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ODZ1 supports glioblastoma progression by enhancing proliferation and migration of cancer stem-like cells

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Abstract
Glioblastoma (GBM) tumors are fast-growing and invasive. We found that ODZ1 which is mainly expressed in fetal brain, plays a crucial role in the migration and proliferation of GBM stem-like cells (GSCs). Introduction of the entire ODZ1 or its intracellular fragment into ODZ1-deficient cells promotes cytoskeletal remodelling, migration and invasion by using 2D and 3D substrates and xenograft models. Moreover, ODZ1 increases the proliferation rate of GSCs in culture and generates bigger tumor masses in the brain of xenografted mice. Consistently, higher levels of ODZ1 are correlated with lower survival both in patients and xenografts. ODZ1 promotes the transcriptional activation of RhoA and ODZ1-induced cytoskeletal remodelling, proliferation and invasion activities were blocked by inhibiting RhoA-ROCK axis. Overall, we describe a novel cancer-associated gene involved in the progression of glioblastoma, providing a putative prognostic marker and a new target for therapeutic strategies.

Statement of significance
ODZ1 is a transmembrane protein involved in embryonic brain development but no evidence exists for its role in cancer. In the present study, we demonstrate that ODZ1 supports the proliferation and invasion capabilities of glioblastoma stem-like cells and its expression is inversely associated with survival in glioblastoma patients and xenografted mice.
Introduction

Glioblastoma is the most common brain tumor in adults and is associated with reduced life expectancy, ranging between 12 and 15 months (1). This aggressiveness is mostly due to the rapid growth and the invasive capacity of tumor cells. Even after complete resection of the tumor, local invasiveness eventually leads to regrowth of a recurrent tumor (2). Current therapeutic regimens do not adequately address the disseminated disease burden and rapid growth associated with infiltrative GBMs. Thus, there is an urgent need to develop novel treatments to specifically target the invasive and proliferative capacities of this tumor. Teneurins are philogenetically conserved type II transmembrane proteins (3). ODZ1 (Teneurin-1, TNM1), which is located on the X-chromosome, has an intracellular domain with two nuclear localization signals and may exert transcriptional regulation functions (4). ODZ1 is mainly expressed in the brain during the embryonic development (reference?). It has been demonstrated that GBM contains hierarchies with highly tumorigenic cells that display stem cell features on top of them (5). These GBM stem-like cells (GSCs) are governed by molecular mechanisms active in brain development, including Notch, Wnt, BMP, TGFβ and receptor tyrosine kinase pathways (6). Moreover, there are many examples of genes that play essential roles in embryonic development and are also involved in promoting or facilitating cancer in adult tissues (7-9). As a representative example, the Hedgehog family of proteins plays an instructional role during the development of many metazoans and is implicated in stem cell maintenance and tissue repair, but also confers growth promoting and survival capabilities to cancer cells (10). So far, expression analyses by using a network of
differentially expressed genes or oligo-based DNA arrays, have suggested an association of ODZ1 with prolactin pituitary tumor metastasis (11) and papillary thyroid carcinoma (12). However, no direct evidence exist that ODZ1 is involved in any process aimed at inducing tumorogenesis or facilitating tumor progression. Recently, we showed that activation of Rho GTPases Rac and RhoA contributed to the invasive capacity of a subpopulation of GBM stem-like cells (GSCs) isolated from the peritumoral tissue (13). Rho GTPases are key regulators of cytoskeleton dynamics and cell polarity, cell cycle progression, cell migration and metastasis (14), which indicates their potential use as therapeutic targets in cancer. Although there is little understanding on the activity of RhoA in primary GSCs, reduced activation of this GTPase correlates with decreased invasive capacity of GSCs (15, 16). Targeting upstream regulators or Rho effectors, alone or in combination with other chemotherapeutic agents might provide effective therapeutic opportunities in different tumors, including glioblastoma.

We have analyzed the role of ODZ1 in GSCs and found that this protein is needed for neurosphere-containing cells to bind to the substrate and emit protrusions that enable them to migrate and invade the surrounding environment. ODZ1 is also key to facilitate proliferation of GSCs. Xenograft animal models show that the presence of ODZ1 increases the spreading and growth of the tumor and reduces survival. We also showed that ODZ1 exerts these tumor-facilitating activities by inducing the expression of RhoA and activation of downstream ROCK kinases.

