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ORIGINAL ARTICLE

Ror1 is expressed inducibly by Notch and hypoxia signaling and regulates stem cell-like property of glioblastoma cells

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Abstract

Ror1 plays a crucial role in cancer progression by regulating cell proliferation and migration. Ror1 is expressed abundantly in various types of cancer cells and cancer stem-like cells. However, the molecular mechanisms regulating expression of *Ror1* in these cells remain largely unknown. *Ror1* and its putative ligand Wnt5a are expressed highly in malignant gliomas, especially in glioblastomas, and the extents of *Ror1* expression are correlated positively with poorer prognosis in patients with gliomas. We show that *Ror1* expression can be upregulated in glioblastoma cells under spheroid culture, but not adherent culture conditions. Notch and hypoxia signaling pathways have been shown to be activated in spheroid-forming glioblastoma stem-like cells (GSCs), and *Ror1* expression in glioblastoma cells is indeed suppressed by inhibiting either Notch or hypoxia signaling. Meanwhile, either forced expression of the Notch intracellular domain (NICD) in or hypoxic culture of glioblastoma cells result in enhanced expression of *Ror1* in the cells. Consistently, we show that both NICD and hypoxia-inducible factor 1 alpha bind to upstream regions within the *Ror1* gene more efficiently in GSCs under spheroid culture conditions. Furthermore, we provide evidence indicating that binding of Wnt5a to Ror1, upregulated by Notch and hypoxia signaling pathways in GSCs, might promote their spheroid-forming ability. Collectively, these findings indicate for the first time that Notch and hypoxia signaling pathways can elicit a Wnt5a–Ror1 axis through transcriptional activation of *Ror1* in glioblastoma cells, thereby promoting their stem cell-like property.

KEYWORDS
glioblastoma, glioblastoma stem-like cells (GSCs), hypoxia, Notch signaling, Ror1

1 | INTRODUCTION

Gliomas can be classified into grades I–IV (WHO; World Health Organisation) based on histological and genomic characteristics.[1] Grade IV gliomas are called glioblastomas and are the most common and aggressive ones. The malignancy of glioblastomas is characterized by glioblastoma stem-like cells (GSCs), which contribute to cancer cell proliferation, invasion, drug resistance, and recurrence.[2–5] Various signaling pathways, including Notch and canonical and/or noncanonical Wnt signaling, as well as environmental factors, such as hypoxia, have been implicated in the regulation of GSCs.[6–8] Any apparently effective therapeutic strategies targeting GSCs are currently unavailable.

Ror1 has been shown to act as a receptor or co-receptor for Wnt5a, thereby mediating noncanonical Wnt signaling.[9] Ror1 plays crucial roles in regulating embryonic morphogenesis, tissue-/organogenesis, tissue repair, and cancer progression.[10–14] Expression of *Ror1* is also detectable at higher levels in various tissues and cells during embryogenesis, but is detectable marginally in adult tissues and cells except for several cell types, including tissue stem cells under physiological conditions.[15] On the other hand, accumulating evidence demonstrates that various types of cancer cells express Ror1 and/or Wnt5a at higher levels, and that Wnt5a-Ror1 signaling, activated in a cell autonomous fashion, promotes cancer cell proliferation and migration.[16,17] In addition, it has been shown that Ror1 plays critical roles in regulating the stemness of cancer stem-like cells, including GSCs.[18–20] These findings suggest that expression of *Ror1* and subsequent Ror1-mediated signaling in GSCs might contribute to the malignant progression of glioblastomas. It has been reported that expression of *Ror1* in lung adenocarcinoma cells is mediated by NKX2-1/TITF1/TTF-1,[21] while expression of *Ror1* in chronic lymphocytic leukemia and gastric cancer cells can be mediated by STAT3.[22,23] However, the molecular mechanism regulating expression of *Ror1* in glioblastomas is still unclear.

