

Activation of the ID/BHLH/RAPIGAP/CDKN1C pathway identifies HGG with poor clinical outcomes that are enriched in mesenchymal tumors. The identification of the 5-marker, ID-based signature predicting clinical outcome of patients with HGG will facilitate the identification of molecularly based subgroups of patients with HGG, who might benefit from targeted therapeutic approaches aimed at extinguishing ID activity in cancer cells. In breast cancer, Oncotype DX is a widely used test that predicts likelihood of recurrence and guides treatment (45, 46). As anti-ID targeting therapies become available, the identification of the high-risk group of glioma patients, with aberrant activity of the ID pathway reported in this study, will be useful for discerning the subset of patients who may receive substantial the subset of patients who may receive substantial benefit from anti-ID approaches.

The mouse model reported in this study, in which Id genes were selectively ablated in glioma cells, has allowed us to define a GIC intrinsic role of ID proteins for the communication with the perivascular niche. However, any therapeutic approach to target ID in human cancer will also target ID functions in nontumor cells (e.g., the tumor endothelium) in which ID inactivation is also expected to generate beneficial antitumor effects (11). It has been suggested that one of the reasons for the limited success of targeted cancer therapies is that they fail to target the key features of cancer stem cells, among which retention of self-renewal and anchorage to the extracellular niche are probably the two most important functional properties (47). Both the cell intrinsic machinery that maintains GICs and the mechanisms orchestrating adhesion of GICs to the niche microenvironment have been independently proposed as promising targets for new therapeutic approaches. The dual master regulatory function of ID proteins unraveled here lends strong support to pharmacological strategies aimed at countering ID function in HGG and other malignancies sustained by cancer stem cells and their interaction with extracellular niches.

Methods

Animals. The Id2<sup>-/-</sup> mouse was used to generate conditional Id2 knockout mice and has been described previously (13). In this floxed mouse, loxP sites flanked the entire protein-coding region of the Id2 gene. These mice were crossed with Id1<sup>-/-</sup> and Id3<sup>-/-</sup> or Id3<sup>+/-</sup> mice to generate Id<sub>3</sub>TKO mice (12, 48, 49).

Histology and immunostaining. Tissue preparation and immunohistochemistry on brain tumors and immunofluorescence staining were performed as previously described (26, 50, 51). Antibodies used in immunostaining are listed in Supplemental Table 3. In histograms, values represent the mean values; error bars are SD or SEM, as indicated in figure legends. Statistical significance was determined by t test (2 tailed) using GraphPad Prism 4.0 software (GraphPad Inc.). Quantification of the IL-6 intensity staining was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/). The histogram of the intensity of fluorescence of each point of a representative field for each condition was generated. The fluorescence intensity of 3 fields from 3 independent tumors was scored and standardized to the number of cells in the field.

Cell culture conditions and cell culture–based assays. Human embryonic kidney 293T and bEnd3 cells (ATCC) were grown in DMEM containing 10% FBS (Invitrogen). GBM-derived GICs were grown as spheres in Neurobasal media containing N2 and B27 supplements and human recombinant FGF-2 and EGF (50 pg/ml each; Peprotech). EGFReIlH-shp53 iGICs were obtained by infecting Id1<sup>-/-</sup>Id2<sup>-/-</sup>Id3<sup>-/-</sup> astrocytes with EGFReIlH-Cre-ER-shp53 lentivirus. After infection, cells were cultured in Neurobasal media (Invitrogen) containing N2 and B27 supplements (Invitrogen) and human recombinant FGF-2 and EGF (50 ng/ml each).

For adhesion assay of pLOC-GFP or pLOC-RAP1-G12V;Q63E-GFP EGFReIlH-Cre-ER-shp53 iGICs spheres were generated by plating cells in low-attachment plates (Corning). Cells were treated for 96 hours with 500 nM tamoxifen or vehicle, and an equal number of iGSC spheres (200 per 18-mm coverslip) were plated on bEND3 cells that had been cultured on coverslips for 36 hours in iGSC-defined medium. After 24 hours, cultures were washed with PBS-1% BSA 4 times, fixed in 4% PFA for 10 minutes, and examined by fluorescent microscopy. The number of GFP-positive spheres was scored by scanning the entire coverslip. Triplicate samples for each infection and treatment were analyzed, and data are presented as the percentage of plated spheres. In histograms, values represent the mean values and error bars are SDs.

