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RHPN2 Drives Mesenchymal Transformation in Malignant Glioma by Triggering RhoA Activation

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Abstract

Mesenchymal transformation is a hallmark of aggressive glioblastoma (GBM). Here, we report the development of an unbiased method for computational integration of copy number variation, expression, and mutation data from large datasets. Using this method, we identified rhophilin 2 (RHPN2) as a central genetic determinant of the mesenchymal phenotype of human GBM. Notably, amplification of the human *RHPN2* gene on chromosome 19 correlates with a dramatic decrease in the survival of patients with glioma. Ectopic expression of RHPN2 in neural stem cells and astrocytes triggered the expression of mesenchymal genes and promoted an invasive phenotype without impacting cell proliferation. Mechanistically, these effects were implemented through RHPN2-mediated activation of RhoA, a master regulator of cell migration and invasion. Our results define *RHPN2* amplification as a central genetic determinant of a highly aggressive phenotype that directs the worst clinical outcomes in patients with GBM. *Cancer Res*; 73(16); 1–11. ©2013 AACR.

Introduction

Glioblastoma (GBM) is the most common malignant brain tumor and is characterized by rapidly dividing cells, resistance to apoptosis, robust angiogenesis, and extensive invasion. The tendency for local invasion leads to wide dissemination within the normal brain tissue surrounding the tumor, and to the formation of new malignant foci (1). As a consequence, complete tumor resection is almost impossible, leading to inevitable recurrence after surgery (2). Growing molecular evidence suggests that effective therapies against GBM should target the deregulated signaling pathways that promote cell migration and invasion (3, 4), highlighting the need to identify specific genes driving these functional abnormalities.

Malignant transformation in glioma results from the accumulation of genetic aberrations, leading to complex and heterogeneous tumor phenotypes (5). Studies of genomic

characterization, including copy number alterations, gene expression, mutations, and methylation have all been used to identify molecular subclasses of malignant glioma that could inform clinical outcome and predict response to therapy (6–8). The reported classifications of GBM have invariably recognized a mesenchymal gene expression signature in patients with poor clinical prognosis (6–8). The mesenchymal signature includes genes related to the extracellular matrix (ECM), cell adhesion, migration, and tumor angiogenesis. A second signature, the proneural signature, was identified in patients with a more favorable clinical outcome (9). The proneural signature is characterized by genes associated with neurogenesis and is negatively correlated with the mesenchymal signature. Further studies of adult and pediatric GBM described the existence of a third signature, the proliferative one, which is enriched for cell proliferation genes, and their expression is also associated with a poor clinical outcome (6, 10, 11). However, the relationship between the proliferative signature and the other 2 signatures is not entirely clear.

Multiple transcription factors have been implicated in controlling the mesenchymal signature. Gene expression network analysis identified the transcription factors *STAT3* and *CAAT/enhancer binding protein β* as 2 genetically normal genes that drive the mesenchymal signature in GBM (12). More recently, it has been shown that the transcriptional coactivator *TAZ* promotes mesenchymal transformation in malignant glioma (13). However, in addition to genetic alterations of *NFI* that are associated with a small subgroup of mesenchymal GBM (7), the genetic drivers of the mesenchymal signature in malignant brain tumors remain largely unknown. Furthermore, although previous studies identified transcription factors triggering mesenchymal gene expression, genetic and/or epigenetic changes in key signaling pathway molecules driving the mesenchymal phenotype in GBM have not been identified.

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Here, we report on the development of Multi-Reg, a new algorithm that integrates copy number aberrations, expression, and mutation data toward identifying driver genes, and describe its application to data of human GBM collected by The Cancer Genome Atlas (TCGA). A key feature of Multi-Reg is that it associates each driver gene with the GBM subclass it induces. This approach identified rhotaxilin 2 (*RHPN2*) as a novel driver gene of the mesenchymal signature. Experimental follow-up established that *RHPN2* promotes the mesenchymal transformation of neural stem cells (NSC) and increases migration and invasion in different glial cell models. Importantly, *RHPN2* amplification and overexpression correlate with a dramatic decrease in the survival of patients with glioma, supporting the involvement of this protein in the most aggressive features of malignant glioma.

Materials and Methods

Identification of drivers

A detailed description of the computational algorithms used can be found in the Supplementary Materials and Methods. The Multi-Reg algorithm was developed for this research, and the software is available from: <http://www.c2b2.columbia.edu/danapeerlab/html/software.html>.

Cell lines and cell culture conditions

SF188, SNB19, and 293T cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS (Gibco/BRL). Primary human astrocytes (Lonza Inc.) were grown in Astrocyte Medium (Sciencell). Mouse NSC (clone C17.2; ref. 14) were cultured in DMEM plus 10% heat-inactivated FBS (Gibco/BRL), 5% horse serum (Gibco/BRL), and 1% L-glutamine (Gibco/BRL). Neuronal differentiation of mouse NSCs was induced by growing cells in DMEM supplemented with 0.5% horse serum.

Lentivirus infection

Lentiviral expression vectors pLOC *RHPN2* and pLOC VEC and lentiviral vectors carrying *RHPN2* short hairpin RNA (shRNA) were purchased from Thermo Scientific Open Biosystems. To generate lentiviral particles, each expression plasmid was cotransfected with pCMV-dR8.91 and pCMV-MD2.G vectors into human embryonic kidney 293T cells using Eugene 6 (Roche). Lentiviral infections were conducted as previously described (15).

Quantitative reverse transcription PCR and microarray analysis

RNA was prepared with RiboPure kit (Ambion), and used for first-strand cDNA synthesis using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative reverse transcription PCR (qRT-PCR) was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems). Primers are listed in Supplementary Table S1. qRT-PCR results were analyzed by the $\Delta\Delta C_t$ method (16) using 18S as a housekeeping gene.

RNA amplification for mouse array analysis was conducted with Illumina TotalPrep RNA Amplification Kit (Ambion). One-and-a-half micrograms of amplified RNA was hybridized on

Illumina Mouse ref8 v2 expression BeadChip according to the manufacturer's instructions. Hybridization data were obtained with an iScan BeadArray scanner (Illumina) and preprocessed by variance stabilization and robust spline normalization implemented in the lumi package under the R-system (17).