In the present study, we first investigated the mechanism regulating expression of *Ror1* in glioblastoma cells using three glioblastoma cell lines, T98G, A172, and U87MG cells. We show that expression of *Ror1* in these cells is upregulated transcriptionally by Notch and hypoxia signaling pathways that are known to be activated in GSCs under spheroid culture condition. Consistently, it was found that Notch intracellular domain (NICD) and hypoxia-inducible factor 1 alpha (HIF1α) bind to upstream regions within the *Ror1* gene, and that ectopic expression of NICD or hypoxic culture conditions result in increased expression of *Ror1* in the cells cultured under adherent conditions. Moreover, it was found that Wnt5a stimulation of T98G and A172 cells promotes their spheroid-forming capacities, supporting a notion that binding of Wnt5a, secreted from tumor microenvironments in patients with glioblastomas, to Ror1 expressed inducibly on glioblastoma cells might regulate the function of GSCs. In summary, our present findings indicate that the Wnt5a–Ror1 axis activated by Notch and hypoxia signaling pathways might play important roles in regulating the functions of glioblastoma cells at least partly in a Dvl2-dependent manner, in particular GSCs, and that Ror1 and/or Ror1-mediated signaling might be novel targets for therapeutic strategies against GSCs.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

The human glioblastoma cell lines, T98G cells, bearing the wild-type *IDH*, mutated *TP53*, *PTEN*, and deletion of *CDKN2A* genes, A172 cells, bearing the wild-type *IDH* and *TP53*, and deletion of *PTEN* genes, and U87MG cells, bearing the wild-type *IDH*, mutated *TP53*, *PTEN*, and *TERT* genes, were obtained from the ATCC and maintained in DMEM (Fujifilm Wako Pure Chemical Corporation) containing 10% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For hypoxia, cells were cultured at 37°C in a humidified atmosphere of 94% N_2, 5% CO_2, and 1% O_2. Cell line authentication was performed on T98G, A172, and U87MG cells by short tandem repeat analysis (Bex Co., Ltd.). Details on patient-derived glioblastoma cells are provided in Appendix S1. In some experiments, cells were treated with DAPT [*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester] (Sigma-Aldrich), a γ-secretase inhibitor, to block Notch signaling. Cells were transfected with the respective siRNAs by using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. The sequences of the siRNA used are listed in Table 1. The siRNAs and their control siRNA (siRNA universal negative control#1) were purchased from Sigma-Aldrich.

The expression plasmids encoding the N-terminally Myc-tagged NICD (pCAG *Myc-NICD*), Ror1 (pcDNA *Ror1*), or their empty plasmids (pCAG or pcDNA), respectively, were co-transfected with the puromycin resistance plasmid (pBABE-puro) into T98G, A172, or U87MG cells by the electroporation method using Super Electroporator NEPA21 (Nepa Gene). To increase proportions of the cells where the expression plasmids were introduced, the transfected T98G, A172, or U87MG cells were cultured for 2 days in culture medium containing 2, 0.75, or 1 μg/ml puromycin, respectively.

2.2 | Immunohistochemistry

Clinical tissue specimens were obtained and analyzed in accordance with procedures approved by the institutional review board of Kobe University Hospital (No. B220027). Primary glioblastoma specimens were resected from 12 patients at Kobe University Hospital, fixed, and embedded in paraffin for sectioning. Tissue sections were incubated with antibody against Ror1 (#4102, 1:50; Cell Signaling

TABLE 1 siRNA sequences used in this study

siRNA	Sense sequence (5′→3′)	Antisense sequence (5′→3′)
si-*Ror1*#1	CCCAGUGAGUAAAUCUCAGU	ACUGAGAUUACUCACUGGG
si-*Ror1*#2	GCAAGCAUCUUUACUAGGA	UCCUAGUAAAGAUGCUUGC
si-*HIF1α*#1	CAAAGUUCACCUGAGCCUA	UAGGCUCAGGUGAACUUUG
si-*HIF1α*#2	GAUUAACUCAGUUUGAACU	AGUUCAAACUGAGUUAAUC
si-*Notch1*#1	CAAUGUGGAUGCCGCAGUU	AACUGCGGCAUCCACAUUG
si-*Notch1*#2	GCUAACAAAGAUAUGCAGA	UCUGCAUAUCUUUGUUAGC
si-*Dvl2*#1	CAUGGAGAAGUACAACUUC	GAAGUUGUACUUCUCCAUG
si-*Dvl2*#2	CUAGUCAACCUGUCUCUCA	UGAGAGACAGGUUGACUAG

Technology [CST]) overnight at 4°C and visualized as described previously.[12] Nuclei were counterstained with hematoxylin.

2.3 | Spheroid culture

T98G, A172, and U87MG cells were seeded in spheroid culture medium [DMEM/F12 containing MACS NeuroBrew-21 without Vitamin A (Miltenyi Biotec Inc.), 20 ng/ml human recombinant EGF, and 20 ng/ml human recombinant bFGF] for 3 days. T98G, A172, and U87MG cells were cultured at densities of 1×10^5, 1×10^5 and 2.5×10^4 cells/ml, respectively. In some experiments, cells were treated with recombinant Wnt5a (R&D Systems).