Adhesion of pLOC-GFP–, pLOC-Rap1GAP-GFP–, or pLOC-p27<sup>Kip1</sup>–, GFP–transduced human GICs to endothelial cells was performed by plating bEnd3 cells on 18-mm coverslips and allowing them to adapt to GSC medium for 36 hours. Dissociated pLOC-GFP–, pLOC-Rap1GAP-GFP–, or pLOC-p27<sup>Kip1</sup>–,GFP–transduced glioma spheres were plated at a density of 25,000 cells per coverslip in quadruplicates. After 30 minutes, plates were vigorously washed with PBS-0.1% BSA 4 times to remove nonadherent cells. Cells were fixed with 4% PFA for 10 minutes and stained with anti-GFP antibody to identify GFP-positive, lentivirus-infected GICs, and the number of GFP-positive cells per field was scored. At least 2,000 GFP-positive cells were counted in each coverslip by scanning multiple fields. Results are presented as the mean ± SD of quadruplicates samples. The assay was repeated twice.

For BrdU incorporation analysis, GICs transduced with pLOC-GFP–, pLOC-Rap1GAP-GFP–, or pLOC-p27<sup>Kip1</sup>–,GFP– were plated on coverslips and cultured in the presence of 10 μM BrdU for 2 hours. Cells fixed in 4% PFA were stained with anti-BrdU antibody, and the number of BrdU-positive cells was scored as a percentage of the total number of cells counterstained with DAPI.

For human and mouse glioma sphere formation, cells were infected with lentiviral particles. Three days later, single cells were plated at density of ≤3 cells per well in triplicate in low-attachment 96-well plates. The number and the size of spheres were scored after 10 to 14 days. Limiting dilution assay was performed as described previously (24). Spheres were dissociated into single cells and plated in 96-well plates in 0.2 ml of medium containing growth factors. Cultures were left undisturbed for 10 days, and then the percentage of wells not containing spheres for each cell dilution was calculated and plotted against the number of cells per well. Linear regres-
sion lines were plotted, and the number of cells required to generate at least 1 sphere in every well (the stem cell frequency) was calculated. The experiment was repeated twice.

**Lentiviral production.** pLKO.1 lentiviral expression vectors carrying shRNAs were purchased from Sigma-Aldrich. The hairpin sequence targeting the RAP1GAP gene is CCTGGATTCTCGCTCAAGTA. pLOC-GFP lentiviral expression vectors carrying RAP1α or Rapp1GAP cDNA were purchased from Open Biosystems. The RAP1-G12V/Q63E mutant was generated using the Phusion Site Direct Mutagenesis Kit (New England Biolabs). Lentivirus preparation and infusions were performed as described previously (26).

**Clinical outcome analysis.** Expression profiles and clinical information for 1,043 newly diagnosed patients with HGG from the data sets were analyzed (20, 38–40). The platform for all 4 data sets was Affymetrix-based and used 2 different chip types: U95Av2 and U133A. Microarray data sets were batch normalized using a previously described method (52). Following batch normalization, we calculated the median value of the bHLH transcription factors and categorized the data for each sample as “0” (if below the median) or “1” (if at or above the median). Since the direction of the survival association was opposite between the bHLH transcription factors and targets compared with the ID genes, we categorized the ID gene expression as “1” if it was below the median and “0” if it was above the median. Gene combinations were then added, with equal weightings for each gene, for each sample. Proportional hazards regression analysis with the 5-gene signature was used to calculate the effect of the integrated tumor score on survival.

**Biochemical methods.** The levels of active GTP-bound RAP1 were determined using the Active RAP1 Pull-Down and Detection Kit (Pierce) according to the manufacturer’s instructions. Proteins were analyzed by immunoblotting using an anti-RAP1 antibody. RAP1 activity in Id1−/−/Id2−/−/Id3−/− astrocytes, transformed by the expression of EGFvBIII-Cre-ER-shp53 and transduced with a pLOC-GFP-RAP1G12V/Q63E or pLOC-vector-GFP lentivirus, was measured as described above.

**RNA preparation and real-time quantitative PCR.** RNA preparation and real-time quantitative PCR were performed as described previously (26, 51). Primers used in real-time quantitative PCR are listed in Supplemental Table 4. The relative amount of specific mRNA was normalized to 18S. Results are presented as the mean ± SD of triplicate amplifications.

