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ODZ1 allows glioblastoma to sustain invasiveness through a Myc-dependent transcriptional upregulation of RhoA

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Running title: ODZ1 promotes invasiveness of glioblastoma

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1 **Abstract**

2 Long-term survival remains low for most patients with glioblastoma which reveals the
3 need for markers of disease outcome and novel therapeutic targets. We describe that
4 ODZ1 (also known as TENM1), a type II transmembrane protein involved in fetal brain
5 development, plays a crucial role in the invasion of glioblastoma cells. Differentiation
6 of glioblastoma stem-like cells drives the nuclear translocation of an intracellular
7 fragment of ODZ1 through proteolytic cleavage by signal peptide peptidase-like 2a. The
8 intracellular fragment of ODZ1 promotes cytoskeletal remodelling of glioblastoma cells
9 and invasion of the surrounding environment both in vitro and in vivo. Absence of
10 ODZ1 by gene deletion or downregulation of ODZ1 by small interfering RNAs
11 drastically reduces the invasive capacity of glioblastoma cells. This activity is mediated
12 by an ODZ1-triggered transcriptional pathway, through the E-box binding Myc protein,
13 that promotes the expression and activation of Ras homolog family member A (RhoA)
14 and subsequent activation of Rho-associated, coiled-coil containing protein kinase
15 (ROCK). Overexpression of ODZ1 in glioblastoma cells reduced survival of
16 xenografted mice. Consistently, analysis of 122 glioblastoma tumor samples revealed
17 that the number of ODZ1-positive cells inversely correlated with overall and
18 progression-free survival. Our findings establish a novel marker of invading
19 glioblastoma cells and consequently a potential marker of disease progression and a
20 therapeutic target in glioblastoma.

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24 **Keywords:** glioblastoma, ODZ1, RhoA, invasiveness, proliferation

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1 **Introduction**

2 Glioblastoma (GBM) is the most common brain tumor in adults and is associated with
3 reduced life expectancy, ranging between 12 and 15 months ¹. This aggressiveness is
4 mostly due to the rapid growth and the invasive capacity of tumor cells. Even after
5 complete resection of the tumor, local invasiveness eventually leads to regrowth of a
6 recurrent tumor ². Current therapeutic regimens do not adequately address the
7 disseminated disease burden and rapid growth associated with infiltrative GBMs. Thus,
8 there is an urgent need to develop novel treatments to specifically target the invasive
9 and proliferative capacities of this tumor. Gene expression profiling of GBMs identified
10 molecular subclasses with prognostic value that were designated proneural, proliferative
11 and mesenchymal ³. Kaplan-Meier plots showed that median survival of the proneural
12 subclass was markedly longer than any of the other two subtypes. It has been
13 demonstrated that GBM contains hierarchies with highly tumorigenic cells that display
14 stem cell features ⁴. These GBM stem-like cells (GSCs) are governed by molecular
15 mechanisms active in brain development, including Notch, Wnt, BMP, TGF β and
16 receptor tyrosine kinase pathways ⁵. Moreover, there are many examples of genes that
17 play essential roles in embryonic development and are also involved in promoting or
18 facilitating cancer in adult tissues ⁶⁻⁸. As a representative example, the Hedgehog family
19 of proteins plays an instructional role during the development of many metazoans and is
20 implicated in stem cell maintenance and tissue repair, but also confers growth
21 promoting and survival capabilities to cancer cells ⁹. Teneurins are phylogenetically
22 conserved type II transmembrane proteins ¹⁰. ODZ1 (Teneurin-1, TNM1), which is
23 located on the X-chromosome, is mainly expressed in the brain during the embryonic
24 development ¹¹. This transmembrane protein has an intracellular region with two
25 nuclear localization signals, which may exert transcriptional regulation functions ^{12, 13}.

1 So far, expression analyses by using a network of differentially expressed genes or
2 oligo-based DNA arrays, have shown increased ODZ1 levels in prolactin pituitary
3 tumor metastasis ¹⁴ and papillary thyroid carcinoma ¹⁵. However, no direct evidence
4 exist that ODZ1 is involved in any process aimed at inducing tumorigenesis or
5 facilitating tumor progression. The *Drosophila* ortholog of ODZ1 interacts with α -
6 spectrin, a cytoskeleton protein that binds to filamentous F-actin, suggesting a
7 mechanism whereby ODZ1 organize the cytoskeleton ¹⁶. Recently, we showed that
8 activation of Rho GTPases Rac and RhoA contributed to the invasive capacity of a
9 subpopulation of GSCs isolated from the peritumoral tissue ¹⁷. Rho GTPases are key
10 regulators of cytoskeleton dynamics and cell polarity, cell cycle progression, cell
11 migration and metastasis ¹⁸, which indicates their potential use as therapeutic targets in
12 cancer. Although there is little understanding on the activity of RhoA in primary GSCs,
13 reduced activation of this GTPase correlates with decreased invasive capacity of GSCs
14 ^{19, 20}. Targeting regulators of cytoskeleton dynamics and invasion might provide
15 effective therapeutic opportunities in glioblastoma.

16 In the current study, we found that ODZ1 is needed for GBM cells to migrate and
17 invade the surrounding environment. Xenograft animal models and tumor specimens
18 from GBM patients indicate that the presence of ODZ1 increases the spreading and
19 growth of the tumor and reduces survival. We also showed that ODZ1 exerts these
20 tumor-facilitating activities by inducing the expression of RhoA and activation of
21 downstream ROCK kinases.

22

23 **Results**

24 **Identification of ODZ1-deficient GSCs**

1 We found that two GSC samples, G104 and G59, did not show the typical
2 morphological changes when they were induced to differentiate (adhesion to the
3 substrate, cytoplasmic projections) but remained forming neurospheres (Figure 1a).
4 However, the expression pattern of differentiation was indistinguishable from other
5 GSC cultures, as determined by analyzing the levels of both GSC and lineage markers
6 (Supplementary Figures S1 and S2). GSCs and GBM tissue from the G104 sample
7 carried a 6 Mb deletion of the long arm of chromosome X that included four genes,
8 SH2D1A, ODZ1, SMARCA1 and BCORL (Figure 1b-d). Among those, ODZ1
9 appeared to be the most interesting gene because it has been associated with
10 cytoskeleton organization and cell shape. Data from the cBioPortal for cancer genomics
11 ^{21, 22} revealed a frequency of ODZ1 gene deletion lower than 1% in glioblastoma
12 samples. Protein expression analyses confirmed the lack of ODZ1 in G104 but also
13 showed very low levels of ODZ1 in G59 cells (Figure 1e). Since ODZ1 gene is not
14 deleted in G59 GSCs (Figure 1d), we studied a potential repression by promoter
15 methylation. Treatment of G59 with the demethylating agent 5-aza-2'-deoxycytidine
16 increased more than 3-fold the expression of ODZ1 (Figure 1f). Although we did not
17 find a canonical CpG island in the ODZ1 promoter, there were three CpG dinucleotides
18 within a fragment of 60 nucleotides located 344 bp upstream of the transcription start
19 site. Analysis of the methylation state at each CpG site in G59 and G63 cells (see Figure
20 1e for comparing levels of ODZ1) revealed an inverse correlation between the level of
21 methylation and the expression of ODZ1 (Supplementary Figure S3). Thus, it is likely
22 that methylation contributes to downregulation of ODZ1 in these cells. Unless
23 otherwise indicated, G104 and G59 cells, named here as ODZ1-deficient GSCs, were
24 used as cell models. Results shown correspond to G104 and were all confirmed in G59
25 cells.

1 **ODZ1 expression promoted spreading of GSCs in vitro and in vivo**

2 Comparison between ODZ1-deficient cells before and after transfection with ODZ1
3 (Figure 1g) by gene expression microarrays revealed that cell morphology and cellular
4 assembly and organization are among the top network functions associated with the
5 expression of ODZ1 (Supplementary Table S1). Interestingly, ODZ1-transfected cells
6 recovered the phenotypic features of differentiated cells, showing adhesion to the
7 substrate and protrusion formation with F-actin location at the edges of projections
8 (Figure 1h-j). Similar pattern was observed in cells cultured on a laminin-coated surface
9 (Supplementary Figure S4a). We also observed that cells were able to migrate out of
10 neurosphere when they expressed ODZ1 (Supplementary Figure S4b). Then, we
11 transfected ODZ1-expressing GSCs with shRNAs targeting this gene. One of them,
12 shRNA-2, significantly downregulated the endogenous levels of ODZ1 protein (Figure
13 2a,b) and promoted a 6-fold increase in the number of non-attached cells that formed
14 neurospheres (Figure 2c,d). Similar effects were observed with the less efficient
15 shRNA-3 (data not shown). Moreover, GSCs with downregulated levels of ODZ1
16 tended to be less dispersed and more aggregated when injected into chicken embryos
17 (Figure 2e). Conversely, ODZ1-deficient cells transfected with ODZ1 acquired the
18 ability to propagate through the surrounding tissue in the embryo (Figure 2f). Consistent
19 with this model, analysis of ODZ1 protein expression in tumor samples from GBM
20 patients revealed the increase of ODZ1 levels in the invading area (Figure 2g,h). When
21 sections of paraffin-embedded neurospheres were immunostained with anti-ODZ1, the
22 staining was mainly localized in cells at the periphery of neurospheres (Figure 2i) likely
23 revealing those cells with higher migratory capacity. Overall, these data strongly
24 suggest that ODZ1 expression enables GBM cells to invade the surrounding
25 environment.