2.4 | Quantitative RT-PCR

RNA isolation and quantitative RT-PCR (qRT-PCR) analysis were carried out as described previously.[24] The amounts of the respective mRNAs were normalized relative to those of *β2M*. The primers used are listed in Table 2.

2.5 | Western blotting

Cells were solubilized in lysis buffer (1% SDS, 0.1% sodium deoxycholate, 20 mM Tris–HCl, pH 8.0). The resultant lysates were subjected to SDS-PAGE, and Western blotting analyses were carried out as described previously.[23] Membranes were immunoblotted with the respective antibodies as follows: anti-Myc mAb (sc-40, 1:1000; Santa Cruz Biotechnology), rabbit anti-HIF1α mAb (#14179, 1:1000; CST), rabbit anti-Ror1 mAb (#16540, 1:1000; CST), rabbit anti-Dvl2 mAb (#3216, 1:1000; CST), and anti-α-tubulin Ab (PM054, 1:1000; Medical and Biological Laboratories), followed by HRP-conjugated secondary antibodies. Immunoreactive bands were visualized with Western Lightning Plus-ECL (Perkin Elmer) and detected using the ECL detection system (LAS-1000; Fujifilm).

2.6 | ChIP assay

ChIP assay was performed using a SimpleChIP® Enzymatic Chromatin IP Kit (#9003; CST) according to the manufacturer's instructions. Briefly, cells (4×10^6) were fixed with 1% (w/v) paraformaldehyde for 10 min at room temperature, followed by incubation with glycine. After washing with ice-cold PBS, cells were digested with micrococcal nuclease. The nuclei were sonicated for four cycles (sonication 20 s, on ice 60 s) by a cell disruptor (UD-201; Tomy) and subjected to immunoprecipitation with rabbit anti-cleaved Notch1 mAb (#4147, 1:100.; CST), rabbit anti-HIF1α mAb (1:50) or isotype-matched control rabbit IgG (#2729, 1:100; CST). After decrosslinking and purification of the DNA fragments, the amounts of the DNA fragments of interest were measured by a LightCycler 480 II System with the respective primer pairs. The primers used are listed in Table 3.

TABLE 2 Primer sequences for qRT-PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Ror1	TGCCAGCCCAGTGAGTAATCT	GCCAATGAAACCAGCAATCTG
Wnt5a	TAAGCCCAGGAGTTGCTTTG	GCAGAGAGGCTGTGCTCCTA
Notch1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
CD15	CCGCTACTACCACCAACTGAG	CTGCGAGTTCTCGAAAGCCA
CD36	TGGGACCATTGGTGATGAG	GCAACAAACATCACCACACC
CD44	TGGACAGGACAGGACCTCTT	GGGTCTCTTCTTCCACCTGTG
Nanog	CCCCAGCCTTTACTCTTCCTA	CCAGGTTGAATTGTTCCAGGTC
Dll1	GATTCTCCTGATGACCTCGCA	TCCGTAGTAGTGTTCGTCACA
Jag1	GTCCATGCAGAACGTGAACG	GCGGGACTGATACTCCTTGA
Hes5	TTCTCAGAGAATGTGTGTGCAGAGT	GGTCAGACACTTGGCAGAAGATG
Hes7	CGGGATCGAGCTGAGAATAGG	GCGAACTCCAATATCTCCGCTT
Hey1	GTTCGGCTCTAGGTTCCATGT	CGTCGGCGCTTCTCAATTATTC
Sox2	GCCGAGTGGAAACTTTTGTTCG	GGCAGCGTGTACTTATCCTTCT
Glut-1	ATTGGCTCCGGTATCGTCAAC	GCTCAGATAGGACATCCAGGGTA
Vegfa	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
Hif1α	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
Dvl2	CTGGAGCCTGAGACAGAAACC	CCATGCTCACTGCTGTCTCTCC
c-Myc	CCCGCTTCTCTGAAAGGCTCTC	CTCTGCTGCTGCTGCTGGTAG
Axin2	AGCCAAAGCGATCTACAAAAGG	AAGTCAAAAACATCTGGTAGGCA
CyclinD1	TCTGTGCCACAGAGATGTGAAG	AGTAGGACAGGAAGTTGTTGG
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

2.7 | Spheroid formation assay

T98G and A172 cells were cultured in spheroid culture medium containing 1.6% (w/v) methylcellulose (Nacalai Tesque). Fresh aliquots of MACS NeuroBrew-21, EGF and bFGF were added at day 3. After 6 days in culture, the living cells were stained with Calcein-AM (Dojindo) and counted using hybrid cell count module (Keyence). In some experiments, cells were treated with Strictinin (Nagara Science Co., Ltd.), a Ror1 inhibitor. The numbers and sizes of spheroids formed were measured, and spheroids were classified into small (<1000 μm^2), medium (1001–2000 μm^2), and large (>2001 μm^2) according to their sizes.