**Results**

**ODZ1 expression in GSCs.** In normal tissues ODZ1 is mainly expressed in the developing brain (Supplementary Fig. S1). We analyzed the expression of ODZ1 in more
than sixty samples of neurosphere cultures derived from GBM patients. Although the mRNA levels of ODZ1 were very heterogeneous among samples, there were two GSC cultures, G104 and G59, with no or very low expression of this gene (Supplementary Fig. S2A and B). Interestingly, these GSCs did not show the typical morphological changes when they were induced to differentiate (adhesion to the substrate, cytoplasmic extensions) but remained forming neurospheres (Supplementary Fig. S2C). However, the gene expression pattern of differentiation was indistinguishable from that of ODZ1-expressing GSCs as determined by upregulation of the astrocytic marker GFAP and downregulation of the stem cell marker CD133 (Supplementary Fig. S2D). Then, we studied the cause for the lack of ODZ1 expression. Microarray-based comparative genomic hybridization revealed that G104 carried a 6 Mb genomic deletion that included the entire ODZ1 gene (Supplementary Fig. S3A). This deletion was present in both GSCs and GBM tissue obtained during surgery (Supplementary Fig. S3B). Data from the cBioPortal for cancer genomics (17, 18) revealed a frequency of ODZ1 gene deletion lower than 1% in glioblastoma samples. However, it has also been detected in other cancers with higher frequencies including adrenocortical carcinoma (9%) and brain low grade gliomas (3.4%) (Supplementary Fig. S3C). Since ODZ1 gene is not deleted in G59 GSCs, we studied a potential repression by promoter methylation. Although we did not find a canonical CpG island spanning the ODZ1 promoter, treatment of G59 with the demethylating agent 5-aza-2'-deoxycytidine increased more than 3-fold the expression of ODZ1 (Supplementary Fig. S3D). Thus, we cannot rule out that methylation contributes to the very low levels of ODZ1 in these cells. Unless otherwise indicated, G59 and G104 cells, named here as ODZ1-deficient GSCs, were used as cell models and all results were confirmed in both models.
ODZ1 expression promoted spreading of GSCs in a chicken embryo model and reduced survival in GBM patients. In order to study the function of ODZ1 in the GBM stem-like cell compartment, we transfected ODZ1-deficient GSCs with ODZ1 (Fig. 1A). Ingenuity Pathway Analysis (IPA) of microarray gene expression data of these cells showed that cell morphology and cellular assembly and organization are among the top network functions associated with the expression of ODZ1 (Supplementary Table S1). Interestingly, ODZ1-transfected cells recovered the phenotypic features of cells undergoing differentiation, showing adhesion to the substrate and protrusion formation with F-actin location at the edges of projections (Fig. 1B and C). There was also an increase in the number of rounded cells with short protrusions and elongated cells with long protrusions, and a reduction in the number of rounded cells with no protrusions (Fig. 1D). Similar pattern was observed in cells cultured on a laminin-coated surface, although there was a significant increase in the number of cell projections (star-shaped cells) (Fig. 1E). We also observed that cells were able to migrate out of the neurosphere when they were forced to express ODZ1 and this migration promoting effect was detected in neurospheres cultured into different matrices made of (collagen I either alone or in combination with hyaluronic acid or matrigel) (Fig. 1F). To further analyze the capacity of ODZ1 to induce migration, we transfected ODZ1-expressing GSCs with shRNAs targeting this gene. One of them, shRNA-2, significantly downregulated the endogenous levels of ODZ1 protein (Fig. 2A) and promoted a 6-fold increase in the number of rounded cells in suspension that formed neurospheres over time (Fig. 2B and C). Moreover, GSCs with downregulated levels of ODZ1 tended to be less dispersed and more aggregated when injected into chicken embryos as compared with the same cells transfected with an irrelevant shRNA (Fig. 2D). Conversely, ODZ1-deficient cells transfected with ODZ1...
acquired the ability to propagate through the surrounding tissue in the embryo (Fig. 2E). Interestingly, patients having GBM tumors with high expression of ODZ1 had a lower mean survival than those with reduced ODZ1 levels as determined by analysing data of the Repository for Molecular Brain Neoplasia Database (Rembrandt) (Fig. 2F).

**ODZ1 promoted more aggressive tumors in xenografted mice.** In order to translate our previous results to a more physiologically relevant in vivo model, we studied the role of ODZ1 in the tumor development and survival of xenografted mice. First, different GBM cell lines were analyzed for their levels of ODZ1. Among those with the lowest expression (Fig. 3A), we chose RG1 because it efficiently and rapidly generates tumors in immunodeficient mice (19). ODZ1-transfected RG1 cells promoted larger tumors than their control counterparts as determined on T2-weighted MRI scans (Fig 3B), and consequently the survival of these animals was significantly reduced (Fig. 3C), which is consistent with the survival data in GBM patients. In a second model, ODZ1-deficient GSCs were transfected with either the entire ODZ1 or its cytoplasmic fragment (Fig. 3D and E), the 45 kDa N-terminal part of the protein. We showed that this fragment (icODZ1) was sufficient to recover the expected morphology of GSCs undergoing differentiation (Fig. 3F). These transfectants were xenografted into the brain of immunodeficient mice and about 5 months later animals were sacrificed. Immunohistochemical analysis of brain slices confirmed bigger tumors in mice grafted with ODZ1- and icODZ1-containing cells as determined by using different markers including vimentin that specifically stained human tissue and served to delimitate the extension of the tumor, human GFAP that is expressed in tumor cells, and Ki67 as a proliferation marker (Fig. 3G).