1 **ODZ1 expression in GBM tumors reduced survival in patients and xenografted**
2 **mice.**
3 **RG1 cell line is highly efficient in promoting rapid development of GBM tumors in**
4 **xenografted mice ²³, and we detected low expression levels of ODZ1 (Figure 3a).**
5 ODZ1-transfected RG1 cells promoted larger tumors than their control counterparts
6 (Figure 3b), and the survival of these animals was significantly reduced (Figure 3c). In a
7 second model, ODZ1-deficient GSCs were transfected with either the entire ODZ1
8 (about 300 kDa) or its cytoplasmic fragment (Figure 3d,e), the 45 kDa N-terminal
9 region of the protein (icODZ1). We showed that icODZ1 was sufficient to recover the
10 morphology of differentiated GSCs (Figure 3f). These transfectants were xenografted
11 into the brain of immunodeficient mice. Immunohistochemical analysis of brain slices
12 confirmed larger tumors in mice grafted with ODZ1- and icODZ1-containing cells as
13 determined by using different markers including vimentin that specifically stained
14 human tissue, human GFAP that is expressed in tumor cells, and Ki67 as a proliferation
15 marker (Figure 3g). Then, we analyzed the expression of ODZ1 in a tissue microarray
16 that included 122 tumor samples from GBM patients (Supplementary Table S2). The
17 anti-ODZ1 antibody that we generated clearly distinguished between tissues that
18 express or do not express ODZ1 (Figure 3h,i). Interestingly, using 35% as a cutoff
19 point, we found that the number of ODZ1-positive cells was inversely correlated with
20 overall survival and disease-free (or progression-free) survival, defined as the time from
21 resection to the first radiological recurrence (Figure 3j,k). The increase in estimated
22 median disease-free survival for patients with lower proportion of ODZ1-positive cells
23 (<35%) was about 45% compared with patients having higher levels of positive tumor
24 cells (7 vs 3.9 months). The increase in median overall survival was 24% (12.5 vs 9.5
25 months). These results were consistent with those determined by analysing data of the

1 Repository for Molecular Brain Neoplasia Database (Rembrandt) (Supplementary
2 Figure S5a). Analysis of a public dataset ³ revealed that grade IV gliomas (GBMs) have
3 significantly higher ODZ1 levels than grade III gliomas (Supplementary Figure S5b).
4 Overall, animal models and patient studies demonstrate that ODZ1 expression in GBM
5 tumors clearly correlates with poorer survival.

6 **The intracellular fragment of ODZ1 is released through proteolytic cleavage**
7 **following differentiation of GSCs.**

8 The signals for cleavage and the proteases that release icODZ1 still remain to be
9 identified. As shown in Figure 4a the expression of ODZ1 mRNA increased 3 to 4-fold
10 after differentiation, and most of the produced protein was cleaved to generate icODZ1
11 (Figure 4b). The increased levels of ODZ1 following differentiation was confirmed by
12 immunofluorescence, which also revealed the nuclear translocation of icODZ1 (Figure
13 4c). ODZ1-deficient cells were transfected with the full-length protein and most of the
14 ODZ1 staining was localized within the nucleus in differentiated cells (Figure 4d).
15 Then, we treated cells with (Z-LL)2 Ketone, an inhibitor of SPP and the homologous
16 SPP-like (SPPL) proteases, that selectively cleave type II integral membrane proteins.
17 (Z-LL)2 Ketone blocked the nuclear localization of icODZ1, giving an ER/Golgi and
18 cell surface staining, similar to the reported distribution of full-length ODZ1
19 overexpressed in HT1080 cells ¹². However, L685,458, an inhibitor of γ secretase, which
20 cleaves type I transmembrane proteins, even at a high concentration (10 μ M), had no
21 effect on ODZ1 nuclear localization (Figure 4d,e). Moreover, the SPP/SPPL inhibitor
22 blocked ODZ1-mediated migration of GSCs in a Boyden chamber-based assay (Figure
23 4f). A search of our gene expression database of GSCs before and after differentiation ²⁴
24 revealed that SPPL2a showed the highest levels among members of this protease family
25 and its expression was even increased in differentiated cells. Based on these data, we

1 knockdown SPPL2a by siRNA in GSCs (Figure 4g). As shown in Figure 4h,i the
2 nuclear staining of icODZ1 observed in control cells or after knocking down another
3 protease such as S2P, a zinc metalloprotease, was drastically reduced in the absence of
4 SPPL2a.

5 **The intracellular fragment of ODZ1 is a key mediator in promoting changes in cell**
6 **shape and invasion capacity of GSCs.**

7 ODZ1-deficient GSCs were transfected with ODZ1, icODZ1 or the extracellular plus
8 the transmembrane regions (ecODZ1) (Figure 5a). As shown in Figure 5b, both ODZ1
9 and icODZ1 promoted long actin-filled projections following differentiation, but cells
10 transfected with ecODZ1 behaved as the control cells and showed little changes in
11 morphology. icODZ1 also promotes localization of CAP/Ponsin to focal adhesions
12 (Figure 5c), a protein that has been shown to interact with the intracellular region of
13 ODZ1¹² and to reorganize F-actin at cell-extracellular matrix contacts²⁵. An epithelial-
14 to-mesenchymal-transition-like (EMT-like) program highlighted by a T-cadherin to N-
15 cadherin switch has been described in GBM cells associated with invasion and a worse
16 prognosis²⁶. Interestingly, N-cadherin along with other mesenchymal markers such as
17 Vimentin and Snail were upregulated whereas T-cadherin was downregulated in
18 icODZ1-transfected GSCs (Figure 5d,e). Moreover, the acquisition of a mesenchymal
19 gene expression pattern associated with a concomitant downregulation of genes that
20 define the proneural GBM subtype³ DLL3, OLIG2, ASCL1 and NCAM1 (Figure 5f).
21 Consistent with the chemoresistance associated with the mesenchymal phenotype, cells
22 expressing icODZ1 were more resistant to temozolomide, a nitrosourea commonly used
23 in GBM patients (Figure 5g). Wound healing assays showed that cells expressing ODZ1
24 or icODZ1 but not ecODZ1 efficiently repopulated the scratch area (Figure 6a,b).
25 Moreover, invasion assays in 3D collagen matrices demonstrated that icODZ1

1 efficiently promoted migration of GSCs (more than 6-fold increase relative to control)
2 (Figure 6c,d). Thus, these results suggest that icODZ1 provides a migration advantage
3 for GSCs to invade the surrounding environment.

4 **The intracellular fragment of ODZ1 induces the transactivation of RhoA through**
5 **E-box binding proteins.**

6 Rho GTPases, mainly Rac1, RhoA and Cdc42, regulate migration and invasion by
7 controlling cytoskeletal dynamics²⁷. RhoA expression is upregulated at the mRNA and
8 protein levels in a number of human malignancies²⁸ and it has been associated with
9 tumor progression²⁹. We showed that induced expression of icODZ1 was followed by a
10 specific increase in the mRNA levels of RhoA but not Rac1 or Cdc42 (Figure 7a).
11 ODZ1-deficient cells transfected with icODZ1 translocates this protein fragment to the
12 nucleus (Figure 7b), where it may regulate transcription^{12, 13}. In a RhoA promoter-
13 luciferase reporter assay, icODZ1 induced a 5-fold increase in luciferase activity (Figure
14 7c). Total RhoA protein expression and RhoA GTPase activity were also upregulated by
15 icODZ1 (Figure 7d,e). Activation of RhoA was further determined by analyzing
16 phosphorylation of MLC2, a downstream mediator of RhoA-ROCK signaling. As
17 shown in Figure 7f, icODZ1 and to a lesser extent ODZ1 but not ecODZ1 increased the
18 level of pMLC2. It has been shown that Myc induces RhoA transcription by recruiting a
19 transcription complex to the noncanonical E-boxes E5 and E6 in the RhoA promoter³⁰.
20 Chromatin immunoprecipitation assays showed significant increases in the binding of
21 Myc to RhoA promoter in the presence of ODZ1 (more than 2-fold) and icODZ1 (4-
22 fold) (Figure 7g). Moreover, as shown in Figure 7h, anti-Myc antibodies
23 coimmunoprecipitated Myc and icODZ1 in cells transfected with entire ODZ1 or
24 icODZ1, but no coimmunoprecipitation was obtained from cells transfected with
25 ecODZ1. Luciferase reporter assays showed that overexpression of Myc increased the

1 RhoA promoter activity (Figure 7j), confirming previous data. Transfection of ODZ1-
2 containing cells with a Myc-specific shRNA that efficiently downregulated Myc mRNA
3 (Figure 7i), reduced the RhoA promoter activity induced by icODZ1 (Figure 7j). In
4 order to eliminate the risk of knocking down genes that are not intended for
5 suppression, we have also used a Myc inhibitory mutant (dominant negative) carrying a
6 deletion in the transactivation domain³¹. Consistently, this blockade strategy resulted in
7 reduction of RhoA promoter activity triggered by icODZ1 (Figure 7k). In support of the
8 role of E-boxes as key promoter elements for ODZ1-induced expression of RhoA,
9 mutation of the E6 binding site abolished activation of the RhoA promoter by icODZ1
10 (Figure 7l). In line with this, mobility shift assays confirmed that E6 efficiently bound
11 E-box binding proteins and this protein-DNA complex was increased in the presence of
12 icODZ1 (Figure 7m). These data show that icODZ1 induces the expression of RhoA
13 through recruitment of Myc to the E6 box within the RhoA promoter.

14 **RhoA-ROCK pathway mediates the invasive and proliferative activities of ODZ1.**

15 The presence of ROCK inhibitor H1152 gave rise to a 4-fold decrease in the invasion
16 capacity of icODZ1-expressing cells (Figure 8a,b). Moreover, we also analyzed the
17 localization of Ponsin at focal adhesions, as a marker of cell-matrix contacts, in the
18 presence of H1152, a RhoA inhibitor or by using siRNAs against ROCK1 and ROCK2
19 (Supplementary Figure S6a-d). In all cases, Ponsin accumulation to projection tips was
20 efficiently blocked by the inhibition strategies.

21 We also described that the proliferation rate of icODZ1-expressing GSCs was about
22 twice higher than control or ecODZ1-transfected cells. ODZ1 also promoted a clear
23 increase in proliferation although at a slightly lower level than icODZ1 (Figure 8c).
24 Consistent with our previous data, ROCK inhibitor reduced the proliferation promoted
25 by icODZ1 and ODZ1 but had no effect on cells expressing ecODZ1 (Figure 8c). This

1 result was confirmed by using a Rho inhibitor which significantly reduced ODZ1-
2 mediated proliferation (Figure 8d). RhoA activation is crucial for the cell cycle G1-S
3 progression through the regulation of CDKN/Cip family of CDK inhibitors. Our
4 expression microarray data analysis showed that among members of this family
5 CDKN1A (p21) and CDKN2C (p18) were downregulated in ODZ1-transfected cells
6 (Figure 8e) which are both known to be modulated by RhoA ³². These results indicate
7 that RhoA-ROCK axis plays a key role in mediating key activities of ODZ1, cell
8 invasion and proliferation, in GSCs.