2.8 | Statistical analysis

Results are presented as the mean ± SD. Significance was determined as $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ compared with control using the Student's *t*-test when two groups were compared, or using one-way ANOVA followed by Dunnett's post hoc test or Tukey's post hoc test when more than three groups were analyzed.

The expression data of *Ror1* and *Wnt5a* in patients with gliomas for TCGA (The Cancer Genone Atlas) were downloaded from the GlioVis data portal.[25] Kaplan–Meier curves for overall survival based on expression levels of *Ror1* and *Wnt5a* were generated using GraphPad Prism 9.0 (GraphPad Inc.) by setting the median expression of *Ror1* and *Wnt5a* as the cut-off. Log-rank test was conducted to assess the significance of the difference between the survival curves.

3 | RESULTS

3.1 | Higher expression levels of *Ror1* are correlated with poorer prognosis of patients with gliomas

To investigate whether Ror1 might be involved in the malignancy of gliomas, we examined the possible correlation between expression levels of *Ror1* in tumor tissues and prognosis of the patients with gliomas (grade II–IV) using the TCGA database. Kaplan–Meier survival analysis revealed that higher expression levels of *Ror1* were associated closely with shorter overall survival (Figure 1A). Expression levels of *Ror1* were also correlated significantly with severer tumor grades and, indeed, *Ror1* was most strongly expressed in grade IV glioblastomas (Figure 1B). We also confirmed expression of Ror1 in clinical specimens from patients with glioblastomas by immunohistochemistry. Among the 12 cases examined, nine cases contained

TABLE 3 Primer sequences for ChIP-qPCR

Name	Forward primer (5′→3′)	Reverse primer (5′→3′)
Ror1 upstream region 1	GTGGCCACGTGATTATTGAG	TAGGCTCTGAAGATGCAAGG
Ror1 upstream region 2	TTCCTCCCATTCCCATACAC	GGAAAAGTGCAAAGGAGCAG
Hes5 promoter	GAGGCCGTAGGAGCAGATAA	AGGTCCCAGTCAGGGAAGGT
Glut-1 promoter	AAGAGGCAAGAGGTAGCAACAG	ACTCCCACTGCGACTCTGAC
Vegfa promoter	TTCGAGAGTGAGGACGTGTG	ATTGGAATCCTGGAGTGACC