**The intracellular fragment of ODZ1 is a key mediator in promoting morphologic changes in differentiating GSCs.** ODZ1-deficient GSCs were transfected with the entire
ODZ1, the intracellular fragment or the extracellular plus the transmembrane regions (ecODZ1) (Fig. 4A). These cells were cultured under differentiation conditions and four days later filamentous actin (F-actin) was stained with phalloidin. As shown in Fig. 4B, both ODZ1 and icODZ1 promoted long actin-filled protrusions but cells transfected with ecODZ1 behaved as the control cells and showed little changes in morphology. icODZ1 also promotes localization of CAP/Ponsin to focal adhesions (Fig. 4C), a protein that has been shown to reorganize F-actin at cell-extracellular matrix contacts (20). An epithelial-to-mesenchymal-transition-like program highlighted by a T-cadherin to N-cadherin switch has been described in GBM cells associated with invasion and a worse prognosis (21). Interestingly, N-cadherin along with other mesenchymal markers such as Vimentin and Snail were upregulated whereas Tcadherin was downregulated in icODZ1-transfected GSCs (Fig. 4D and E). Moreover, consistent with the chemoresistance associated with the mesenchymal phenotype, cells expressing icODZ1 were more resistant to temozolomide, a nitrosourea commonly used in GBM patients (Fig. 4F). We further evidenced that icODZ1 was not only able to promote morphological changes but also to induce or facilitate migration in both 2D and 3D substrates. Wound healing assays showed that ODZ1 and icODZ1 but not ecODZ1 efficiently repopulated the scratch area (Fig. 5A and B). Moreover, invasion assays in 3D collagen matrices, which is more representative of the tumor niche than 2D surfaces, demonstrated that although ODZ1 promoted migration of GSCs through the gel (about 3-fold increase relative to control), icODZ1 was significantly more efficient (more than 6-fold increase relative to control) (Fig. 5C and D). Thus, these results suggest that GSCs with higher levels of ODZ1 would have an advantage to migrate and invade the surrounding environment.
The intracellular fragment of ODZ1 mediates its activity on GSCs by transcriptional activation of RhoA. Rho GTPases regulate the morphologic changes associated with motility and invasion by controlling the actin polymerization and actomyosin contraction (22). Thus, we asked whether ODZ1 could mediate its activity through a Rho protein. First, ODZ1-deficient GSCs were transfected with an inducible icODZ1-containing vector. Induced expression of icODZ1 was followed by a specific increase in the mRNA levels of RhoA but not Rac1 or Cdc42 (Fig. 6A). icODZ1 is directly targeted to the nucleus (Fig. 6B), where it may exert transcriptional regulation activities. This could explain the stronger effects of icODZ1 when compared with the membrane-associated protein. We showed that icODZ1 was able to activate the RhoA promoter. In a RhoA promoter-luciferase reporter assay, icODZ1 induced a 5-fold increase in luciferase activity (Fig. 6C). Total RhoA protein expression and RhoA activity, as determined by the levels of GTP-bound RhoA, were also upregulated by icODZ1 (Fig. 6D). Activation of RhoA was further determined by analyzing phosphorylation of MLC2, a downstream mediator of RhoA-ROCK signaling. As shown in Fig. 6E, icODZ1 and to a lesser extent ODZ1 but not ecODZ1 increased the level of pMLC2 as detected by immunofluorescence. These results indicate that icODZ1 promotes expression of RhoA and activation of the RhoA-ROCK axis. To confirm this, we analyzed the localization of Ponsin at focal adhesions, a hallmark of ODZ1 activity, in the presence of a RhoA inhibitor, a ROCK inhibitor or by using siRNAs against ROCK1 and ROCK2 (Fig. 7A-D). In all cases, Ponsin accumulation to projection tips was efficiently blocked by the inhibition strategies. Moreover, icODZ1-promoted invasion of GSCs in a 3D collagen matrix, another hallmark of ODZ1 activity, was blocked with ROCK inhibitor. The
presence of H1152 gave rise to a 4-fold decrease in the invasion capacity of icODZ1-expressing cells (Supplementary Fig. S4A and B).

**ODZ1 increases the proliferation rate of GSCs.** A consistent observation in cultures of ODZ1-deficient GSCs transfected with ODZ1 or icODZ1 is that they grow faster than control or ecODZ1-transfected cells. This effect was quantitated over a period of 48 h and found that the proliferation rate in icODZ1 was about twice higher than control or ecODZ1-transfected cells. ODZ1 also promoted a clear increase in proliferation although at a slightly lower level than icODZ1 (Fig. 7E). Consistent with our previous data, ROCK inhibitor reduced the proliferation promoted by icODZ1 and ODZ1 but had no effect on cells expressing ecODZ1 (Fig. 7E). This result was confirmed by using a Rho inhibitor which significantly reduced ODZ1-mediated proliferation, although in this case the effect was detected at 72 h (Supplementary Fig. S5A). RhoA activation is crucial for the cell cycle G1-S progression through the regulation of CDKN/Cip family of CDK inhibitors. Microarray analysis of gene expression showed that among members of this family CDKN1A (p21) and CDKN2C (p18) were downregulated in ODZ1-transfected cells (Supplementary Fig. S5B) which are both known to be regulated by RhoA (23).