9

10 **Discussion**

11 We have focussed this work on ODZ1 gene, which is predominantly expressed in the
12 developing brain. Two GSC samples with no or very little expression of ODZ1 were
13 used as ODZ1-deficient GSCs in this work. GSCs grow in suspension forming
14 neurospheres and in the presence of serum, they differentiate to proliferating
15 intermediate precursors due, at least in part, to activation of the NFκB pathway ³³.
16 Morphological changes are driven by cytoskeletal reorganization, which is a key feature
17 of cell migration. We showed that ODZ1 promotes actin cytoskeletal remodelling,
18 migration and invasion of GSCs as determined by using 2D and 3D in vitro systems, a
19 xenograft model of chicken embryo and tumor specimens, in which ODZ1-expressing
20 GSCs efficiently invade the surrounding environment. Previous work in *Drosophila*
21 suggested that the ODZ1 homolog Ten-m is involved in remodelling of the postsynaptic
22 cytoskeleton, physically linking the synaptic membrane to the cytoskeleton ¹⁶.
23 Moreover, it has been described that chicken ODZ1 interacts with ponsin, which in turn
24 binds to vinculin that could anchor the intracellular region of ODZ1 to the actin
25 cytoskeleton ¹². Consistently, we found that ODZ1 promoted long actin-filled

1 protrusions and the localization of ponsin at projection tips in GSCs, which strengthen
2 the role of ODZ1 as a regulator of cytoskeletal remodelling. Actin cytoskeleton has key
3 roles in cell cycle progression ³⁴. In line with this, ODZ1 increases the proliferative
4 capacity of GSCs. Invasiveness and proliferation are two hallmarks of GBM and
5 strategies aimed at targeting pathways that control these processes, mainly in GSCs, are
6 the focus of intensive research ³⁵⁻³⁷. Rho GTPases are key players in controlling
7 cytoskeleton dynamics, cell migration and cell division ¹⁸. Elevated levels of RhoA
8 have been described in clinical samples of high-grade gliomas ³⁸. Interestingly, the
9 intracellular fragment of ODZ1 (icODZ1) is able to induce the expression and activation
10 of RhoA in GSCs. We found that icODZ1 is released following differentiation of GSCs
11 by a proteolytic mechanism triggered by SPPL2a, a type II transmembrane protein
12 specific protease. SPPL2a is overexpressed in GBM compared with other brain tumors
13 including oligodendrogliomas and low grade astrocytomas, as determined by searching
14 the Rembrandt database. Consistent with the role of SPPL2a in ODZ1 processing, this
15 protease has been shown to release the intracellular FasL domain, which translocates to
16 the nucleus and regulates gene transcription³⁹. Myc is a well known oncoprotein that
17 control the expression of a variety of genes involved in cell proliferation and
18 differentiation⁴⁰, and it has been described that Myc stabilization may be linked to the
19 pathogenesis of GBM⁴¹. Our data strengthen the role of Myc in GBM by showing that
20 icODZ1 binds to Myc and recruits this transcription factor to an E-box site in the
21 promoter region of RhoA, which is in line with previous results where it is
22 demonstrated that Myc induces RhoA transcription by recruiting a transcription
23 complex to E-boxes within the RhoA promoter³⁰. By using different inhibition
24 strategies, we demonstrated that RhoA signaling mediates the cytoskeletal remodelling
25 and the increase in cell migration, invasion and proliferation promoted by ODZ1. In line

1 with our data, high levels of RhoA activity have been associated with protrusion
2 formation in different cancer models ^{42, 43}. The relevance of Rho-ROCK pathway in
3 promoting migration and growth of GBM cells has been mostly described by using
4 established cell lines ^{44, 45}. However, other models have strengthened this functional
5 correlation. Although it has been described that invading GBM cells have low RhoA
6 activity by using a rat GBM cell line⁴⁶, many studies using primary human tumor cells
7 support the key role of RhoA to provide an invasive phenotype to GBM cells^{47, 48}.
8 Moreover, activation of RhoA in neural stem cells has also been shown to be key for
9 cell migration and invasion⁴⁹. Our data provides a novel ODZ1-RhoA-ROCK axis for
10 therapeutic strategies against a pathway controlling key activities in GSCs. Blockade of
11 RhoA expression or activation through its upstream regulator ODZ1 has the advantage
12 that this protein is located at the cell membrane which facilitates inhibition strategies
13 with small molecules or antibodies. Additionally, contrary to other integral membrane
14 proteins associated with GBM such as EGFR that is widely expressed in a number of
15 adult tissues, or RhoA which is ubiquitously expressed across tissues, ODZ1 expression
16 is mostly restricted to fetal brain, which could make ODZ1-targeted therapies more
17 tumor-specific limiting their side effects.

18 In summary, our study shows for the first time that ODZ1, a protein that participates in
19 the embryonic development of the brain, is also involved in cancer progression by
20 promoting the growth and invasion capabilities of glioblastoma stem-like cells via a
21 transcriptional pathway that induces the expression of RhoA and activation of
22 downstream ROCK. Thus, these data provide a novel and promising target to develop
23 therapeutic strategies aimed at blocking the two features that make glioblastoma so
24 aggressive and lethal.

25

1 **Materials and methods**

2 **Primary cell cultures.**

3 Cells were maintained as neurospheres in serum-free DMEM/F12 medium (Invitrogen,
4 Carlsbad, CA) as previously described ²⁴, and plated at a density of 3×10^6 live cells/60-
5 mm plate. Neurospheres were dissociated every 4-5 days to facilitate cell growth. Cells
6 were used between passages 10 and 20. To promote differentiation, neurospheres were
7 cultured in the same medium but in the presence of 10% FCS for four days. Tumor
8 samples were obtained from patients after informed written consent had been given, as
9 approved by the Research Ethics Board at the Valdecilla Hospital. Established GBM
10 cell line RG1 (L0627), kindly provided by Dr. Rosella Galli, was cultured as previously
11 described ²³. Cell proliferation and cell survival were evaluated with Alamar Blue
12 bioassay (Life Technologies). **Unless otherwise indicated, experiments were performed**
13 **with undifferentiated GSCs. All cells used in this work were mycoplasma-free.**

14 When indicated, cells were treated with 5 μ M ROCK inhibitor H1152 (Tocris
15 Bioscience, Bristol, UK), 1 μ g/ml Rho Inhibitor I (Cytoskeleton, Inc., Denver, CO), 100
16 μ M Temozolomide (Merck, Whitehouse Station, NJ), 50 μ M (Z-LL)2-Ketone
17 (Calbiochem, MA) or 10 μ M L685,458 (Tocris Bioscience, Bristol, UK).

18 DNA copy number changes were evaluated using Affymetrix GeneChip 250-NspI/StyI
19 SNP microarrays as previously described ¹⁷. DNA methylation was analyzed by a
20 pyrosequencing method ⁵⁰ which accurately measures the degree of methylation at
21 several CpGs in close proximity with high quantitative resolution.

22 **In vivo models.**

23 Tumor xenografts in chicken and mice ⁵¹ were established as previously described.
24 Stereotactically guided intracranial injections in athymic Nude-Foxn-1^{nu} male mice,
25 aged 8-9 weeks, were performed injecting 50.000 cells resuspended in 2 μ l of culture

1 medium. Survival of mice was analyzed by the Kaplan-Meier method. No
2 randomization was used. Images in chicken embryos are the integration of all Z-stacks
3 taken along the dorsal-ventral plane at 15 μm intervals to cover the whole limb. The
4 model of tumor xenografts in mice was reviewed and approved by the Research Ethics
5 and Animal Welfare Committee at the Instituto de Salud Carlos III, Madrid. Magnetic
6 resonance imaging analysis was performed in mice injected IP with Gd-DOTA
7 (Dotarem, Bloomington, IN) by using a 4.7 TBiospec BMT 47/40 spectrometer (Bruker,
8 Billerica, MA). No blinding experiments were done.

9 **Immunofluorescence analysis.**

10 GSCs were grown on glass cover slips previously coated with 10 $\mu\text{g}/\text{ml}$ fibronectin
11 (Sigma- Aldrich, St Louis, MO), or 10 $\mu\text{g}/\text{ml}$ laminin (Sigma). Cells were incubated
12 with antibodies against phospho-myosin light chain 2 (pMLC2) (#3671, Cell Signaling,
13 Danvers, MA), Ponsin (SC-25496, Santa Cruz Biotechnology, Santa Cruz, CA), Sox2
14 (MAB2018, R&D Systems, Minneapolis, MN), GFAP (Z0334, DAKO, Glostrup,
15 Denmark) or Tuj1 (T-8660, Sigma). Then, the cells were incubated with Texas red-
16 conjugated or fluorescein isothiocyanate-conjugated goat anti-rabbit secondary
17 antibodies (#111-035-144, Jackson ImmunoResearch, Cambridgeshire, UK). Actin
18 cytoskeleton was stained with tetramethylrhodamine isothiocyanate- or fluorescein
19 isothiocyanate-conjugated Phalloidin (Sigma) and nuclei visualized with 4',6-
20 diamidino-2-phenylindole (Life Technologies). Confocal images were taken with a Ti-
21 Eclipse microscope (Nikon, Tokyo, Japan). When indicated, fluorescence intensity was
22 determined by measuring pixel intensity in a defined area (nucleus or the entire cell)
23 using ImageJ software. Single plane confocal images were acquired with the same
24 settings for each experiment.

25 **Tissue Microarray.**

1 Demographic, clinical and therapeutic data of patients included in the tissue microarray
2 are presented in Supplementary Table S2. Replicate formalin-fixed paraffin-embedded
3 tissue samples of representative tumor regions from 122 patients with GBM were
4 collected for the preparation of 4 tissue microarrays. No samples were excluded from
5 the analysis. Tissue microarrays were reviewed by an expert pathologist (NTV) blinded
6 to all sample identifiers. A semiquantitative scoring system for the percentage of
7 positive cells was used. Patients were divided into two groups of low (<35%) and high
8 (>35%) number of ODZ1-positive cells. Differences in overall and disease-free survival
9 between both groups were estimated by a Kaplan-Meier survival plot.

10 **Immunohistochemical staining.**

11 Vibratome sections (200 µm thick) of mouse brains were paraffin-embedded and then
12 consecutive semithin sections were processed and incubated with primary antibodies
13 against Vimentin, glial fibrillary acidic protein (GFAP) and Ki67 (M7248, DAKO)
14 followed by horseradish peroxidase-conjugated secondary antibodies (K4065, K5007,
15 DAKO) and visualized by a chromogen or stained with hematoxylin-eosin. Slides
16 containing tumor tissue from GBM patients or neurospheres from GSC cultures
17 embedded in paraffin were stained with antibodies against the N-terminal region of
18 ODZ1.