**Intracranial injections, tamoxifen treatment, and tumor volume evaluation.** Intracranial injection of Ras-V12-ires-Cre-ER-shp53 lentivirus was performed in 4-week-old IdcTKO mice in accordance with guidelines of the National Institute for Research on Cancer’s Animal Care and Use Committee. Briefly, 1.3 µl purified lentiviral particles in PBS (1 x 10⁹/ml) were injected 1.45 mm lateral and 1.6 mm anterior to the bregma and 2.3 mm below the skull using a stereotaxic frame ( Kopf Instruments). Orthotopic implantation of mouse glioma cells was performed as described previously using 50,000 cells in 2 µl phosphate buffer (26). Tamoxifen was administered using a feeding needle for 4 days at 9 mg/40 g of mouse weight, starting 12 days after surgery and at biweekly intervals thereafter for 3 additional cycles. Mice were monitored daily and sacrificed when neurological symptoms appeared. Tumor volume was obtained using the formula for an ellipsoid: length x width x height x (π/6). The dimensions were derived from a complete, H&E-stained, histological sectioning of the mouse brain. The length was determined by counting the number of sections containing tumor cells and multiplying it by the section thickness. The width and height measurements were taken from the section that showed the largest tumor area (53). Kaplan-Meier survival curve was generated using the DNA Starview software package (AbacusConcepts).

**Mouse tumor microarrays and computational analyses.** Total RNA was extracted from quadruplicate samples of mouse HRasV12-shp53-Cre-ER-IdcTKO–induced tumors treated with tamoxifen or vehicle and used for analysis on Illumina MouseRef-8 version 2.0 expression BeadChip. The raw array data were normalized using the Bioconductor package Lumi using quantile normalization. In order to classify mouse samples according to the human GBM phenotypes, we used data obtained from TCGA Data Portal (54). Description of TCGA data, platforms, and analyses are available at http://tcga-data.nci.nih.gov/. The specific data sources were (according to “Data Levels and Data Types”) as follows: expression data, “Level 2” normalized signals per probe set (Affymetrix HT_HG-U133A). First, we analyzed data to select samples that could be categorized as mesenchymal or proneural according to 3 published GBM phenotypic signatures (20, 26, 27). Differentially expressed genes were selected based on fold change ≥2 and P < 10⁻⁵. Using these stringent criteria, we identified 29 proneural and 41 mesenchymal samples. Then, we applied the R package (55) to the raw array data to normalize mouse and human data and remove batch effects. Finally, we applied the LDA implemented in the MASS package (56) to classify the mouse samples. LDA is a well-established method to obtain a reduced-dimension representation of the data. LDA computes an optimal transformation (projection) by minimizing the within-class distance and maximizing the between-class distance simultaneously, thus achieving maximum class discrimination. In our study, class labels are available (proneural and mesenchymal), and supervised approaches such as LDA are more effective than unsupervised ones such as principal component analysis for classification. In this analysis, the 70 human GBM samples were treated as the training set. LDA returns a value ranging between 0 (not belonging) and 1 (belonging) to each sample in a data set, indicating the membership in each class. Thus, the values returned provide an indication of the likelihood of a sample belonging to each class. Each mouse tumor was then allocated to the class to which it most belongs. Probability equal to 1 indicates the maximum probability. The complement of the probability (1-p) can be interpreted as a P value of the test to indicate how data are consistent with the null hypothesis (the sample does not belong to the predicted subgroup). Significant functional annotation clusters enriched in differentially expressed genes between control and tamoxifen-treated HrasV12-shp53-Cre-ER-IdcTKO tumors were predicted using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Bioinformatics Resources at the National Institute of Allergy and Infectious Diseases, NIH). The microarray expression data have been deposited in the ArrayExpress database (accession no. E-MTAB-1303).

**Statistics.** Results are expressed as mean ± SD or mean ± SEM, as indicated in figure legends, for the indicated number of observations. Statistical significance was determined by the unpaired 2-tailed Student’s t test using GraphPad Prism 4.0 software (GraphPad Inc.). P values are indicated in figure legends.

**Study approval.** All animal studies were reviewed and approved by the IACUC at Columbia University.

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