**Discussion**

Although in recent years a great effort has been made to understand the molecular features of GBM through tumor genetics and mouse modelling, few of these findings have entered into clinical practice to date. Thus, there is still a need to deepen into the mechanisms used by GBM cells to become invasive and resistant to chemotherapy.

We have focussed this work on ODZ1 gene, which is predominantly expressed in the developing brain. There are no previous evidences to support the role of ODZ1 in cancer.
However, Ingenuity Pathway Analysis (IPA) of gene expression microarray data generated by our group revealed that 149 cancer-associated genes are differentially expressed in GSCs transfected with ODZ1. Two GSC samples with no or very little expression of ODZ1 were used as ODZ1-deficient GSCs in this work. GSCs grow in suspension forming neurospheres and they attach to the substrate and acquire a flat and extended morphology under differentiation conditions (24). We found that ODZ1-deficient GSCs do not acquire the morphology of differentiating cells, although the genetic program of differentiation appears to be normal. The capacity of ODZ1 to control adherence to extracellular matrix and morphological changes of GSCs is in line with the IPA network analysis which showed that cell morphology, cellular assembly and cell-cell interactions are among the top cellular functions of ODZ1. Morphological changes are driven by cytoskeletal reorganization, which is a key feature of cell migration. We showed that ODZ1 promotes actin cytoskeletal remodelling, migration and invasion of GSCs as determined by using 2D and 3D in vitro systems and a xenograft model of chicken embryo, where ODZ1-expressing GSCs efficiently invade the surrounding environment. Previous work in Drosophila suggested that the ODZ1 homolog Ten-m is involved in remodelling of the postsynaptic cytoskeleton, physically linking the synaptic membrane to the cytoskeleton (25). Moreover, it has been described that chicken ODZ1 interacts with CAP/ponsin, which in turn binds to vinculin that could anchor the intracellular region of ODZ1 to the actin cytoskeleton (4). Consistently, we found that ODZ1 promoted long actin-filled protrusions and the localization of ponsin at projection tips in GSCs, which strengthen the role of ODZ1 as a regulator of cytoskeletal remodelling and provides the first evidence of this ODZ1 activity in cancer cells. Actin cytoskeleton has key roles in cell cycle progression (26). Thus, not surprisingly, ODZ1 increases the proliferative
capacity of GSCs. Invasiveness and proliferation are two hallmarks of GBM and strategies aimed at targeting pathways that control these processes, mainly in GSCs, are the focus of intensive research (2729). Rho GTPases are key players in controlling cytoskeleton dynamics, cell migration and cell division (14). Elevated levels of RhoA have been described in clinical samples of high-grade gliomas (30). Interestingly, ODZ1 is able to induce the expression and activation of RhoA in GSCs. Moreover, RhoA is transcriptionally activated by the intracellular fragment of ODZ1 (icODZ1) that is translocated to the nucleus. icODZ1, which lacks a DNA binding domain, is likely to interact with transcriptional regulators to control gene expression. In line with this, it has been described that icODZ1 binds to the transcriptional repressor MBD1 (4). Further studies will be needed to decipher how icODZ1 activates gene expression. By using different inhibition strategies, we demonstrated that RhoA signaling mediates the cytoskeletal remodelling and the increase in cell migration, invasion and proliferation promoted by ODZ1. Although RhoA has been typically associated with rounded morphology and amoeboid motility, there is insufficient functional evidence for the role of RhoA in promoting this type of cell migration in GBM cells (31). In line with our data, high levels of RhoA activity have been associated with elongated morphology and mesenchymal-type invasion in different cancer cell lines (32, 33). Shifts towards a mesenchymal phenotype appear to be a common pattern of disease progression in GBM patients and are reminiscent of epithelial-mesenchymal transitions (EMT) of epithelial tumors (34). We demonstrate that ODZ1-induced changes in GSCs are associated with the acquisition of a mesenchymal phenotype characterized by an EMT-like gene expression signature. Mesenchymal GBM show aggressive dissemination, often with multifocal presentation as well as resistance against chemotherapy. Consistently, ODZ1-
expressing GSCs are more invasive and more resistant to temozolomide. The relevance of Rho-ROCK pathway in promoting migration and growth of GBM cells has been described by using established cell lines (35, 36). Our data provides a novel ODZ1-RhoA-ROCK axis for therapeutic strategies against a pathway controlling key activities in GSCs. Blockade of RhoA expression or activation through its upstream regulator ODZ1 has the advantage that this protein is located at the cell membrane which facilitates inhibition strategies with small molecules or antibodies. Additionally, contrary to other integral membrane proteins associated with GBM such as EGFR that is widely expressed in a number of adult tissues, or RhoA which is ubiquitously expressed across tissues and is involved in a number of cellular functions, ODZ1 expression is mostly restricted to fetal brain, which could make ODZ1-targeted therapies more tumor-specific limiting their side effects.

In summary, our study shows for the first time that ODZ1, a protein that participates in the embryonic development of the brain, is also involved in cancer progression by promoting the growth and invasion capabilities of glioblastoma stem-like cells. Thus, these data provide a novel and promising target to develop therapeutic strategies aimed at blocking the two features that make glioblastoma so aggressive and lethal.