19 **Expression analyses.**

20 To assess the expression of individual genes, a cDNA was generated and amplified
21 using the following primers: CD133, Tuj1, glial fibrillary acidic protein (GFAP), β-
22 Actin (16),

23 T-Cadherin (5'TTCTGTGCGTTCTCCTGTCC^{3'} and
24 5'TCTCAGAGCAACTAAGCCGC^{3'}),

1 N-Cadherin (5'GACAATGCCCTCAAGTGTT3' and
2 5'CCATTAAGCCGAGTGATGGT3'),
3 Vimentin (5'TCGGCGGGACAGCAGG3' and 5'GGTGGACGTAGTCACGTAGC3'),
4 Snail (5'TAGCGAGTGGTTCTTCTGCG3' and 5'AGGGCTGCTGGAAGGTAAAC3'),
5 RhoA (5'ACCCGCCTTCGTCTCCGAGT3' and
6 5'GGCAGCCATTGCTCAGGCAAC3'),
7 Rac1 (5'AGGCCATCAAGTGTGTGGTG3' and 5'CTTGTCCAGCTGTGTCCCAT3'),
8 Cdc42 (5'GCCTATTACTCCAGAGACTGC3' and
9 5'GTTTCATAGCAGCACACCTG3'),
10 icODZ1 (5'ACTCAAGAGATGGAATTCTGTG3' and
11 5'CTTAGTGCATGGTCAGGTG3'),
12 ecODZ1 (5'ACAATGATGGACGGTGCCTT3' and 5'GTGTCCCTCCCCTCTATGGT3')
13 ODZ1 exon 1 (5'GGACCAATTGTGAATCTGCC3' and
14 5'CCTACAACCTCAGCTGGGC3'),
15 ODZ1 exon 20 (5'GGGAAGGTTTTGCAGGC3' and
16 5'CCACTGTGCTAGAGGCTGG3'),
17 ODZ1 exon 32 (5'CTTGCAAGCCTGTCCTTTCC3' and
18 5'CCCAGTGTTACCGATGAGC3'),
19 Stag2 (5'CACGCTGGCTAATTTTTGT3' and 5'CAATACAGGGCAGGTGTGCT3'),
20 S2P (5'GTTGCTGAGGACTCTCCTGC3' and 5'AGCACACATCTGTGAGGCTG3'),
21 SPPL2a (5'ATGAAGACAGGTGGGCTTGG3' and
22 5'AGCTGCGAGTTCAACCATGA3'), SMARCA1
23 (5'CGTGGTCATAGAGGACGAGC3' and 5'TAGGCGCTTTAGCAGCAAGT3'),
24 BCORL1 (5'GCATGTGTGGCATCAACGAG3' and
25 5'GGGATCGTCAGGCTTGTCTT3'), SH2D1A (5'TACCGAGTGTCCAGACAGA3'

1 and ^{5'}TCAGGATCTTCTCTTATCCCTGT^{3'}). Quantitative real-time PCR was
2 performed in a 7000 sequence detection System (Life Technologies).

3 **Transfections, Gene Silencing and Gene Reporter Assays.**

4 ODZ1-deficient GSCs were transfected with the entire ODZ1 cDNA, the extracellular
5 (plus transmembrane) fragment (both from Origene, Rockville, MD) or the intracellular
6 fragment, that was PCR-amplified from ODZ1 cDNA and cloned into pCMV6. Stable
7 transfectants were selected with 500 µg/ml geneticin (Life Technologies). icODZ1 was
8 subcloned in a tetracycline-regulated retroviral vector ⁵². Cells were transiently
9 transfected with this vector and the expression of ODZ1 was induced in the presence of
10 2 µg/ml doxycycline (Sigma).

11 GSCs were transfected with ODZ1-specific shRNAs (Thermo Fisher Scientific,
12 Waltham, MA) by using nucleofection or with ROCK1, ROCK2, S2P or SPPL2a
13 siRNAs (Dharmacon's SmartPools, Thermo Fisher Scientific) by Lipofectamine
14 RNAiMax (Life Technologies). Knockdown of Myc was achieved by using specific
15 shRNAs cloned in lentiviral pLKO (Sigma) and retroviral pRS ⁵³ vectors. For functional
16 inactivation of Myc, a construct containing mutant c-Myc (pMLV-D106-143) that
17 carries a deletion in the transactivation domain³¹, was used.

18 Cells were co-transfected with 1 µg of human RhoA promoter cloned into pGL3 vector
19 ³⁰ and 50 ng of pRSV-β-galactosidase by nucleofection. When indicated, site-directed
20 mutagenesis of E5 and E6 boxes, CA(C>A)(G>T)CG, of the RhoA promoter was
21 conducted by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) as
22 previously described ⁵⁴.

23 **Expression Microarray.**

24 Total RNA from empty vector- and ODZ1-transfected cells was extracted using the
25 RNeasy mini kit (Qiagen, Valencia, CA) and microarray gene expression analysis was

1 performed with the Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara,
2 CA). The selection of those genes differentially expressed was performed using a
3 criteria based on the fold-change value. Probe sets were selected as significant using a
4 logFC cut-off of 1.5. The raw data have been deposited in a MIAME compliant
5 database (GEO accession number, GSE65526).

6 **Chromatin Immunoprecipitation (ChIP)**

7 Purification of sonicated nuclear lysates and immunoprecipitation were performed as
8 described . Precipitates using anti-Myc (SC-40, Santa Cruz Biotechnology) or irrelevant
9 IgG were heated at 65°C to reverse the cross-linking. Quantitative PCR was performed
10 using primers (5'CGTGAAGAGTTGGCAGTTCG^{3'} and
11 5'ACGCCCTAAAAGCAAACC^{3'}) specific for a region (187 bp) of the RhoA
12 promoter containing the functional cMyc binding sites ³⁰, or primers
13 (5'CAACCGACCAGTCACATCC^{3'} and 5'ATGTTGTTGAGGGCTTCCAG^{3'}) that
14 generate a fragment from exon 5 of SKP2, used as a negative control.

15 **Electrophoretic mobility shift assay.**

16 Nuclear extracts (10 µg of total protein) were incubated with a ³²p-labeled double-
17 stranded DNA probe corresponding to E-boxes E5 (5'CCGCGCACGCGCACCTAA^{3'})
18 and E6 (5'ATGCCCCACGCGGCTGCA^{3'}). Samples were run on a 5% non-denaturing
19 polyacrylamide gel. Gels were dried and visualized by autoradiography.

20 **Western Blot, Immunoprecipitation and Pulldown assays.**

21 Total protein extracts were separated on 8 or 12% polyacrilamide gels or 4-20%
22 gradient gels (BioRad, Hercules, CA) and transferred to nitrocellulose. Blots were
23 incubated with antibodies against α-Tubulin (SC-23948, Santa Cruz Biotechnology,
24 Santa Cruz, CA), RhoA (#2117, Cell Signaling Technology, Danvers, MA), ODZ1
25 (AF6324, R&D Systems) and N-Cadherin (C-2542, Sigma) followed by incubation with

1 secondary anti-rabbit, anti-mouse or anti-sheep antibodies conjugated to horseradish
2 peroxidase (SC-2004, SC2005, SC-2473, Santa Cruz Biotechnology). For protein
3 immunoprecipitation, cells were treated as previously described⁵⁴. Cleared lysates were
4 incubated with anti-cMyc antibodies and proteinA/G conjugated to agarose beads
5 (Santa Cruz Biotechnology). Proteins were then electrophoresed, transferred to
6 nitrocellulose membranes and incubated with anti-ODZ1 antibodies. For pulldown
7 experiments, glutathione S-transferase-conjugated Rhotekin was expressed from pGEX-
8 Rhotekin and assays were performed as described¹⁷. Protein band quantification was
9 carried out using ImageJ software.

10 **Migration and Invasion Assays.**

11 For the wound-healing assay, cells were seeded into collagen I coated 24-well plates
12 and grown to confluence. The cell monolayers were scratched with a pipette tip. Cell
13 migration was expressed by the percentage of wound closure. For 3D assays, cells were
14 included in collagen-based matrices and processed as previously described¹⁷. The effect
15 of protease inhibitors on the migration capacity of GSCs was analysed by using a
16 modified Boyden chamber assay in 24-well plates (Transwell, Corning Incorporated,
17 NY). Cells were placed in the upper compartment and following 24 h of incubation,
18 migratory cells in the lower face of the membrane were fixed and stained.

19 **Statistical analysis.**

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

24

25 **Conflict of interest**

1 The authors declare that no conflict of interest exists

2

3 **Acknowledgements**

4 This work was supported by the Instituto de Salud Carlos III (ISCIII), grants
5 PI13/01760 (JLF-L), PI12/00775 (PS-G) and SAF2014-53526R (JL), and program Red
6 Temática de Investigación Cooperativa en Cáncer (RTICC) grants RD12/0036/0022
7 (JLF-L), RD12/0036/0027 (PS-G), RD12/0036/0063 (JAM-C), RD12/0036/0003 (AP)
8 and RD12-0036-0033 (JL), Instituto de Investigación Valdecilla (IDIVAL) grant
9 APG/03 to JLF-L, and Cancer Research UK C33043/A12065 and Royal Society
10 RG110591 to VS-M. We thank R. Galli for kindly providing RG1 cell line. We also
11 thank M.E. Fernández-Valle for helping with magnetic resonance imaging experiments.

12

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6

7

8 **Figure legends**

9 **Figure 1. Transfection of ODZ1-deficient GSCs with ODZ1 restores the**
10 **morphology of differentiating cells.** (a) Neurospheres were disaggregated and cultured
11 in the presence of FBS. Morphological changes of differentiating cells were visualized
12 after 4 days of culture. Scale bar: 30 μ m. (b) Representation of the X-Chromosome
13 showing the size of the deleted genomic fragment and the deleted genes in G104 GSCs.
14 (c) Amplification of genes included in the chromosomal deletion found in G104 cells.
15 Actin is included as a positive amplification control. (d) Amplification of different
16 exons of the ODZ1 gene from GSC-derived genomic DNA. Stag2 was used as a
17 positive control of amplification. (e) ODZ1 protein expression in undifferentiated GSC
18 samples. α Tubulin was used to assure equal loading. (f) G59 GSCs were treated with
19 the DNA demethylating agent 5-aza-2'-deoxycytidine (3 μ M 5-Aza) for 3 days and the
20 ODZ1 expression was determined by quantitative RT-PCR. Histograms show the
21 mean \pm S.D. *p<0.01, Student's *t* test. Data representative of 3 separate experiments. (g)
22 ODZ1 protein levels in ODZ1-transfected GSCs. The levels of α -tubulin were analysed
23 to assure equal loading. Transfected cells were cultured in differentiation medium and 4
24 days later their morphology (h) and the distribution of F-actin (i) were assessed by
25 using phase contrast or fluorescence microscopy respectively. (h, Scale bar: 50 μ m; i,

1 Scale bar: 5 μ m). (j) GSC transfectants cultured in differentiation medium were counted
2 as a function of their morphology. NP, no protrusions; SP, small protrusions; LP, long
3 protrusions. Histograms show the mean \pm S.D. * p <0.01, ** p <0.05, Student's t test. Data
4 representative of 3 separate experiments counting at least 100 cells per condition each
5 time.