Immunofluorescence

Cells were grown on polylysine (Sigma) treated glass cover slips and fixed with PBS 4% paraformaldehyde for 15 minutes. Then, they were permeabilized (with PBS, 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 2% fetal calf serum) for 5 minutes and saturated with the blocking buffer (PBS, 1% BSA, and 2% goat serum) for 30 minutes. The primary and the following secondary antibodies were incubated at room temperature for 1 hour. These were: smooth muscle actin (SMA; mouse monoclonal, Sigma), β III-tubulin (mouse monoclonal, Promega), fibronectin and paxillin (mouse monoclonal, BD Biosciences), phospho-cofilin (pCofilin; rabbit monoclonal, Cell Signaling Technology), and goat anti-mouse and anti-rabbit Cy3 conjugated (Life Technologies). Actin cytoskeleton was stained with Alexa-Fluor 568 Phalloidin (Life Technologies), and nuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired with Nikon A1R MP confocal microscope. Quantification of the fibronectin intensity staining in mouse NSCs was conducted using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

Cell assays

Wound-healing assay. Exponentially growing cells were seeded (1.5×10^5) in a 24-well plate to create a dense monolayer and then scratched with a 200 μ L tip. Serum-free medium was added after washing in PBS, and wound closure was monitored by taking pictures over time for 24 hours.

Invasion assay. A total of 2×10^4 cells were added to the upper compartment of a 24-well BioCoat Matrigel Invasion Chamber (BD Biosciences) in serum-free DMEM. After 24 hours, invading cells were fixed, stained with crystal violet 0.1%, and counted. In invasion inhibition assays, the RhoA inhibitor I (C3 exoenzyme; Cytoskeleton, Inc.) at a concentration of 1 μ g/mL was used.

Proliferation assay. Cell proliferation was evaluated by MTT assay. Five thousand cells per well were seeded in 96-well plates. At the indicated times, MTT solution (Sigma) in complete medium (0.28 mg/mL final concentration) was added and incubated at 37°C for 4 hours. The medium was discarded, and the formazan salts were dissolved in 4 mmol/L HCl, with 0.1% NP40 in isopropanol. The colorimetric substrate was measured and quantified at 560 nm in an ELISA plate reader.

RhoA activity assay

Exponentially growing cells were serum-starved for 24 hours, detached with Accutase solution (Innovative Cell Technologies Inc.), and adhered to fibronectin-coated dishes (10 μ g/mL; Sigma) for 30 minutes. Then, cells were lysed and 300 μ g of protein was tested in a rhotekin-RBD bead pull-down assay (Rho Activation Assay Biochem Kit, Cytoskeleton Inc.) for 1 hour at 4°C. After thorough washes, the samples were boiled for 5 minutes in Laemmli buffer to

detach active GTP-bound Rho and then loaded on 4% to 20% SDS-PAGE gels (Invitrogen) and immunoblotted using an anti-RhoA antibody.

Results

The Multi-Reg algorithm

An emerging trend in cancer treatment is drugs that target genes and signaling pathways that are only activated in specific cancer cells (18, 19). However, genomics has revealed incredible heterogeneity in cancer, which makes identification of specific genes contributing to cancer progression (driver genes) difficult. Targeting novel drivers is especially important in GBM, because the median survival with conventional therapy is 12 to 15 months (5). A key challenge in identifying driver genes from DNA copy number is that amplification and deletions frequently involve large regions of DNA, each consisting of multiple genes. To pinpoint the driver genes within such

genomic regions, we previously developed CONEXIC (20), a computational algorithm that integrates copy number and gene expression data, to identify driver genes and connect these to their expression signatures. A key limitation to our previous approach is that CONEXIC can only identify the one dominant driver for each expression signature. However, multiple drivers can contribute to the same effect, sometimes acting in parallel. For example, almost all patients with GBM have activated receptor tyrosine kinase signaling and disrupted *p53/RB* signaling, but each patient could have a different combination of deletions, amplifications, and mutations in some of the many genes known to influence these signaling pathways (21).

We therefore developed Multi-Reg, based on a similar framework as CONEXIC, but seeking multiple regulators for each phenotype. This improvement was achieved by a change in the main statistical model. Although CONEXIC