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Materials and Methods

Primary cell cultures. Cell cultures from tissue specimens used in this study have been previously characterized and described by our group (13, 37). GSCs were maintained in serum-free DMEM/F12 medium (Life Technologies, Paisley, UK) as described and plated at a density of $3 \times 10^6$ live cells/60-mm plate. Primary neurospheres were dissociated every 4 days to facilitate cell growth. To promote differentiation, neurospheres were cultured in the same medium but in the presence of 10% FBS for four days. Established GBM cell line RG1 (L0627), kindly provided by Dra. Rosella Galli, was cultured as previously described (19). Cell proliferation and cell survival were evaluated with Alamar Blue bioassay (Life Technologies) using a Synergy HT reader (BioTek, Winooski, VT).

When indicated, cells were treated with 5 μM ROCK inhibitor H1152 (Tocris Bioscience, Bristol, UK), 1 μg/ml Rho Inhibitor I (Cytoskeleton, Inc., Denver, CO) or 100 μM Temozolomide (Merck, Whitehouse Station, NJ).

In vivo models. Tumor xenografts in chicken were established as previously described (13). Briefly, GSCs were labeled with CFDA SE Cell Tracer (Invitrogen, Carlsbad, CA) and microinjected into the embryos limb bud. After 24 hrs of incubation, embryos were fixed in 4% PFA, and the dissected limbs monitored by confocal microscopy. Images shown are the integration of all Z-stacks taken along the dorsal-ventral plane at 15 μm intervals to cover the whole limb.

For tumor xenografts in mice we followed a procedure described elsewhere (38). This model was reviewed and approved by the Research Ethics and Animal Welfare
Committee at the Instituto de Salud Carlos III, Madrid, in agreement with the European Union and national directives. Briefly, $5 \times 10^4$ GBM cells were resuspended in 2 ml of culture medium and stereotactically injected in athymic nude Foxn1<sup>nu</sup> mice (Harlan Laboratories, Indianapolis, IN). The injections were made into the striatum and the animals were sacrificed at onset of symptoms.

**Mouse magnetic resonance imaging (MRI).** MRI analysis was performed in mice injected IP with Gd-DOTA (Dotarem, Bloomington, IN). T1W images were acquired in a 4.7 TBiospec BMT 47/40 spectrometer (Bruker, Billerica, MA) with a spin-echo sequence (Centro de Apoyo a la Investigación, CAI, Universidad Complutense, Madrid, Spain).

**Generation of anti-ODZ1 antibodies.** Two anti-ODZ1 antibodies were generated in rabbits. The anti-N-terminus antiserum was raised against a GST fusion protein containing 40 amino acids of the N terminal region of ODZ1, and the anti-C-terminus included 68 aminoacids of the C terminal region. Both antibodies were purified from the antiserum by affinity chromatography using GST-ODZ1 fusion protein-Sepharose columns, after preclearing of anti-GST antibodies.

**Immunofluorescence analysis.** GSCs were grown on glass cover slips previously coated with 10 µg/ml fibronectin (Sigma- Aldrich, St Louis, MO), 0.1% gelatin (Sigma) or 10 µg/ml laminin (Sigma). After 2-4 days, cells were fixed in 4% PFA for 15 min, washed in PBS and blocked with 0.3% Triton X-100, 1% BSA for 1 hr. Cells were incubated with anti-phospho-myosin light chain 2 (Ser19) (Cell Signaling, Danvers, MA), or anti-Ponsin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. Then, the cells were washed with PBS and incubated with Texas red-conjugated or FITC-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch,
Cambridgeshire, UK) for fluorescence detection. Actin cytoskeleton was stained with TRITC- or FITC-conjugated Phalloidin (Sigma) and nuclei visualized with DAPI (Life Technologies). Samples were mounted with ProLong Gold Antifade Reagent (Life Technologies). Confocal images were taken with a Ti-Eclipse microscope (Nikon, Tokyo, Japan).

**Immunohistochemical staining.** Mouse brains containing tumors generated by injection of ODZ1-transfected GSCs were fixed in 4% PFA. Vibratome sections (200 μm thick) were paraffin-embedded and then consecutive semithin sections were processed and incubated with primary antibodies against Vimentin, GFAP and Ki67 (DAKO, Glostrup, Denmark) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by a chromogen or stained with hematoxilin-eosin.