6 **Figure 2. ODZ1 expression is sufficient to induce cell spreading in vitro and in**
7 **vivo. (a,b)** Western blot and quantification of ODZ1 after transfection of G63 GSCs
8 with specific shRNAs. The levels of α -tubulin were analysed to assure equal loading.
9 * p <0.001, Student's t test. Data representative of 3 separate experiments. (c) GSCs were
10 transfected with ODZ1-specific shRNA (sh-2) and the number of nonattached living
11 cells were quantitated. sh-C, irrelevant shRNA. Histograms show the mean \pm S.D.
12 * p <0.001, Student's t test. Data representative of 3 independent experiments. (d) GSCs
13 transfected with ODZ1-specific shRNA-2 and EGFP formed neurospheres under
14 differentiation conditions. (e) CFDA SE-labeled GSCs with downregulated levels of
15 ODZ1 were injected into the chicken embryo limb and 24 h later cell spreading was
16 determined by confocal microscopy. (f) ODZ1-deficient GSCs were transfected with
17 ODZ1 or empty vector and xenografted in chicken embryo as above. Dotted lines
18 delimitate the area of tumor cell spreading. Paraffin sections from ODZ1-deficient (g)
19 and ODZ1-expressing (h) GBM tissue specimens were immunostained with anti-ODZ1
20 antibodies. The following histological areas were identified: the tumor core, with many
21 ODZ1(-) cells; the tumor border, with a similar cellular density but a higher proportion
22 of ODZ1(+) cells and the infiltrated parenchyma, with low cellularity but most cells
23 showing a faint staining for ODZ1. (i) Sections of paraffin-embeded neurospheres were
24 immunostained with anti-ODZ1. Note that immunostaining is mainly localized at the
25 periphery of neurospheres.

1 **Figure 3. ODZ1-expressing tumors are more aggressive and reduce survival.** (a)
2 ODZ1 mRNA levels in different GBM cell lines by RT-PCR. β Actin expression was
3 used for signal normalization. (b) Representative T2-weighted nuclear magnetic
4 resonance (NMR) images showing tumors in the brain of mice xenografted with ODZ1-
5 transfected RG1 GBM cells. (c) Kaplan-Meier survival curves of mice harboring RG1-
6 derived tumors with high or low levels of ODZ1. (d) Expression of ODZ1 and icODZ1
7 in transfected GSCs. The levels of α -tubulin were analyzed to assure equal loading. (e)
8 Schematic representation of the ODZ1 protein showing the intracellular (IC)
9 transmembrane (TM) and extracellular (EC) parts of the protein. NLS, nuclear
10 localization signal. (f) ODZ1-deficient cells were transfected with icODZ1 and cultured
11 under differentiation conditions. Cell morphology was assessed after 4 days of culture.
12 Scale bar: 50 μ m. (g) ODZ1-deficient GSCs transfected with ODZ1 or icODZ1 were
13 xenografted in mice and brain sections were obtained for immunohistochemical analysis
14 Note the intracranial tumor staining (brown) by using markers that reveal the presence
15 of tumor cells. HE: hematoxylin-eosin staining. (h) Anti-ODZ1 antibodies identify
16 negative or positive staining in GBM specimens. (i) Representative examples showing
17 different levels of ODZ1 staining on a tissue microarray with specimens from 122
18 patients with GBM. Kaplan-Meier curves comparing overall survival (j) and disease-
19 free survival (k) between two groups of patients with low and high proportion of
20 ODZ1-positive GBM cells.

21 **Figure 4. icODZ1 is translocated to the nucleus following differentiation of GSCs.**

22 (a) RNA was obtained from two GSC cultures before and after differentiation, and the
23 expression of ODZ1 was determined by qRT-PCR. Values show the mean \pm S.D.
24 * p <0.01, Student's t test. (b) ODZ1 protein expression in GSCs before and after
25 differentiation. Note the increase in the levels of the intracellular fragment. α -tubulin

1 was analyzed to assure equal loading. (c) Immunofluorescence of GSCs and
2 differentiated GSCs with anti-ODZ1 antibodies. Scale bar, 5 μm . (d)
3 Immunofluorescence of differentiated ODZ1-deficient cells overexpressing full-length
4 ODZ1 in the absence or in the presence of protease inhibitors by using anti-ODZ1.
5 Scale bar, 10 μm . NOTE: morphological changes following treatment with the inhibitor
6 were observed in some GSC models but not in others, whereas reduction of nuclear
7 levels of ODZ1 was always detected. (e) Quantification of the fluorescence intensities
8 of immunofluorescence images exemplified in (d) by using ImageJ software. RFI,
9 relative fluorescence intensity. (f) The same cells as in (d) were analyzed for their
10 capacity to migrate with or without the protease inhibitor (ZLL)2-Ketone by using a
11 modified Boyden chamber. (g) mRNA expression levels of SPPL2a and S2P proteases
12 following transfection with specific siRNAs. C, irrelevant siRNA. Values show the
13 mean \pm S.D. * $p < 0.01$, Student's t test. (h) Subcellular localization of ODZ1 in
14 differentiated GSCs transfected with the indicated siRNAs. Scale bar, 10 μm . (i)
15 Quantification of the fluorescence intensities of immunofluorescence images
16 exemplified in (h).

17 **Figure 5. ODZ1 promotes actin cytoskeleton remodeling and induces a**
18 **chemotherapy resistant mesenchymal-like phenotype.** (a) RT-PCR showing the
19 expression of ODZ1 in ODZ1-deficient GSCs transfected the intracellular fragment
20 (icODZ1), the extracellular plus transmembrane fragment (ecODZ1) or the entire ODZ1
21 with oligonucleotides specific for the ic or ec regions of the cDNA. β Actin expression
22 was used for signal normalization. (b) Representative confocal images of the different
23 GSC transfectants showing F-actin staining. Scale bar, 10 μm . (c) ODZ1-deficient
24 GSCs expressing icODZ1 accumulates the focal adhesion protein ponsin at projection
25 tips as determined by immunofluorescence. Scale bar: 10 μm . (d) icODZ1-dependent

1 mRNA expression levels of the epithelial-like marker T-Cadherin (T-Cad) and the
2 mesenchymal specific markers N-Cadherin (N-Cad), Vimentin (Vim) and Snail as
3 determined by qRT-PCR. Histograms show the mean \pm S.D. * p <0.01, ** p <0.001,
4 Student's *t* test. Data representative of 3 separate experiments. (e) N-Cadherin protein
5 expression in transfectant GSCs. The levels of α -tubulin were analyzed to assure equal
6 loading. (f) Fold-change (FC) in the expression of genes associated with the proneural
7 GBM subtype in icODZ1-transfected cells relative to control transfected cells. Data
8 from the expression microarray. logFC indicates the log₂ of FC. (g) icODZ1-transfected
9 GSCs were treated with 100 μ M Temozolomide (TMZ) and 48 h later cell viability was
10 determined by using alamarBlue bioassay. Values show the mean \pm S.D. * p <0.001,
11 Student's *t* test. Data representative of 3 independent experiments.

12 **Figure 6. ODZ1 promotes cell migration and invasion.** (a) Micrographs of a wound-
13 healing assay showing the migration of different transfectant GSCs towards the
14 scratched area. (b) Quantification of migration as percentage of wound closure (marked
15 by the dashed lines). Histograms show the mean \pm S.D. * p =0.01 compared with pCMV6
16 control, Student's *t* test. Data representative of 3 separate experiments. (c)
17 Representative images of a cell invasion assay in a 3D collagen matrix. Nuclei were
18 stained with DAPI. (d) Quantification of the invasion assay as percentage of cells
19 detected at 30 μ m from the bottom. Histograms show the mean \pm S.D. * p <0.05,
20 ** p <0.001 compared with pCMV6 control, Student's *t* test. Data representative of 3
21 separate experiments.

22 **Figure 7. ODZ1 triggers the transcriptional activation of RhoA.** (a) The mRNA
23 levels of Rho GTPases were analyzed by qRT-PCR in GSCs transfected with an
24 icODZ1-containing doxycycline-inducible construct. * p <0.01. (b) Nuclear localization
25 of the intracellular fragment of ODZ1 protein after transfection, as determined by

1 immunofluorescence. Scale bar: 5 μ m. (c) ODZ1-deficient GSCs were cotransfected
2 with a RhoA promoter-luciferase reporter construct and icODZ1 and luciferase activity
3 was determined 48 h later. * p <0.01. (d,e) GTP-bound RhoA and total RhoA protein
4 expression were determined in ODZ1-deficient cells transfected with icODZ1. C, empty
5 vector; IC, icODZ1. * p <0.01, ** p <0.001. (f) ODZ1 transfectants were analyzed for the
6 expression of phosphorylated MLC2 by immunofluorescence. Scale bar: 10 μ m. Cell
7 fluorescence intensities (mean \pm S.D.) were determined by analyzing 12 cells for each of
8 three independent experiments. (g). A ChIP assay was performed to study the ODZ1-
9 mediated binding of Myc to the promoter of RhoA. Immunoprecipitates were analyzed
10 by qPCR using primers specific to the target site or an irrelevant region. * p <0.01. (h).
11 ODZ1-Myc binding complexes were assessed in ODZ1-transfectants by
12 immunoprecipitation with anti-Myc antibodies and subsequent blotting with anti-ODZ1
13 antibodies. The input represents the starting material. (i) Downregulation of Myc in
14 cells transfected with shRNAs as determined by qRT-PCR. * p <0.01. (j) RhoA
15 promoter-luciferase activity in the presence of Myc-specific shRNAs. * p <0.001. (k)
16 RhoA promoter-luciferase activity in the presence of a dominant negative form of Myc.
17 * p <0.001. (l) Activity of the RhoA promoter carrying mutated E5 or E6 binding sites.
18 * p <0.02. (m) Mobility shift assay using E-boxes, E5 and E6 from the RhoA promoter,
19 as DNA probes. All histograms show the mean \pm S.D., and statistical significance is
20 determined by using the Student's *t* test. All data are representative of 3 separate
21 experiments.