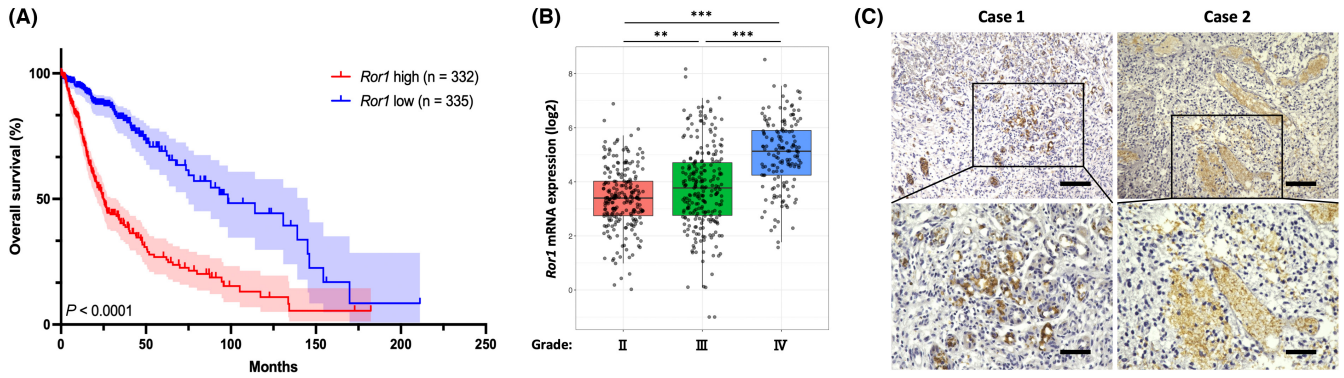

FIGURE 1 Higher expression levels of *Ror1* are correlated with poorer overall survival and severer tumor grade of patients with gliomas. (A) Kaplan–Meier survival analysis of *Ror1* high (red line) and low (blue line) expression groups in patients with gliomas. The cut-off point was determined at median expression of *Ror1*. The number of patients is shown as *n*. *P* values are obtained from the log-rank test. (B) Expression levels of *Ror1* in glioma specimens were analyzed in correlation with classified tumor grades in patients with gliomas. Statistical significance was analyzed using the Tukey's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$. (C) Immunohistochemical analyses of Ror1 with surgical specimens from patients with glioblastomas. Representative images from two cases are shown. Lower images show magnified views of boxed regions. Scale bars, 100 μm (upper panels) and 50 μm (lower panels).

tumor cells that were positive for Ror1 (Figure 1C). These findings indicate that elevated expression of *Ror1* in gliomas might be implicated in their malignant characteristics.

3.2 | Notch signaling is required for the expression of *Ror1* in the spheroid-forming glioblastoma cells

Recent studies have reported that GSCs contribute to the malignant characteristics of glioblastomas, therefore we first examined whether GSCs express *Ror1* at higher levels. In accordance with the previous reports that GSCs can be enriched within the spheroid-forming glioblastoma cells,[26] we cultured glioblastoma cell lines, T98G, A172, and U87MG cells, under spheroid condition (Figures 2A and S1A,C), and analyzed expression levels of *Ror1*, *Wnt5a* and stem cell marker genes (*CD15*, *CD36*, *Nanog*, or *CD44*) using qRT-PCR. We found that expression levels of *Ror1* as well as several sets of stem cell marker genes were increased in all three cell lines under spheroid culture conditions compared to adherent culture conditions (Figures 2B and S1B,D). Interestingly, increased expression of *Wnt5a* in spheroid-forming cells was detected in U87MG cells (Figure S1D), while expression levels of *Wnt5a* in T98G and A172 cells were decreased markedly or unaffected under spheroid culture conditions compared to adherent culture conditions, respectively (Figures 2B and S1B). These results indicate that *Ror1* is likely to be expressed predominantly in GSCs, but that expression levels of *Wnt5a* in GSCs are regulated differentially in a cell-line dependent manner.

Because the cell-to-cell interaction was enhanced in spheroid-forming cells, we next examined the possible involvement of Notch signaling, a major signaling pathway regulating cell-to-cell interaction and stemness, in spheroid-forming cells by analyzing the expression levels of Notch signaling-related genes, encoding its ligands and transcriptional target genes. qRT-PCR analysis revealed that expression levels of *Notch1* and/or genes encoding its ligands, *Jag1* or *Dll1*, were increased in all three cell lines examined under spheroid culture conditions compared to adherent culture conditions (Figures 2B and S1B,D). The Notch target genes *Hes5* and *Sox2* were also expressed highly in spheroid-forming T98G and A172 cells (Figures 2B and S1B), while distinct Notch target genes (*Hey1* and *Hes7*) were increased significantly under spheroid culture conditions compared to adherent culture conditions in U87MG cells (Figure S1D). These results suggest that Notch signaling might be activated in the GSCs within spheroids.

Notch signaling plays critical roles in regulating stem cell-like properties of GSCs.[27] Indeed, spheroid formation assay, which is an in vitro assay to evaluate the stemness, demonstrates that inhibition of Notch signaling with DAPT, a γ-secretase inhibitor, or suppressed expression of *Notch1* by transfecting siRNAs against *Notch1* (si-*Notch1*#1 or si-*Notch1*#2) decrease the numbers of spheroids in T98G cells (Figure 3A–C). Since it can be envisaged that the sizes of spheroids formed might reflect some, if not all, of the effect of DAPT or *Notch1* knockdown on differential stages of spheroid formation, we further dissected the sizes of spheroids formed in these treatments during spheroid formation assay. It was found that DAPT treatment and *Notch1* knockdown inhibit formation of spheroids with small and medium sizes rather than the large size in T98G cells (Figure 3A,C), indicating that DAPT and *Notch1* knockdown might affect earlier stages of spheroid formation. We then analyzed the effect of DAPT on expression levels of *Ror1* in T98G cells and found that DAPT treatment decreases expression levels of *Ror1*, stem cell marker genes, and Notch target genes in the cells under spheroid culture, but not adherent culture conditions (Figure 3D). On the other hand, expression levels of *Wnt5a* were unaffected by the