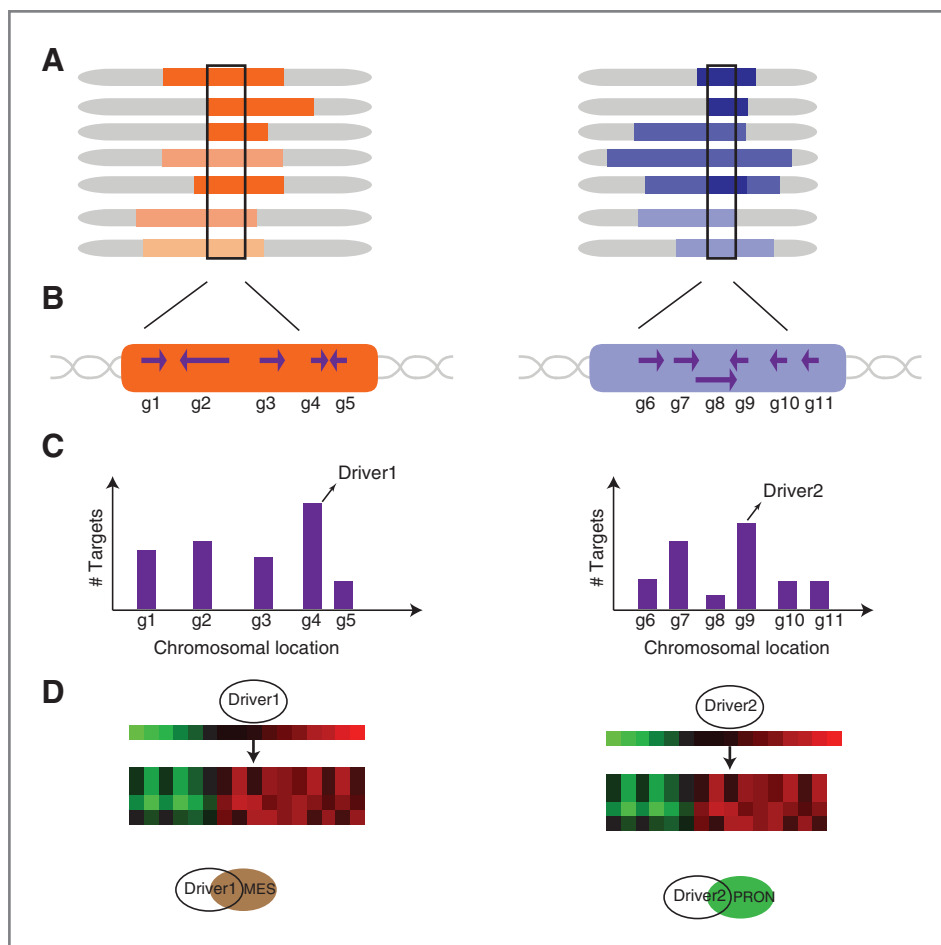


Figure 1. Principles of the algorithm Multi-Reg. A, copy number allows us to identify specific amplified (orange) or deleted (blue) chromosomal regions. B, each chromosomal region includes multiple genes (genes are identified as g1 to g5 for the amplified region and g6 to g11 for the deleted region). C, combining expression allows us to focus on fewer drivers in each region. We can measure the effect each gene has on expression (see Supplementary Materials and Methods for more details) and identify candidate drivers. The purple bars represent the number of targets identified. Genes in the region with more than a minimum of targets are identified as the candidate driver for that region (arrows). D, the overlap between the targets of each driver with known GBM signatures allows us to identify the function of the driver. If most of the targets of a driver overlap with the mesenchymal signature (MES; left), we will assume that the driver contributes to the mesenchymal phenotype. Similarly, if the targets of the driver overlap with the proneural signature (PRON; right), we will assume that it contributes to the proneural phenotype.

and other methods begin from gene expression signatures and attempt to find a driver for each one (22), Multi-Reg begins from candidate drivers and then finds a signature associated with each driver.

Multi-Reg begins from regions that are significantly altered in copy number, either amplified or deleted (Fig. 1A). It identifies all genes in each region as candidate driver genes (Fig. 1B). Then, for each candidate driver, it generates its gene expression signatures, that is, the list of candidate target genes associated with this driver. Comparing the expression signatures between drivers from the same region allows us to focus on significant drivers (Fig. 1C). The final step of the analysis involves assigning the expression signature of each predicted driver to a distinct subtype of GBM (mesenchymal, proliferative, or proneural). This leads to the testable hypothesis that some genes altered in copy number are drivers of distinct biologic functions and the same region may contain multiple drivers that effect distinct GBM subtypes (Fig. 1D). More details on the algorithm can be found in the Supplementary Materials and Methods.

Identifying drivers in glioblastoma

We analyzed gene expression and copy number measurements from 136 samples (corrected for batch effects) of primary GBM from the TCGA cohort (21). See Supplementary Materials and Methods for details. We identified 238 regions that are recurrently altered in copy number using a modified version of GISTIC called JISTIC (23) and generated a list of 747 candidate drivers contained within these regions. Next, we applied Multi-Reg to integrate copy number with gene expression and mutation data to pinpoint the top-ranking candidate drivers and their targets. Applying Multi-Reg resulted in the identification of 83 high-scoring drivers, which associate with a total of 12,125 targets.

Multi-Reg identified many of the well-known oncogenes and tumor suppressors in GBM, including *EGFR*, *NF1*, *CKDN2B*, *p53*, *PIK3CA*, *RB1*, *PTEN* (21), and more (see Supplementary Table S2 for complete list). The successful identification of known key brain tumor drivers increases our confidence in the novel predictions discussed below. To

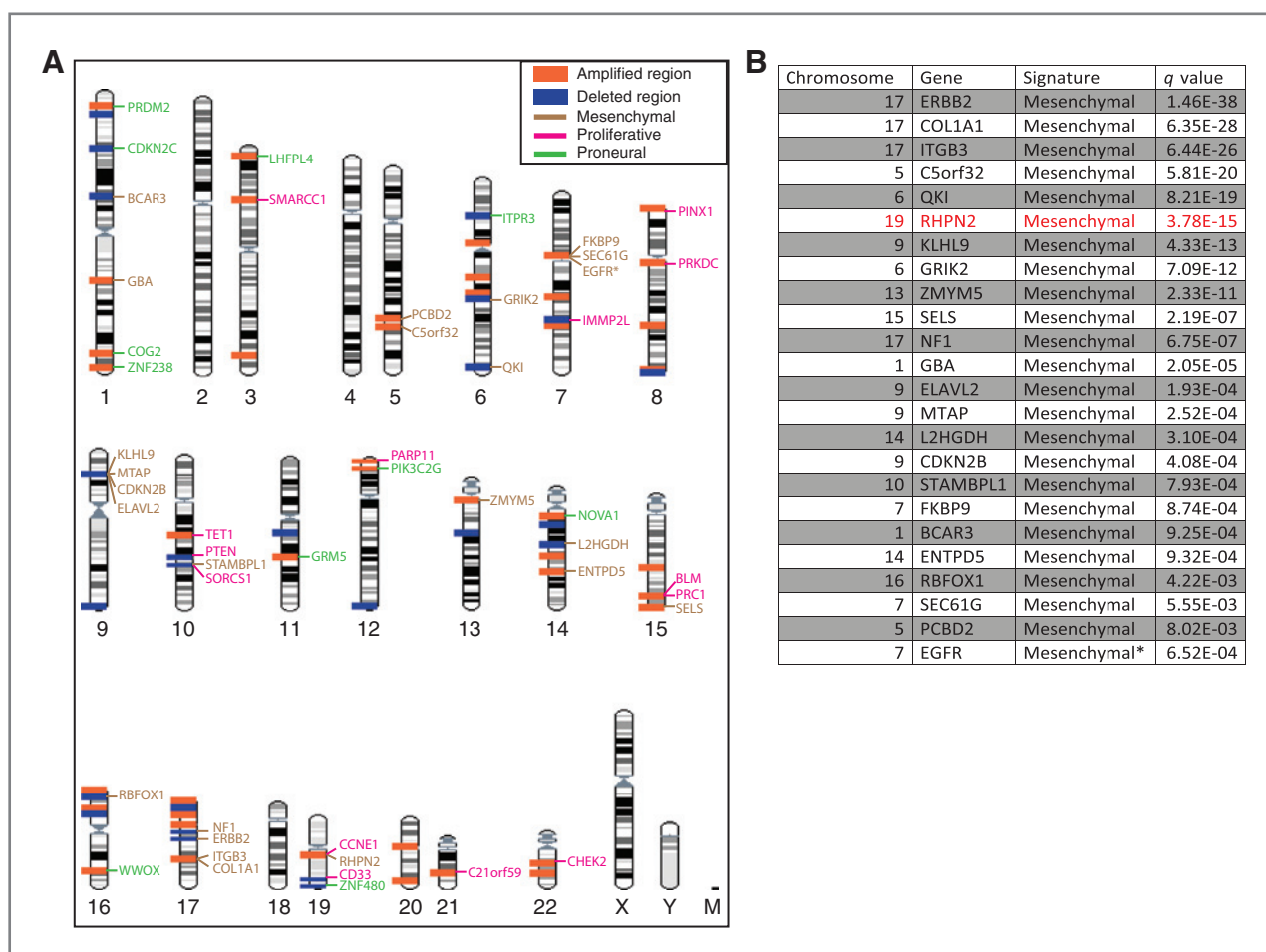


Figure 2. Multi-Reg identifies multiple regions and genes. A, graphical representation of chromosomes with amplified and deleted regions (orange and blue, respectively). Genes identified as candidate drivers are noted and colored according to their signature, mesenchymal (brown), proneural (green), and proliferative (pink). B, list of driver genes identified by Multi-Reg and significantly associated with a GBM signature class, sorted by class and *q* value. In the table, chromosomal location, gene symbol, GBM signature, and *q* value are reported. *, EGFR represses proneural with the *q* value listed.