22 **Figure 8. ODZ1 promotes invasion and proliferation through RhoA-ROCK axis.**

23 (a) Invasion assay of GSC transfectants in a 3D collagen matrix with or without ROCK
24 inhibitor H1152. DAPI-stained cells were visualized by confocal microscopy at
25 different focal planes. (b) Quantification of the invasion assay as percentage of cells

1 detected at 20 μm from the bottom. Histograms show the mean \pm S.D. * $p=0.0005$,
2 Student's t test. Data representative of 3 separate experiments. (c) GSC transfectants
3 were cultured with or without H1152 and proliferation was assessed by Alamar Blue
4 assay. Histograms show the mean \pm S.D. * $p=0.001$ compared with untreated control,
5 Student's t test. Data representative of 3 independent experiments. (d) GSC transfectants
6 were cultured with or without Rho inhibitor I and proliferation was assessed as above.
7 Histograms show the mean values \pm SD. * $p=0.005$ compared with untreated control,
8 Student's t test. Data representative of 3 separate experiments. (e) Gene expression
9 microarray data showing the fold change of CDKN/Cip family of CDK inhibitors in
10 ODZ1-transfected cells compared with control cells. Dashed line marks the fold change
11 cut-off ($\log_2\text{FC}>0.5$) used in the analysis.