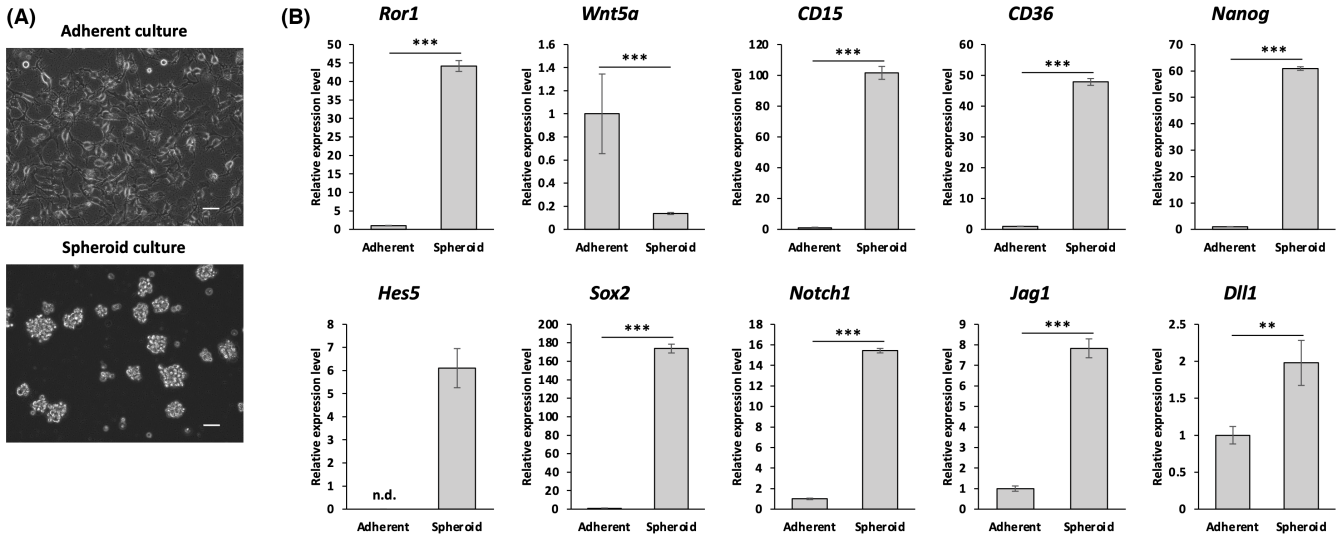

FIGURE 2 Upregulated expression of *Ror1*, stem cell marker genes, and Notch signaling-related genes in T98G cells cultured under spheroid conditions. (A) Representative images of T98G cells cultured under adherent or spheroid conditions. Scale bars, 50 μm. (B) mRNA levels of the indicated genes in T98G cells cultured under either adherent or spheroid conditions were determined by qRT-PCR. Data are expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. n. d., not determined.

treatment with DAPT under both culture conditions. We further assessed the effects of forced expression of Myc-tagged NICD (pCAG *Myc-NICD*), a gain-of-function Notch mutant, on the expression levels of *Ror1* in T98G (Figure 3E) and U87MG cells (Figure S2A). Expression levels of *Ror1* as well as Notch target genes were increased in T98G and U87MG cells expressing Myc-NICD ectopically even under adherent culture conditions, whereas expression levels of stem cell marker genes were unaffected by ectopic expression of Myc-NICD (Figures 3F and S2B). These results suggest that activation of Notch signaling in glioblastoma cells is required to induce expression of *Ror1*. Interestingly, expression levels of *Wnt5a* were increased in U87MG cells, but not in T98G cells, under spheroid