determine the functional properties of the identified drivers, we compared the gene expression signatures previously associated with mesenchymal, proneural, and proliferative GBM subtypes (12) with the gene modules associated with each driver identified by Multi-Reg, using hypergeometric enrichment.

In addition to well-known drivers of GBM, we identified new drivers that regulate the GBM signatures. Overall, out of the 83 drivers we identified, 23 drivers were associated with the mesenchymal, 14 with the proliferative, and 11 with the proneural signature (Supplementary Table S2 for all drivers; see Fig. 2A and B for a schematic representation of these 48 drivers and the chromosomal location of the drivers associated with the mesenchymal signature). Thus, our algorithm is able to identify drivers for each of the 3 crucial GBM subtypes.

Multi-Reg identifies *RHPN2* as an amplified driver of the mesenchymal subclass

We focused on the genes implicated in the mesenchymal subtype, as this subtype has the worst clinical prognosis. We sorted the 23 genes associated with the mesenchymal signature according to *q* value. The top 3 genes, *ERBB2*, *COL1A1*, and *ITGB3*, were mutated, but they did not harbor copy number changes. The next 2 top genes, *C5orf32* and *RHPN2*, were both amplified and overexpressed. The *RHPN2* gene is located on Chr19q12-q13 and, according to data from TCGA and Rembrandt database, it is amplified and overexpressed in more than 30% of gliomas (Supplementary Fig. S2). This gene codes for a RhoA-binding protein, called RHPN2, which is not well characterized. We focused further experiments on *RHPN2*, given its potential biologic function as regulator of Rho GTPases (24), which are key factors for

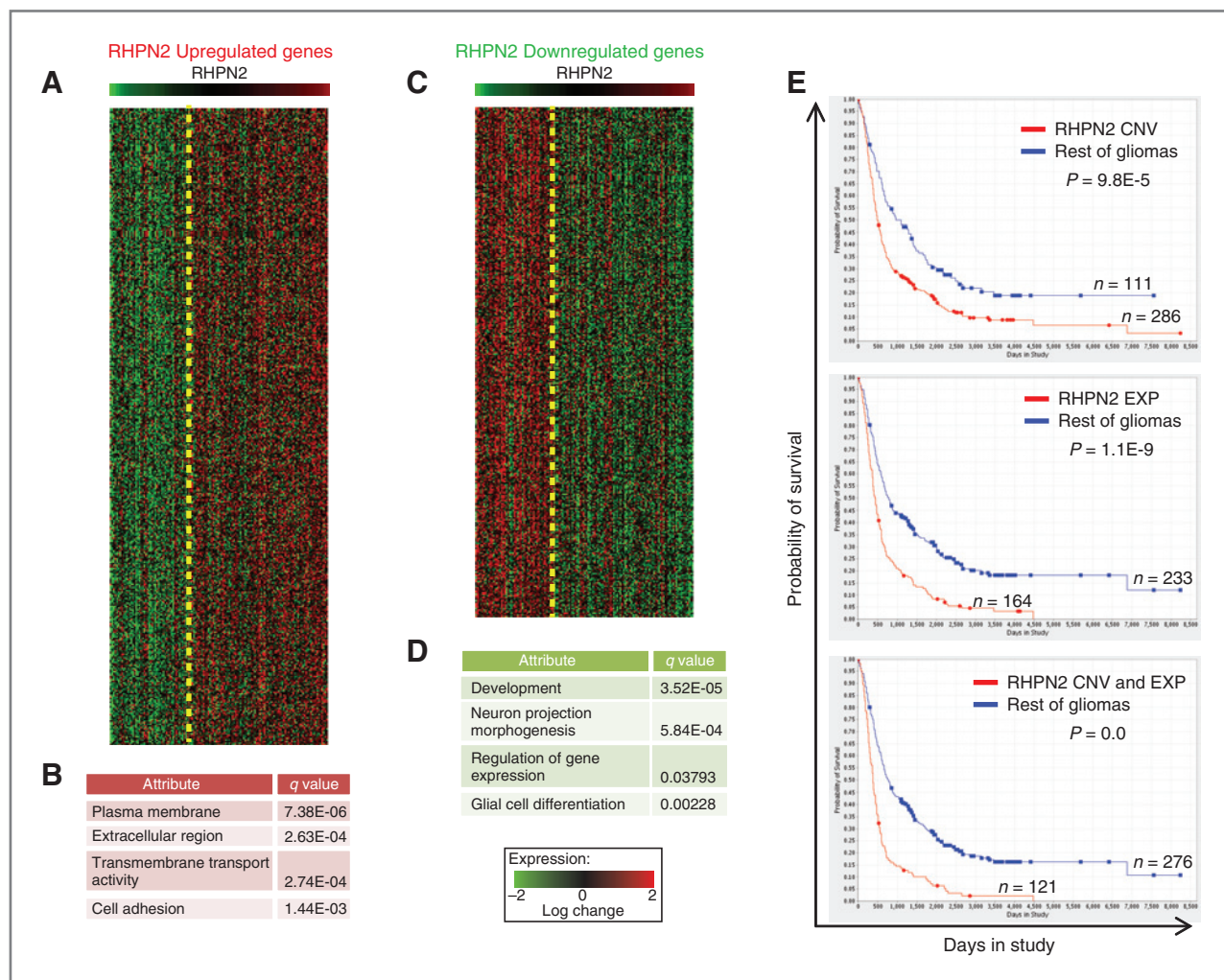


Figure 3. *RHPN2* module analysis. A, module of *RHPN2* upregulated genes generated by Multi-Reg. B, GO enrichment for *RHPN2* upregulated genes. *RHPN2* upregulated genes are related to cell-ECM interactions. C, module of *RHPN2* downregulated genes generated by Multi-Reg. D, GO enrichment for *RHPN2* downregulated genes. *RHPN2* downregulated genes are related to glial cell differentiation. E, survival analysis of patients with glioma from Rembrandt database. Each plot represents the probability of survival of patients with glioma in correlation with CNV (copy number variation ≥ 2), EXP (gene expression ≥ 2), and a combination of both.