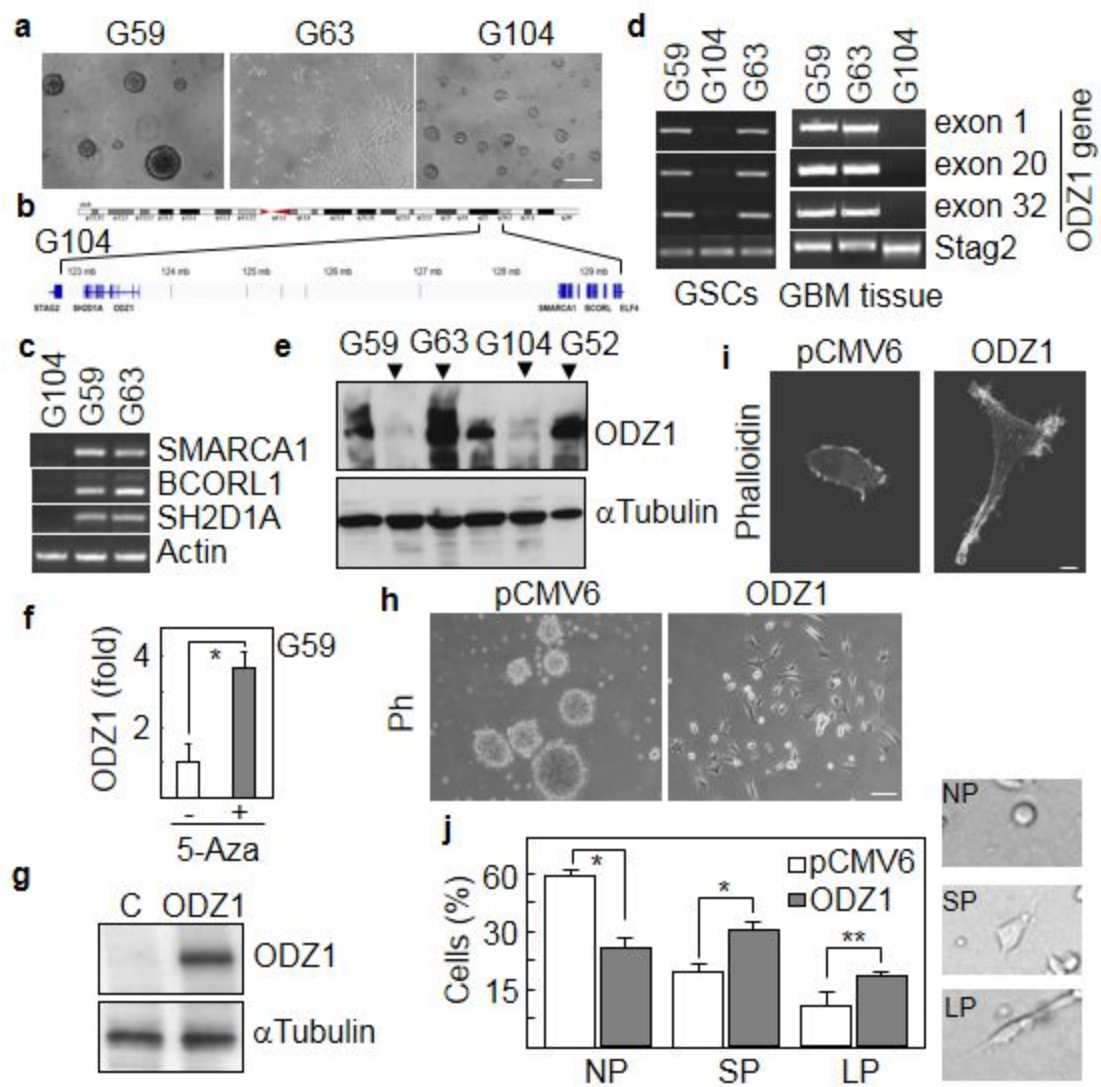


Figure 1

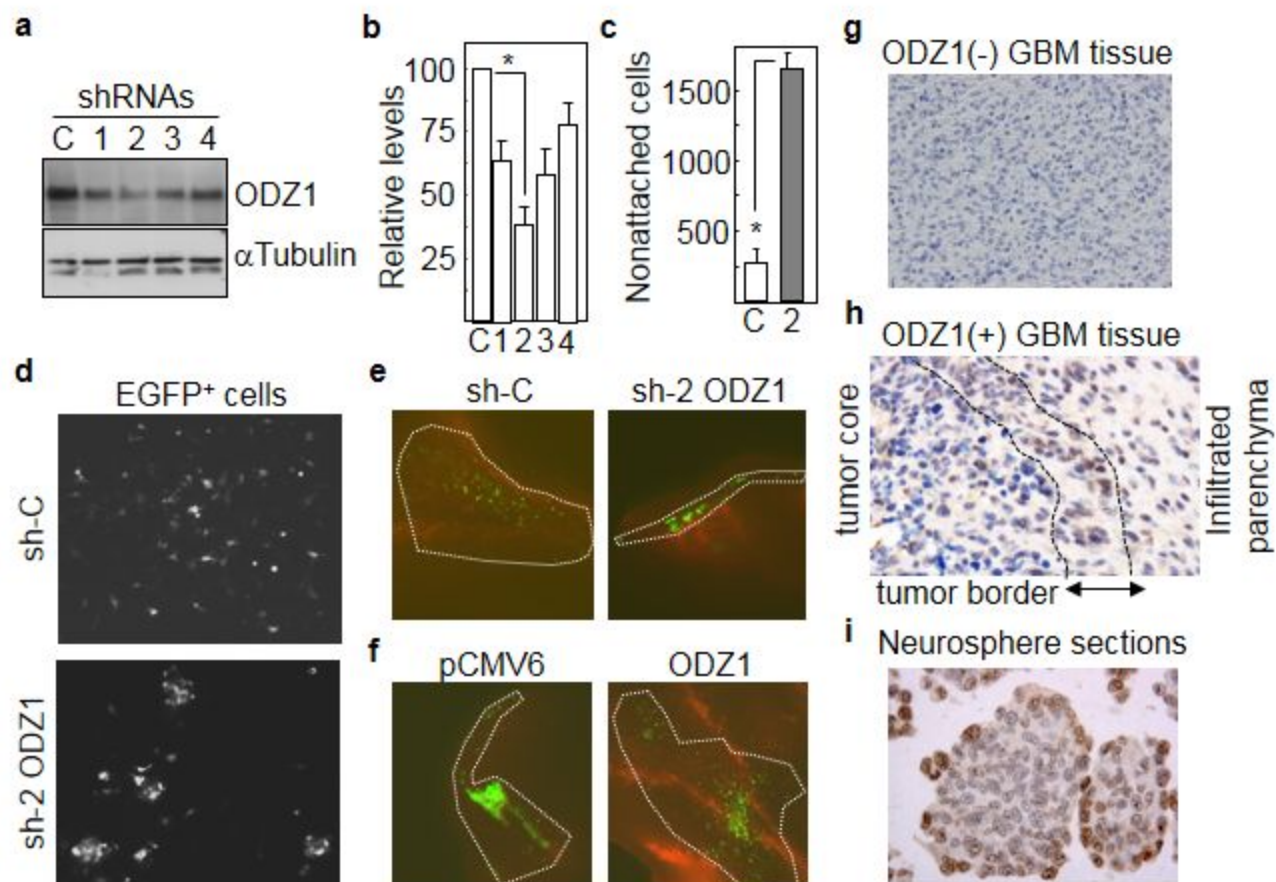


Figure 2

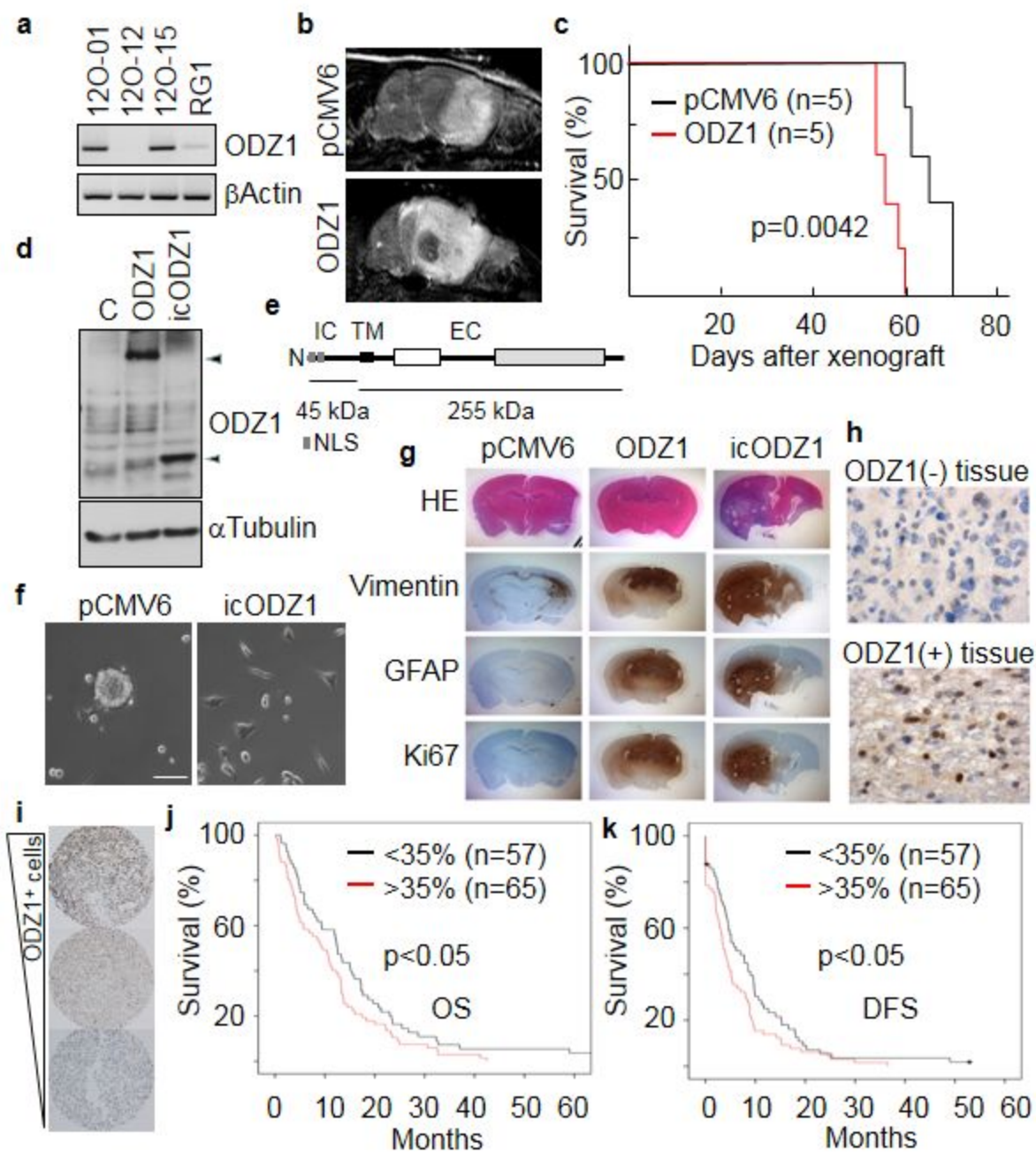


Figure 3

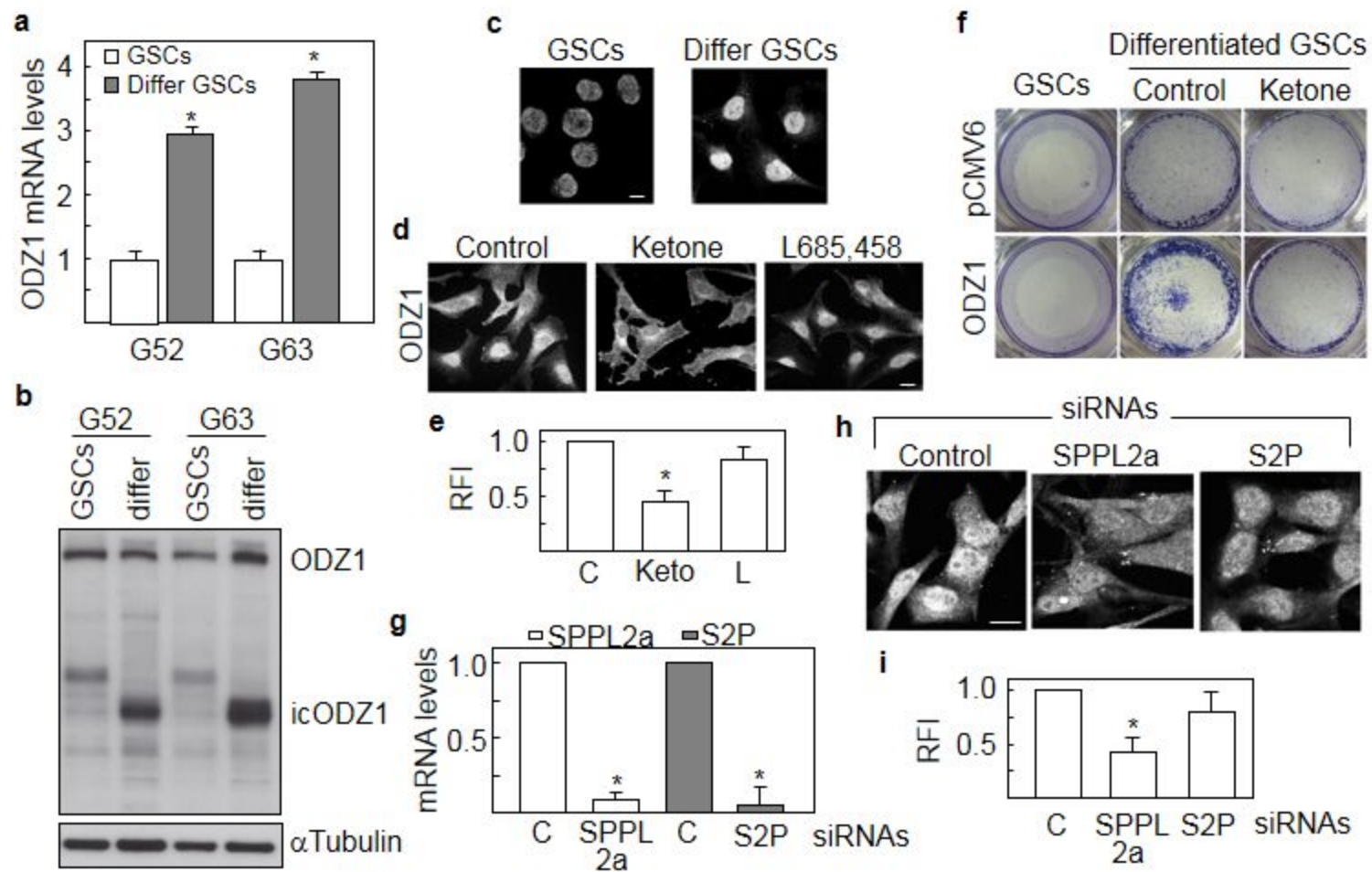


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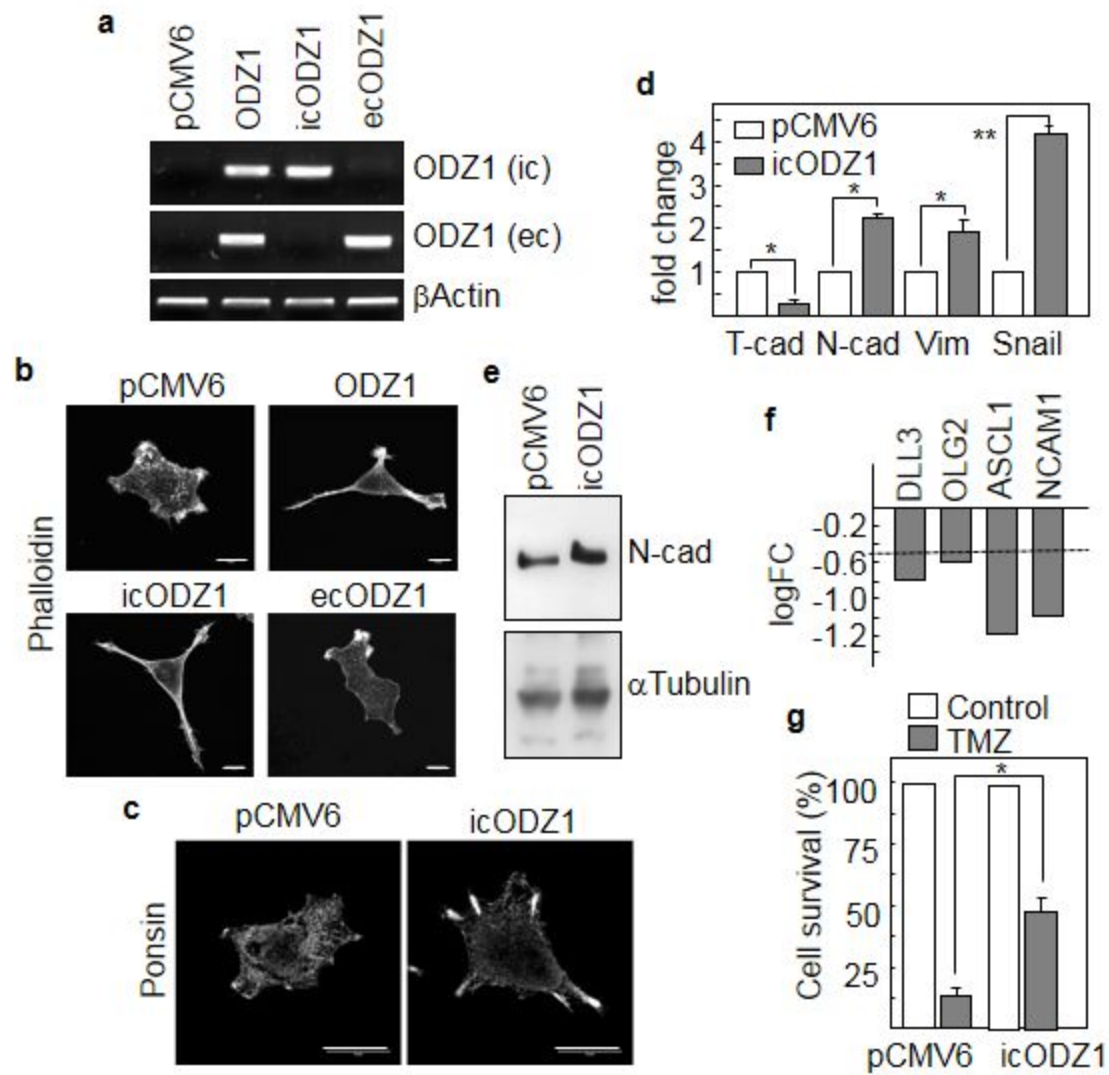