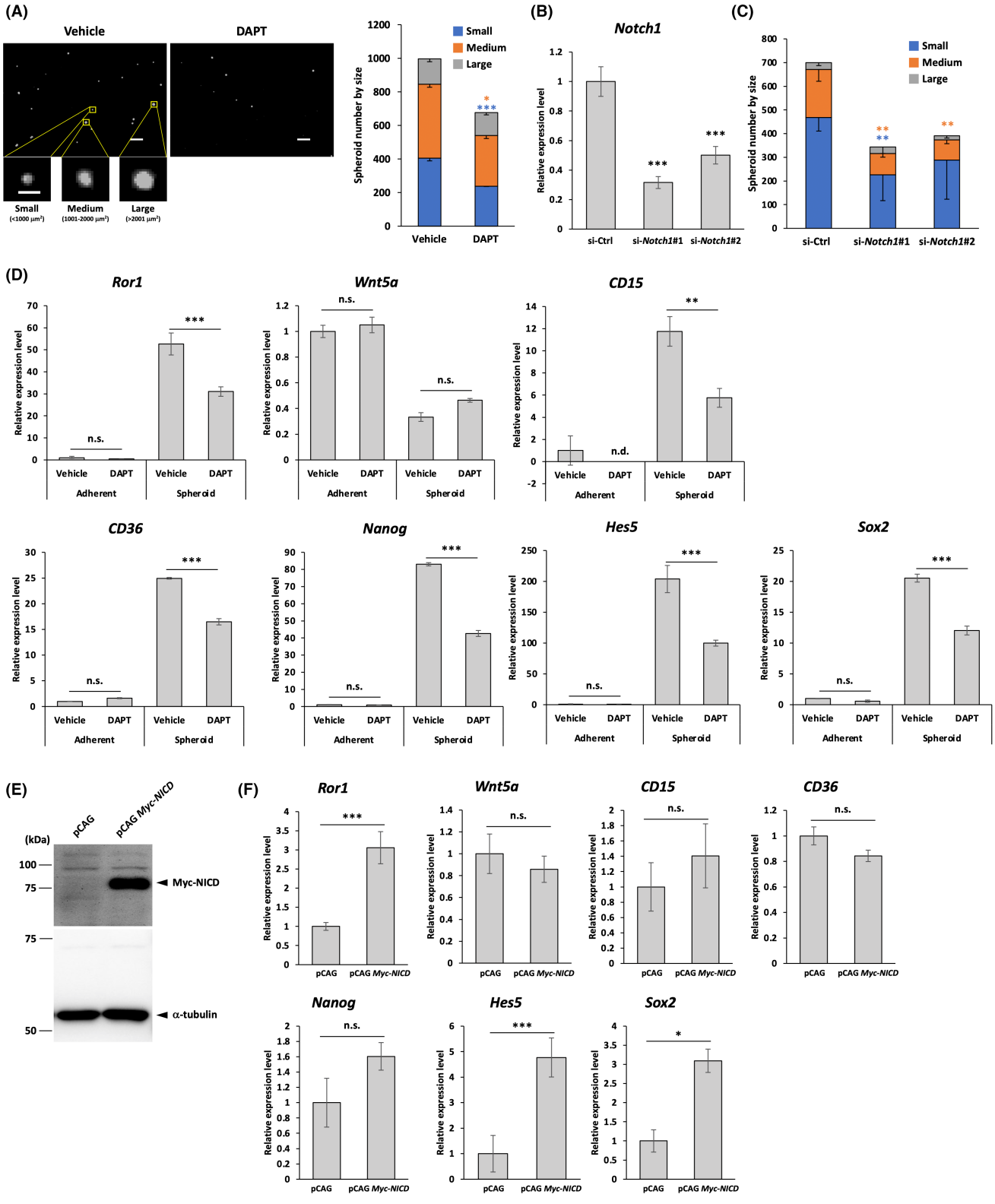

FIGURE 3 Notch signaling is required for spheroid-forming capacities and expression of *Ror1* in T98G cells. (A) Representative images of spheroid formation assay. The numbers of spheroids formed by T98G cells treated with either DAPT (10 μM, a γ-secretase inhibitor to block Notch signaling) or vehicle (DMSO) alone were evaluated at day 6. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test. Scale bars, 200 μm (upper panels) and 50 μm (lower panels). (B) The graph shows relative expression levels of *Notch1* mRNA in T98G cells transfected with the indicated siRNAs as assessed by qRT-PCR. Data are expressed as mean ± SD. $^{***}P < 0.001$, Dunnett's post hoc test. (C) The graph shows the spheroid-forming capacities of T98G cells transfected with the indicated siRNAs. Data are expressed as mean ± SD. $^{**}P < 0.01$, Dunnett's post hoc test. (D) T98G cells cultured under either adherent or spheroid conditions were treated with DAPT (10 μM) or its vehicle alone for 3 days. mRNA levels of the indicated genes were determined by qRT-PCR. Data are expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey's post hoc test. n.s., not significant. n.d., not determined. (E) Cell lysates from T98G cells transfected with either empty plasmid (pCAG) or the plasmid encoding Myc-tagged NICD (pCAG *Myc-NICD*) were subjected to Western blotting with the anti-Myc-tag mAb. (F) mRNA levels of the indicated genes in transfected T98G cells were determined by qRT-PCR. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test. n.s., not significant.

culture (Figure S1D) or ectopic expression of NICD (Figure S2B), suggesting that Notch signaling might upregulate expression of *Wnt5a* in a cellular context-dependent manner.