**Quantitative RT-PCR and RT-PCR.** To assess the expression of individual genes, a cDNA was generated and amplified using primers for human CD133, Tuj1, glial fibrillary acidic protein (GFAP), α-Actin (Nogueira et al., 2011), T-Cadherin

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\begin{align*}
5'\text{TTCTGTGC}A\text{CCTCCTGTCC}^3' & \quad \text{and} \quad 5'\text{TTCAGAGCTAAGC}^3' \text{C}
\end{align*}
\]

NCadherin \(5'\text{GACAATGCCCTCAAG}GTT^3'\) and
\[
5'\text{CCATTAAGCGA}G\text{GTGATGG}^3'
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Vimentin \(5'\text{TCGGCGGGACAGC}^3'\) and
\[
5'\text{GGTGACGTA}G\text{TCACGTC}^3'
\]

Snail \(5'\text{TAGCGAGTGGTTCTTCTC}G^3'\) and
\[
5'\text{AGGGCTGCTGA}G\text{AGTG}A^3'
\]

icODZ1 \(5'\text{ACTCAAGAGATGGAATTCTCTG}^3'\) and \(5'\text{CTTAGTGCA}G\text{TCAGGG}^3'\), ecODZ1 \(5'\text{ACAATGATGGACGGTG}CTT^3'\) and \(5'\text{GTGTCCTC}CCTCCCTCTATG}^3'\), ODZ1 exon1 \(5'\text{GGACCAATTGTG}A\text{ATCTGCC}^3'\) and
\[
5'\text{CCTACAACCTCAGC}^3'
\]

ODZ1 exon20 \(5'\text{GGGAAGGT}TTTTGCG}^3'\) and
\[
5'\text{CCACTGTGCTA}G\text{AGGC}^3'
\]

ODZ1 exon32
(5′CTTGCAAGCCTGTCCTTTCC3′ and 5′CCCAGTGGTACCGATGAGC3′), and Stag2 (5′CACGCCTGGCTAATTTTTGT3′ and 5′CAATACAGGGCAGGTGTGCT3′).

Quantitative real-time PCR was performed in a 7000 sequence detection System (Life Technologies). At least three independent experiments for each quantitative PCR were performed.

**Expression Microarray.** Total RNA from empty vector- and ODZ1-transfected cells was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) and microarray gene expression analysis was performed with the Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA). Both background correction and normalization were done using Robust Multichip Average algorithm. The selection of those genes differentially expressed was performed using a criteria based on the fold-change value. Probe sets were selected as significant using a logFC cut-off of 1.5. The raw data have been deposited in a MIAME compliant database (GEO accession number, GSE65526).

**Copy number variation analysis.** DNA copy number changes were evaluated using Affymetrix GeneChip 250-NspI/StyI SNP microarrays as previously described (13). Bioinformatic analysis was performed using Affymetrix Genotyping Console 3.0.2 and Partek 6.5 software (Partek Incorporated, St Louis, MO).

**2D Wound Healing Assay.** For the wound-healing assay, cells were seeded into collagen I coated 24-well plates and grown to confluence. The cell monolayers were carefully scratched with a 200μl pipette tip and cells were incubated in fresh medium supplemented with serum. To monitor the migration of cells into the wounded area, cell cultures were photographed under the phase contrast microscope at 0 and 24 h post wounding. Cell migration was expressed by the percentage of wound closure, n= 4.  **3D Invasion Assays.** Cells (10^5 cells/ml) were included in serum-free matrix composed of 1.7 mg/ml collagen
I alone (Nutacon BV, Leimuiden, The Netherlands), or collagen I combined with 2.3 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ) or hyaluronic acid (Sigma) into 96-well ViewPlates (PerkinElmer, Waltham, MA) and processed as previously described (Sanz-Moreno et al., 2008; Ruiz-Ontañón et al., 2013). Confocal Z slices were collected at 50 μm, 30 μm and 3 μm (bottom of the well) with a Ti-Eclipse microscope.

Transfections, Gene Silencing and Gene Reporter Assays. ODZ1-deficient GSCs were transfected with the entire ODZ1 cDNA, the extracellular (plus transmembrane) fragment (both from Origene, Rockville, MD) or the intracellular fragment, that was PCR-amplified from ODZ1 cDNA and subsequently cloned into pCMV6. Stable transfectants were selected with 500 μg/ml geneticin (Life Technologies). icODZ1 was subcloned in a tetracycline-regulated retroviral vector (39). Cells were transiently transfected with this vector and the expression of ODZ1 was induced in the presence of 2 μg/ml doxycycline (Sigma).

For ODZ1 downregulation, GSCs were transfected with ODZ1-specific shRNAs (Thermo Fisher Scientific, Waltham, MA) by using nucleofection. GSCs were transfected with ROCK1 and ROCK2 siRNAs (Dharmacon's SmartPools, Thermo Fisher Scientific) by Lipofectamine RNAiMAX (Life Technologies).

Cells were co-transfected with 1 μg of human RhoA promoter cloned into pGL3 vector (40) and 50 ng of pRSV-Δ-gal by nucleofection. 48h post-transfection, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems, Foster City, CA). Results were normalized for tranfection efficiency with values obtained with pRSV-Δ-gal.
**Western Blot.** Total protein extracts were separated on 8 or 12% polyacrylamide gels or 4-20% gradient gels (BioRad, Hercules, CA) and transferred to nitrocellulose. Blots were blocked with 5% bovine serum albumin (BSA) and incubated with antibodies against GAPDH, β-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), RhoA (Cell Signaling Technology, Danvers, MA), ODZ1 (R&D Systems, Minneapolis, MN) and NCadherin (Sigma) followed by incubation with goat anti-rabbit or anti-mouse or antisheep conjugated to alkaline phosphatase (Sigma). Bound antibody was detected by a chemiluminiscence system (Applied Biosystems).