Figure 5

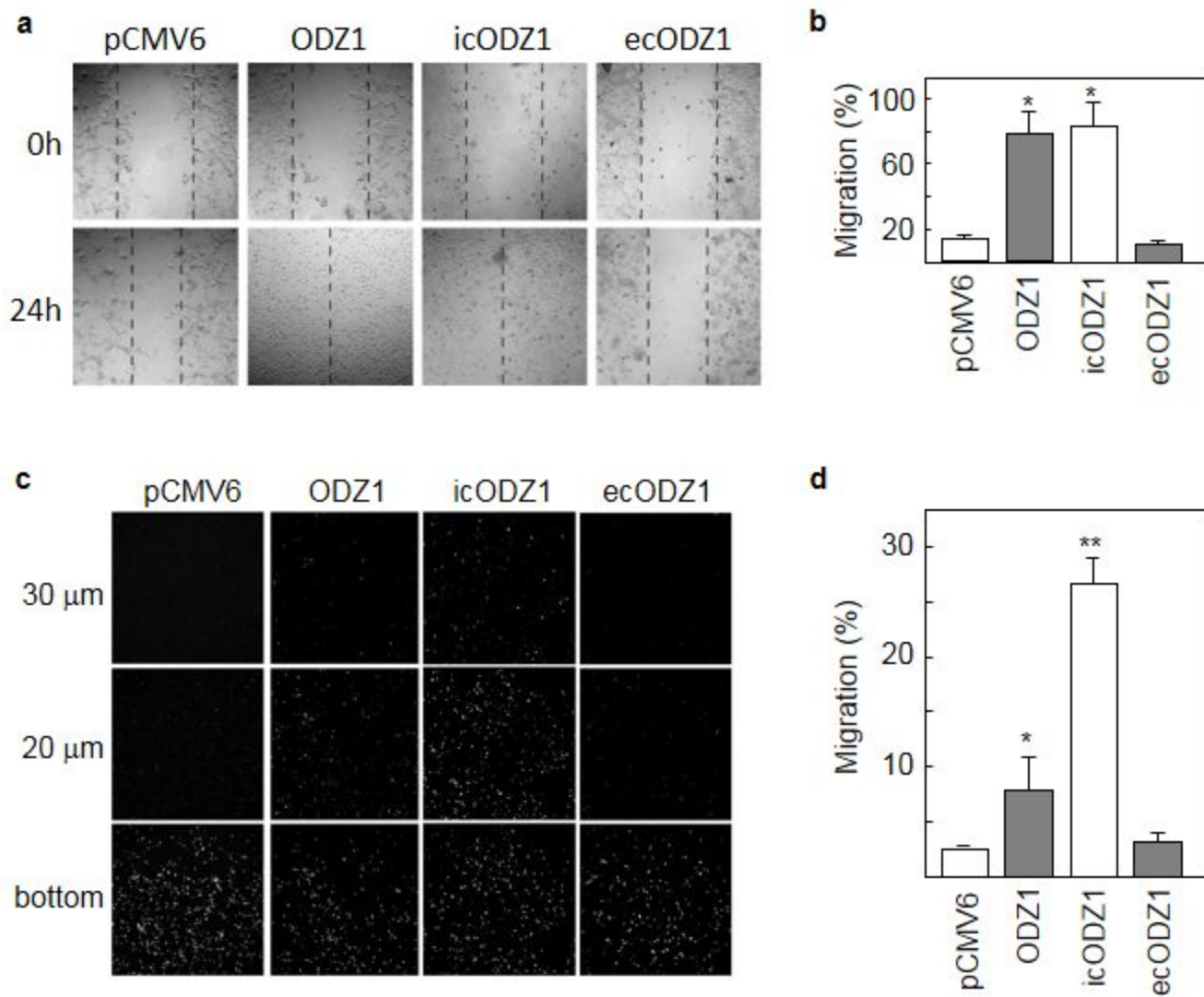


Figure 6

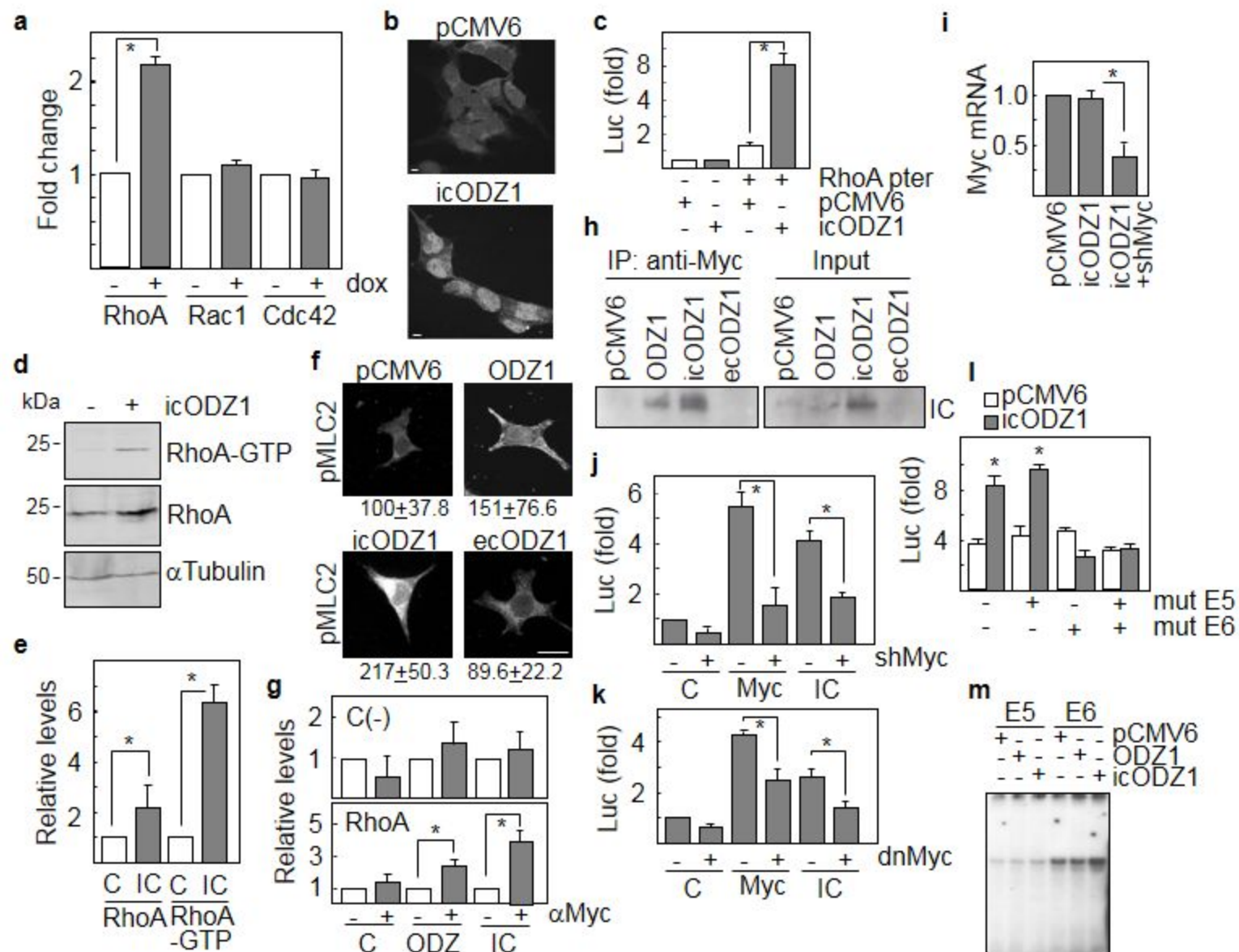


Figure 7

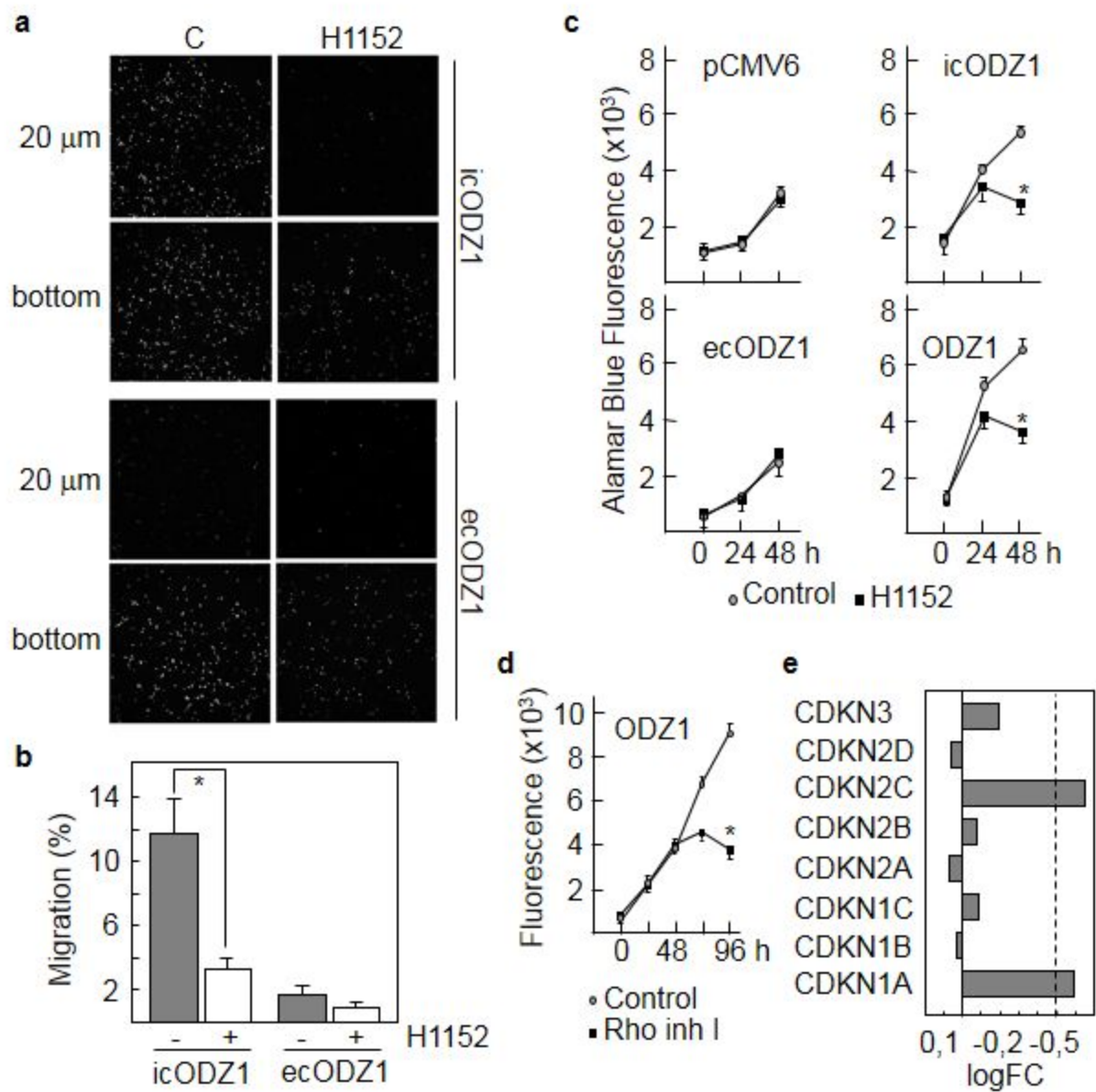


Figure 8