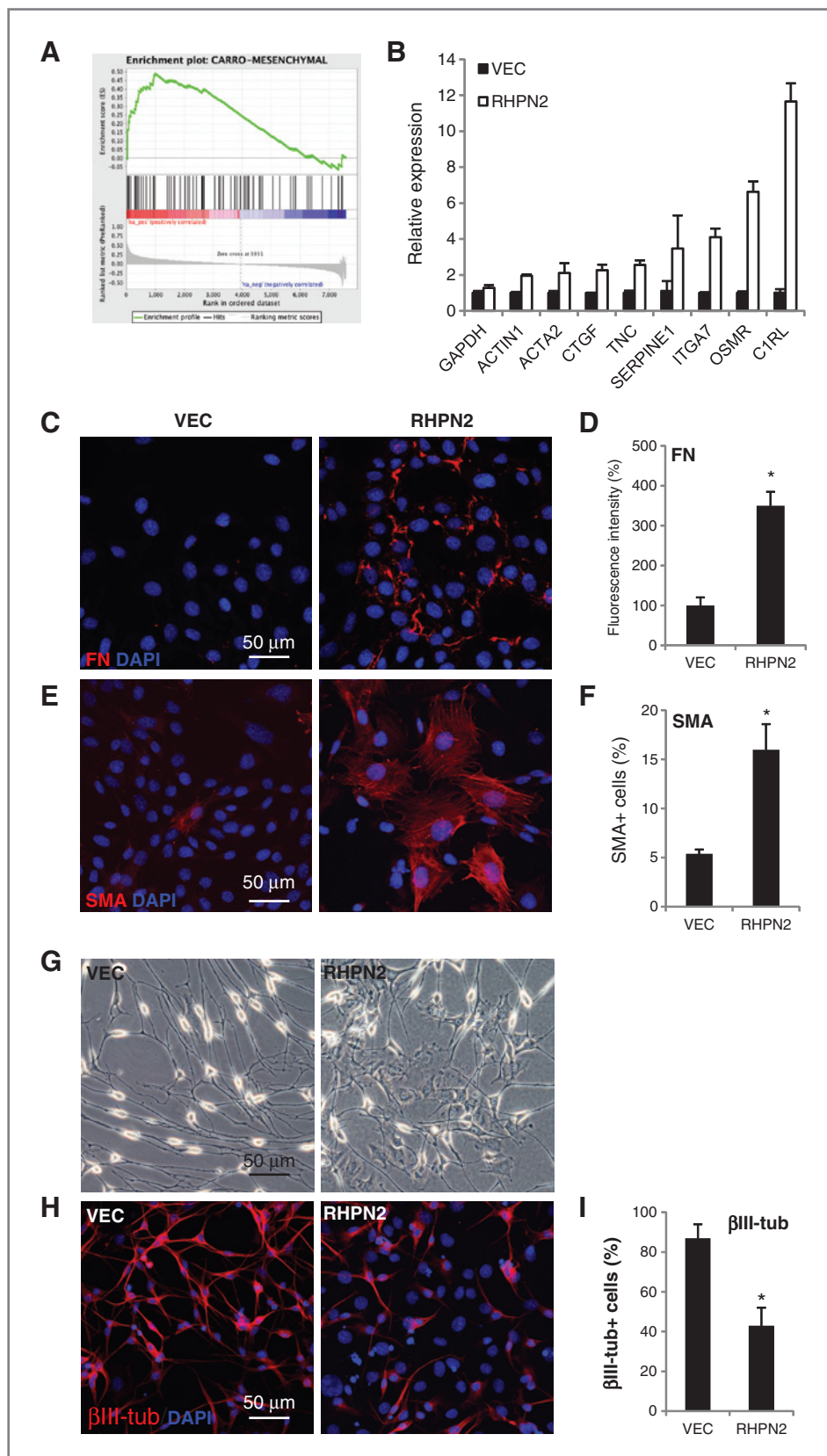


Figure 4. *RHPN2* induces mesenchymal transformation. A, GSEA of mesenchymal gene enrichment on the gene expression profile of C17.2 upon *RHPN2* overexpression. The barcode plot indicates the position of the mesenchymal genes; red and blue indicate a positive and a negative correlation, respectively ($P = 0.0003$). B, qRT-PCR of mesenchymal targets in C17.2 cells. Mean value \pm SD of 3 experiments are reported. C, immunofluorescence analysis of fibronectin (FN, red) on C17.2 expressing the control pLOC vector (VEC) and *RHPN2*. Nuclei are stained with DAPI (blue). Scale bar, 50 μ m. D, ImageJ quantification analysis of C17.2 produced FN. Mean value \pm SD of 20 fields per each condition are reported. *, $P < 0.0003$. E, immunofluorescence analysis of SMA (red) on C17.2. Nuclei are stained with DAPI (blue). Scale bar, 50 μ m. F, quantification analysis of SMA-positive C17.2 cells. Mean value \pm SD of 10 fields per each condition are reported. *, $P < 0.0001$. G, morphology of C17.2 cultured in absence of mitogens for 5 days. Scale bar, 50 μ m. H, immunofluorescence for β III-tubulin (red) of C17.2 cells after 5 days of differentiation. Scale bar, 50 μ m. I, quantification of C17.2 β III-tub+ cells. Mean value \pm SD of 5 fields per each condition are reported. *, $P < 0.0001$.