**Statistical analysis.** All statistics were calculated with the SPSS statistical package (version 13.0). Data are presented as mean ± S.D. of at least three independent experiments. Differences between groups were tested for statistical significance using the unpaired 2-tailed Student’s *t* test. The significance level was set at *p*<0.05.

**Author’s Contributions**

**Conception and design:** JL Fernandez-Luna

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References


**Figure legends**

**Figure 1.** Transfection of ODZ1-deficient GSCs with ODZ1 restores the morphology of differentiating GSCs. (A) ODZ1 protein levels in ODZ1-transfected GSCs. The levels of
□-tubulin were analysed to assure equal loading. Transfected cells were cultured in differentiation medium and 4 days later their morphology (B) and the distribution of Factin (C) were assessed by using phase contrast or fluorescence microscopy respectively. (D) GSC transfectants cultured in differentiation medium were counted as a function of their morphology as rounded, R, rounded with protrusions, R+P, and elongated, E. Histograms represent the mean ± S.D. (n=3; *p<0.01, **p<0.05). (E) Factin staining of cells differentiated onto a laminin-coated surface. (F) Phase contrast images of ODZ1-transfected neurospheres cultured in collagen-based matrices.

**Figure 2.** ODZ1 expression is sufficient to induce cell spreading in vitro and in vivo. (A) GSCs were transfected with different shRNAs targeting ODZ1 and the expression levels of this protein were determined 72 h later. The levels of □-tubulin were analysed to assure equal loading. (B) GSCs were transfected with the most effective ODZ1-specific shRNA (sh-2) and the number of living cells floating in the culture medium were quantitated. sh-C, irrelevant shRNA. Values represent the mean±S.D. (n=3; *p<0.001). (C) GSCs transfected with ODZ1-specific shRNA-2 and EGFP formed neurospheres under differentiation conditions as determined by fluorescence microscopy. (D) CFDA SE-labeled GSCs with downregulated levels of ODZ1 were injected into the chicken embryo limb and 24 h later cell spreading was determined by confocal microscopy. (E) ODZ1-deficient GSCs were transfected with ODZ1 or empty vector and xenografted in chicken embryo as above. (F) Rembrandt data set showing the survival of GBM patients with differential expression of ODZ1.

**Figure 3.** ODZ1-expressing tumors are more aggressive. (A) ODZ1 mRNA levels in different GBM cell lines by RT-PCR. □Actin expression was used for signal normalization. (B) Representative T2-weighted nuclear magnetic resonance (NMR)
images showing tumors in the brain of mice xenografted with ODZ1-transfected RG1 GBM cells. (C) Kaplan-Meier survival curves of mice harboring RG1-derived tumors with high or low levels of ODZ1. (D) Schematic representation of the ODZ1 protein showing the intracellular (IC) transmembrane (TM) and extracellular (EC) parts of the protein. NLS, nuclear localization signal. (E) Expression of ODZ1 and icODZ1 in transfected GSCs. The levels of β-tubulin were analyzed to assure equal loading. (F) ODZ1-deficient cells were transfected with icODZ1 and cultured under differentiation conditions. Cell morphology was assessed after 4 days of culture. Scale bar: 50 μm. (G) ODZ1-deficient GSCs transfected with the entire ODZ1 or its intracytoplasmic fragment (icODZ1) were xenografted in mice and brain sections were obtained for immunohistochemical analysis. Note the intracranial tumor staining (brown) by using markers that reveal the presence of tumor cells. HE: hematoxylin-eosin staining. Figure 4. ODZ1 promotes actin cytoskeleton remodeling and induces a chemotherapy resistant mesenchymal-like phenotype. (A) RT-PCR showing the expression of ODZ1 in ODZ1-deficient GSCs transfected the intracellular fragment (icODZ1), the extracellular plus transmembrane fragment (ecODZ1) or the entire ODZ1 with oligonucleotides specific for the ic or ec regions of the cDNA. β-Actin expression was used for signal normalization. (B) Representative confocal images of the different GSC transfectants showing F-actin staining. Scale bar, 10 μm. (C) ODZ1-deficient GSCs expressing icODZ1 accumulates the focal adhesion protein ponsin at projection tips as determined by immunofluoreescence. Scale bar: 10μm. (D) icODZ1-dependent mRNA expression levels of the epithelial-like marker T-Cadherin (T-Cad) and the mesenchymal specific markers N-Cadherin (N-Cad), Vimentin (Vim) and Snail as determined by qRT-PCR. Histograms
represent the mean±S.D. (n=3; *p<0.01, **p<0.001). (E) N-Cadherin protein expression in transfectant GSCs. The levels of β-tubulin were analyzed to assure equal loading. (F) icODZ1-transfected GSCs were treated with 100μM TMZ and 48 h later cell viability was determined by using alamarBlue bioassay. Values represent the mean±S.D. (n=3; *p<0.001).