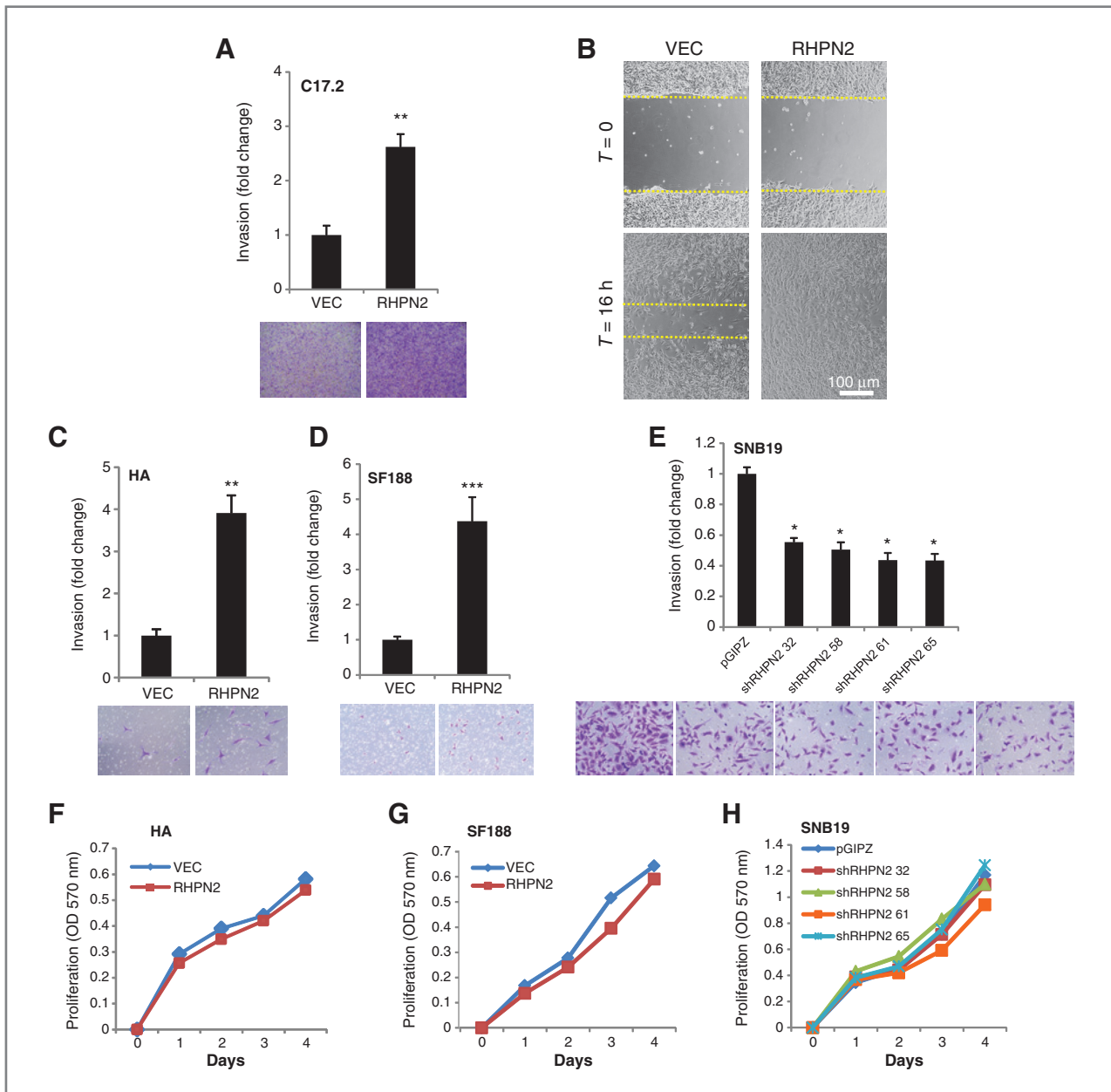


Figure 5. *RHPN2* promotes cell invasion. **A**, quantification of C17.2 invading cells. **, $P < 0.001$. **B**, microphotographs of C17.2 wound-healing assay. Scale bar, 100 μ m. **C** and **D**, quantification of primary human astrocyte (HA) and SF 188 invading cells at 24 hours. **, $P < 0.001$; ***, $P < 0.0001$. **E**, quantification of SNB19 invading cells at 24 hours upon *RHPN2* silencing. *, $P < 0.005$. **F–H**, proliferation of HA, SF188, and SNB19 cells upon overexpression or silencing of *RHPN2*. All graphs in this figure show mean value \pm SD of 3 repeats.

cell migration and invasion, 2 hallmarks of the mesenchymal phenotype.

To further investigate the genes associated with this driver, we used Gene Ontology (GO) enrichment to analyze the function of predicted up- or downregulated target genes identified by Multi-Reg for *RHPN2* (Fig. 3). We found that *RHPN2*-upregulated genes were significantly enriched for plasma membrane, extracellular region, transmembrane transporter activity, regulation of protein kinase cascade, and cell adhesion (Fig. 3A and B), indicating that *RHPN2* regulates the

expression of genes involved in cell–ECM interactions, which is consistent with the induction of a mesenchymal phenotype. Genes downregulated by *RHPN2* were significantly enriched for the GO terms such as development, neuron projection morphogenesis, regulation of gene expression, and glial cell differentiation (Fig. 3C and D) as well as for the proneural signature (q value = 5.33×10^{-13}). Thus, the GO results match the predictions based on GBM signatures, and *RHPN2* induces a mesenchymal phenotype while repressing neuronal and glial cell differentiation.