**Figure 5.** ODZ1 promotes cell migration and invasion. (A) Micrographs of a woundhealing assay showing the migration of different transfectant GSCs towards the scratched area. (B) Quantification of migration in the wound healing assay as percentage of wound closure. Histograms represent the mean±S.D. (n=3; *p=0.01 compared with pCMV6 control). (C) Representative images of a cell invasion assay in a 3D collagen matrix. Nuclei were stained with DAPI. (D) Quantification of the invasion assay as percentage of cells detected at 30 μm from the bottom. Histograms represent the mean±S.D. (n=3; *p<0.05, **p<0.001 compared with pCMV6 control).

**Figure 6.** ODZ1 triggers the transcriptional activation of RhoA. (A) The mRNA expression levels of Rho GTPases RhoA, Rac1 and Cdc42 were analyzed by qRT-PCR in ODZ1-deficient GSCs transfected with an icODZ1-containing doxycycline-inducible construct. Histograms represent the mean±S.D. (n=3; *p<0.01). (B) The intracellular fragment of ODZ1 protein was localized within the nucleus after transfection with icODZ1, as determined by immunofluorescence with antibodies against the N-terminal part of the protein. (C) ODZ1-deficient GSCs were cotransfected with a RhoA promoter-luciferase reporter construct and icODZ1 and luciferase activity was determined 48 h later. Histograms represent the mean±S.D. (n=3; *p<0.01). (D) GTPbound RhoA and total RhoA protein expression in GSC transfectants were determined in ODZ1-deficient cells transfected with icODZ1. The levels of β-tubulin were analyzed to assure equal...
loading. (E) ODZ1 transfectants were analyzed for the expression of phosphorylated MLC2 by immunofluorescence. Single plane confocal micrographs were taken under the same intensity settings. Scale bar: 10 μm.

**Figure 7.** ODZ1 increases focal adhesions and promotes proliferation through RhoA activation. (A-D) The expression of the focal adhesion protein Ponsin is shown in icODZ1-transfected GSCs in the presence or in the absence of different inhibitors of the RhoA-ROCK pathway, Rho inhibitor I, ROCK inhibitor H1152 and siRNAs against ROCK1 and ROCK2. Single plane confocal micrographs were taken under the same intensity conditions. Scale bar: 5 μm. (E) GSC transfectants were cultured in the presence or in the absence of ROCK inhibitor H1152 for the indicated time intervals and proliferation was assessed by Alamar Blue assay. Histograms represent the mean±S.D. (n=3; *p=0.001, **p<0.001 compared with untreated control).

**Supplementary Figure S1.** ODZ1 is mainly expressed in fetal brain. Microarray data for ODZ1 expression in different tissues were taken from the BioGPS data set (http://biogps.org).

**Supplementary Figure S2.** Morphological changes in GSCs undergoing differentiation depend on ODZ1. (A) mRNA levels of ODZ1 in different GSC samples were determined by quantitative RT-PCT. (B) ODZ1 protein expression in GSC samples. Tubulin was used to assure equal loading. (C) Neurospheres were disaggregated and cultured in the presence of FBS. Morphological changes of differentiating cells were visualized after 4 days of culture. (D) Gene expression levels of markers associated with differentiation of GSCs were determined by quantitative RT-PCR. S, GSCs. D, GSCs undergoing differentiation. Histograms represent the mean±S.D. (n=3; *p<0.01, **p<0.05).
Supplementary Figure S3. Genetic basis for the lack of ODZ1 expression. (A) Representation of the X-Chromosome showing the size of the deleted genomic fragment and the deleted genes in G104 GSCs. (B) Amplification of different exons of the ODZ1 gene from GSC-derived genomic DNA. Stag2 was used as a positive control of amplification. (C) ODZ1 gene deletion frequency in different tumors was obtained from the cBioPortal for cancer genomics. (D) G59 GSCs were treated with the DNA demethylating agent 5-aza-2'-deoxycytidine (3 μM 5-Aza) for 3 days and the ODZ1 expression was determined by quantitative RT-PCR. Histograms represent the mean±S.D. (n=3; *p<0.01).

Supplementary Figure S4. Inhibition of ROCK blocks icODZ1-promoted invasiveness. (A) Invasion assay of GSC transfectants in a 3D collagen matrix with or without H1152. DAPI-stained cells were visualized by confocal microscopy at different focal planes. (B) Quantification of the invasion assay as percentage of cells detected at 20 μm from the bottom. ecODZ1-expressing cells were included here for comparison. Histograms represent the mean±S.D. (n=3; *p=0.0005).

Supplementary Figure S5. Rho inhibition reduces cell proliferation induced by ODZ1. (A) GSC transfectants were cultured in the presence or in the absence of Rho inhibitor I for the indicated time intervals and proliferation was assessed by Alamar Blue assay. Histograms represent the mean values±SD. (n=3; *p=0.005 compared with untreated control). (B) Gene expression microarray data showing the fold change of CDKN/Cip family of CDK inhibitors in ODZ1-transfected cells compared with control cells. Dashed line marks the fold change cut-off (log2FC>0.5) used in the microarray analysis.

Supplementary Table S1. Ingenuity cellular functions and disorders associated with
ODZ1.