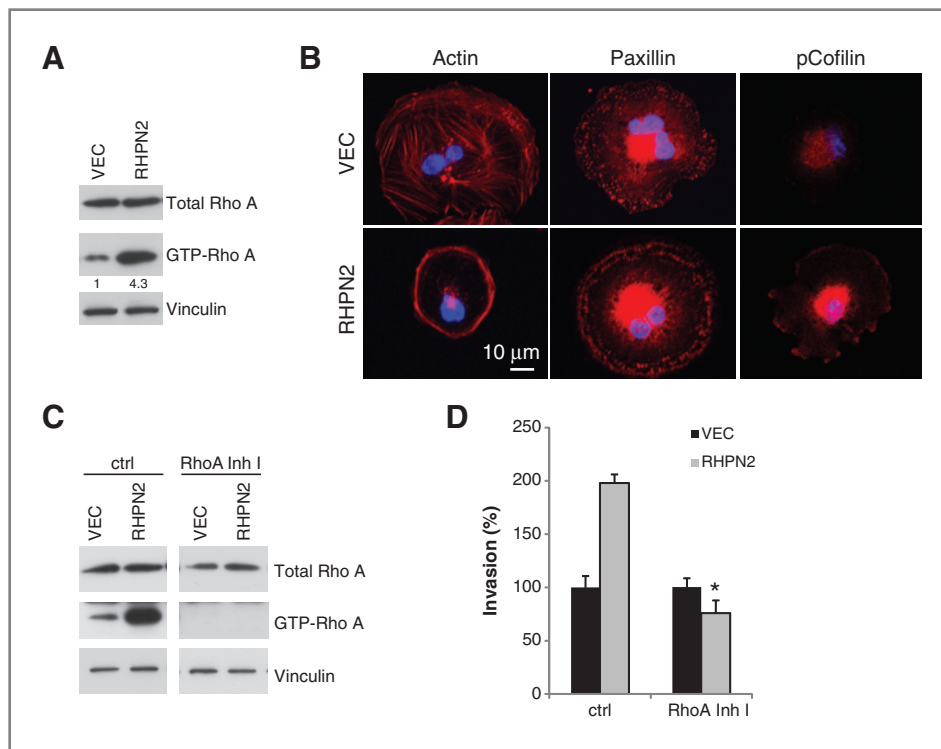


Figure 6. *RHPN2* induces a mesenchymal phenotype by triggering RhoA activation. **A**, Rho activity assay of human astrocytes (HA) after 30 minutes of adhesion on fibronectin. **B**, actin, paxillin, and pCofilin immunofluorescence staining (red) of HA after 30 minutes of adhesion on fibronectin. Nuclei are stained in blue. Scale bar, 10 μ m. **C**, Rho activity assay of HA. Cells were serum starved for 24 hours and treated with 1 μ g/mL of the RhoA inhibitor I for 6 hours. Untreated (ctrl) and treated (RhoA inh I) HA were then plated on fibronectin for 30 minutes and harvested for RhoA analysis. **D**, quantification of HA invading cells at 24 hours. Cells were treated with 1 μ g/mL of the RhoA inhibitor I. Mean value \pm SD; $n = 3$; *, $P < 0.00003$.

A survival study conducted in the Rembrandt database supported the importance of *RHPN2* in GBM and encouraged us to investigate the role of this protein. According to this analysis, *RHPN2* amplification and overexpression predicts a markedly poor clinical outcome of patients with glioma (Fig. 3E). Among the top-ranked drivers selected by Multi-Reg and associated with the mesenchymal genes, *RHPN2* displayed the strongest statistical association with patient survival (data not shown).

To experimentally validate the new driver for GBM aggressiveness inferred by the Multi-Reg algorithm, we conducted biologic assays, overexpressing and/or silencing *RHPN2* in different cell models. We started by testing the expression of specific genes and proteins that are representative of GBM phenotypes, conducted functional assays, and finally explored, in detail, the mechanism of action of *RHPN2*.

***RHPN2* triggers mesenchymal transformation of neural stem cells**

We used recombinant lentiviruses to express *RHPN2* in C17.2 (Supplementary Fig. S3A), a mouse immortalized NSC line previously used to investigate mesenchymal transformation of high-grade glioma (12). Consistent with the computational predictions, microarray gene expression analyzed by gene set enrichment analysis (GSEA) showed that the expression of *RHPN2* in C17.2 led to significant enrichment of mesenchymal genes (Fig. 4A; $P = 0.0003$). This result was validated by qRT-PCR of a representative panel of mesenchymal genes. *Acta1*, *Acta2*, *Ctgf*, *Tnc*, *Serpine1*, *Itga7*, *Osmr*, and *C1rl* were upregulated after ectopic expression of *RHPN2* (Fig. 4B). In addition, *RHPN2* triggered the expression

of mesenchymal proteins as shown by immunostaining for the mesenchymal markers fibronectin and SMA (encoded by the *Acta2* gene; Fig. 4C–F; $P < 0.0001$).

Next, we asked whether *RHPN2* altered the default neuronal differentiation pathway of NSCs. Normally, C17.2 NSCs undergo neuronal differentiation upon mitogen removal (14). However, ectopic expression of *RHPN2* conferred a fibroblast-like morphology (Fig. 4G) and inhibited neuronal differentiation, as evidenced by a decrease in β III-tubulin immunostaining (Fig. 4H and I; $P < 0.0001$). These findings suggest that *RHPN2* blocks neuronal differentiation by reprogramming NSCs toward an aberrant mesenchymal lineage.

***RHPN2* increases invasion in neural stem cells and glioma cell lines**

To elucidate the full scope of the biologic phenotypes triggered by *RHPN2*, we used both gain- and loss-of-function experiments. On the basis of the Multi-Reg predictions about the mesenchymal phenotype, we focused on invasion and migration assays. In addition to mouse NSCs, we used primary human astrocytes, candidate cells-of-origin for GBM, and 2 human glioma cell lines, SF188 and SNB19, which display the lowest and highest *RHPN2* expression levels among several glioma cell lines, respectively (Supplementary Fig. S3B). One of the most distinguishing features of mesenchymal transformation is increased invasiveness (12). Indeed, expression of *RHPN2* in C17.2 cells promoted invasion through the extracellular matrix in a Matrigel invasion assay (Fig. 5A) and enhanced cell migration in a wound assay (Fig. 5B). Interestingly, ectopic expression of *RHPN2* (Supplementary Fig. S3C) resulted in dramatic changes of the morphology of human astrocyte.

Compared with control vector infected cells, human astrocyte expressing *RHPN2* acquired an elongated and spindle-shaped morphology (Supplementary Fig. S3D). Notably, these changes were associated with increased ability to invade through Matrigel (Fig. 5C; $P < 0.001$). A significant gain of invasion was observed also when *RHPN2* was introduced in SF188 cells (Fig. 5D and Supplementary Fig. S3E; $P < 0.00001$). Conversely, silencing of *RHPN2* in the human glioma cell line SNB19 by 4 different shRNA sequences (Supplementary Fig. S3F) significantly decreased the invasive capacity through Matrigel (Fig. 5E; $P < 0.005$). Neither the ectopic expression of *RHPN2* in human astrocyte and SF188 or its silencing in SNB19 affected cell proliferation (Fig. 5F–H).

Taken together, these data indicate that *RHPN2* overexpression is sufficient and necessary to drive transformation of glioma cells along the mesenchymal lineage.

***RHPN2* promotes invasion by activation of RhoA**

We sought to identify the molecular mechanisms by which *RHPN2* amplification/overexpression generates mesenchymal transformation of glial cells. To address this question, we first asked whether *RHPN2* affects RhoA activity in primary human astrocytes. We selected astrocytes as appropriate cellular models for the next series of experiments, as they are candidate cells-of-origin for GBM and display low rates of invasion. Ectopic expression of *RHPN2* (Supplementary Fig. S3C) resulted in more than 4-fold increase of RhoA activity (measured as RhoA-GTP), when compared with control cells (Fig. 6A).

Next, we asked whether the increase in RhoA activity by *RHPN2* affected organization of the actin cytoskeleton, a key determinant of cell migration and invasion. After 30 minutes of adhesion on fibronectin, vector-transduced human astrocyte displayed a well-organized network of actin stress fibers that were distributed in the entire cell body (Fig. 6B, top). *RHPN2*-expressing cells manifested a profound reorganization of the actin cytoskeleton, characterized by lack of stress fibers and accumulation of actin at the cell border in a ring-shaped manner, resembling a nonpolarized lamellipodium (Fig. 6B, bottom). Immunostaining for paxillin, a protein implicated in cell migration (25), showed the expected localization of this protein at the focal contacts in control cells (Fig. 6B, top). However, in human astrocyte transduced with the *RHPN2*-expressing lentivirus, paxillin formed concentric circles at the cell edge, colocalizing with actin (Fig. 6B, bottom). Moreover, the aberrant activation of the RhoA pathway by *RHPN2* resulted in the accumulation of membrane speckles of pCofilin (Fig. 6B), a downstream effector of RhoA (26).

Finally, we asked whether the increased RhoA activity induced by *RHPN2* was responsible for the enhanced cell invasion. Treatment of vector and *RHPN2*-expressing cells with a specific RhoA inhibitor (named RhoA inhibitor I or C3 exoenzyme) completely abrogated RhoA activity after 6 hours (Fig. 6C). An invasion assay revealed that inhibition of RhoA did not affect the basal invasion capacity of human astrocyte transduced with the control lentivirus, but it completely reversed the *RHPN2*-induced invasion (Fig. 6D; $P < 0.00003$). Together, these results indicate that activation of RhoA is

specifically recruited to enhance invasion of human astrocyte after ectopic expression of *RHPN2*.

Discussion

Malignant transformation in gliomas results from the accumulation of genetic aberrations and the deregulation of several key signaling pathways (5). The advent of genome-wide profiling studies of one data type at a time led to the identification of certain driver genes involved in glioma malignancy, but additional important insights can be gained by integrating multiple data. Existing methods for integration have generally found one regulator for a given gene expression signature (20, 22). Recent studies have shown success in identifying drivers based on copy number and expression, but only resulted in a limited number of targets (~500 targets in total; ref. 27).

Here, we presented a new algorithm called Multi-Reg, which improves on these existing methods, finds multiple regulators for genes identified as targets, and associates each driver gene with a relevant phenotype. Finding multiple regulators for each phenotype is a more accurate reflection of the biology of cancer where many amplifications, deletions, and mutations can influence the same signaling pathway (21).

We applied the Multi-Reg algorithm to a GBM dataset, and related our results to known subtypes of glioma. In addition to novel drivers, we correctly identified previously known drivers, thus increasing our confidence in our results. The novel drivers we identified were involved in all 3 glioma subtypes, including proliferative, proneural, and mesenchymal.

Among all the genes selected by Multi-Reg, we focus on those that induce the mesenchymal phenotype, because they should correlate with a bad prognosis and could represent ideal potential therapeutic targets. In particular, we decided to deeply investigate *RHPN2*, a gene located on chromosome 19q12-13, which has been described as a RhoA-binding protein, but its biologic function remained obscure (24, 28, 29). Importantly, Multi-Reg identified *RHPN2*, correctly predicted its resulting phenotype as a mesenchymal subclass inducer and let us shed light on its novel functional role as regulator of invasion in glioma. In accordance with the computational prediction, *RHPN2* ectopic expression in mouse NSCs induced the expression of mesenchymal genes, prevented neuronal differentiation, and promoted invasion and migration, thus operating as a bona fide master regulator of mesenchymal transformation. Notably, *RHPN2* expression was not sufficient for neoplastic transformation and did not affect other cell functions, such as proliferation. Taken together, these results indicate that *RHPN2* amplification and overexpression are not implicated in glioma tumorigenesis, but, most likely, they represent a late event in glioma progression and significantly contribute to worse prognosis of the patients harboring this genetic aberration, as confirmed by a survival analysis (Fig. 3E).

It is important to note that pooled RNA interference screens (30), a popular genome-wide technique that is focused on growth and proliferation would not detect *RHPN2* as a driver because *RHPN2* does not effect proliferation. The ability of Multi-Reg to connect drivers with their phenotypes was a crucial feature in identifying the correct follow-up experiments and, importantly, it unveiled a new biologic function of *RHPN2*.

We investigated in depth the mechanism of action of RHPN2 and showed that this protein promotes mesenchymal transformation by activating RhoA. Increased levels of RhoA-GTP and pCofilin, a downstream effector of RhoA, were detected in *RHPN2*-expressing cells. Notably, the invasive phenotype was reversed upon treatment with a specific RhoA inhibitor, indicating that the *RHPN2*-induced mesenchymal transformation resulted from aberrant RhoA activation.

RhoA is a member of the Rho GTPases, a family of proteins that play essential roles in multiple biologic processes. In particular, their ability to regulate cytoskeletal dynamics, cell adhesion, and cell migration points to a central role in cancer cell invasion and metastasis (31). The involvement of Rho family GTPases in glioma malignancy and invasion has been previously described, but the specific role of RhoA has remained controversial (reviewed by Khalil and El-Sibai; ref. 32). Goldberg and Kloog (3) showed that glioma cell migration was blocked following treatment with a Ras inhibitor, and that this effect was associated with decreased Rac-1 and increased RhoA activity. In contrast, Manning and colleagues (33) reported that blocking RhoA signaling could inhibit lysophosphatidic acid-induced migration in glioma cells. Our findings showed that RhoA activation is necessary for *RHPN2*-induced glioma invasion, matching more closely the role of RhoA described in other cancer models (34) and highlighting RhoA as a potential therapeutic target of GBM. Our study also emphasizes the critical need to enhance the efforts to produce specific RhoA inhibitors for therapeutic applications, especially for tumors that lack effective therapies, such as GBM.

In conclusion, by developing a new integrated analysis and applying it to GBM, we identified a very frequent genetic aberration in this cancer that drives a gain in malignancy through the activation of a key regulator for the mesenchymal phenotype. Notably, this is the first report describing a mes-

enchymal driver that does not belong to the transcription factor category and unveiling the related molecular pathway. By disclosing the role of RhoA in mesenchymal transformation and glioma invasiveness, our work sets the stage for new therapeutic tools in mesenchymal GBM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Danussi, U.D. Akavia, A. Lasorella, D. Pe'er, A. Iavarone

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Danussi, A. Jovic, A. Lasorella, A. Iavarone

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Danussi, U.D. Akavia, D. Pe'er, A. Iavarone

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Jovic, A. Lasorella

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