3.3 | Expression of *Ror1* in glioblastoma cells is upregulated via HIF1α under hypoxic conditions

It has been reported that a hypoxic tumor microenvironment contributes to the acquisition and maintenance of stem cell-like properties in glioblastomas.[28,29] As expected, HIF1α protein, a master regulator of hypoxic response, was accumulated in spheroid-forming T98G cells, and suppressed expression of *HIF1α* resulted in decreased numbers of spheroids, especially those of small and medium size (Figure 4A–C). The findings indicate that the interior of the spheroid might be in a hypoxic condition, and that HIF1α might contribute to the spheroid-forming capacities of T98G cells. We then examined whether a hypoxic environment is involved in regulating expression of *Ror1* in glioblastoma cells. When adherent cultured T98G cells were incubated under hypoxic conditions, amounts of HIF1α proteins were increased (Figure 4A) and expression levels of *Ror1*, HIF1α target genes (*Glut-1* and *Vegfa*), and stem cell marker genes, but not Notch target genes, were increased (Figure 4D). We next examined the effect of *HIF1α* knockdown on hypoxia-induced expression of *Ror1* and found that suppressed expression of *HIF1α* inhibited expression of *Ror1*, in particular under hypoxic conditions (Figure 4E,F). These results indicate that hypoxic conditions might contribute to induce expression of *Ror1*, possibly without affecting Notch signaling.

3.4 | Both NICD and HIF1α bind to upstream regions of the *Ror1* gene

Next, we investigated the possible involvement of NICD and HIF1α in the transcriptional regulation of *Ror1* gene. We retrieved publicly available data from the ChIP-Atlas database concerning the binding of Notch activation complex, including NICD and RBP-J, and HIF1α to the *Ror1* gene promoter, and identified two candidate *Ror1* upstream regions where putative binding sites of NICD, RBP-J, and HIF1α are located in close proximity (Figure 5A). Thus, we performed ChIP assay with anticleaved Notch1 and anti-HIF1α antibodies to detect the binding of NICD and HIF1α to these regions within the *Ror1* gene (*Ror1* upstream regions 1 and 2, indicated in Figure 5A) in T98G cells under adherent and spheroid culture conditions. ChIP assay revealed that bindings of NICD to these upstream regions of the *Ror1* gene as well as *Hes5* promoter (used as a positive control for NICD binding) and HIF1α to similar regions of the *Ror1* gene as well as *Glut-1* and *Vegfa* promoter (used as positive controls for HIF1α binding) were enriched under spheroid culture conditions compared to adherent culture conditions (Figure 5B,C). These results suggest that NICD and HIF1α can activate transcription of *Ror1* gene through their bindings to these *Ror1* promoter regions in glioblastoma cells.

We further investigated the possible association among *Ror1*, Notch target genes, and/or HIF1α target genes in glioma specimens using the TCGA database. Coexpression analysis of *Ror1*, Notch transcriptional target genes,[30] and/or HIF1α transcriptional target genes[31] revealed that expression levels of *Ror1* in gliomas are correlated positively to those of several Notch target genes (Figure S3A) and HIF1α target genes (Figure S3B).

3.5 | Expression of *Ror1* is required for spheroid-forming capacities of glioblastoma cells

Finally, we investigated the role of induced expression of *Ror1* in regulating the stemness of glioblastoma cells. When expression levels of *Ror1* were suppressed in T98G and A172 cells by transfecting siRNAs against *Ror1* (si-*Ror1*#1 or si-*Ror1*#2) (Figures 6A,B and S4A), the numbers of spheroids, especially of small size, were decreased (Figures 6C,D and S4B). Similar results were obtained by treating T98G cells with Strictinin, a Ror1 inhibitor[32] that prevents ligand interaction with Ror1 (Figure 6E). Moreover, suppressed or forced expression of *Ror1* in T98G cells resulted in decreased or increased expression of several stem cell marker genes, respectively (Figure S5A,B). Although expression levels of *Wnt5a* in T98G, but not A172 and U87MG cells, were decreased under spheroid culture conditions compared to adherent culture conditions (see Figure 2B), a previous study reported that *Wnt5a* was

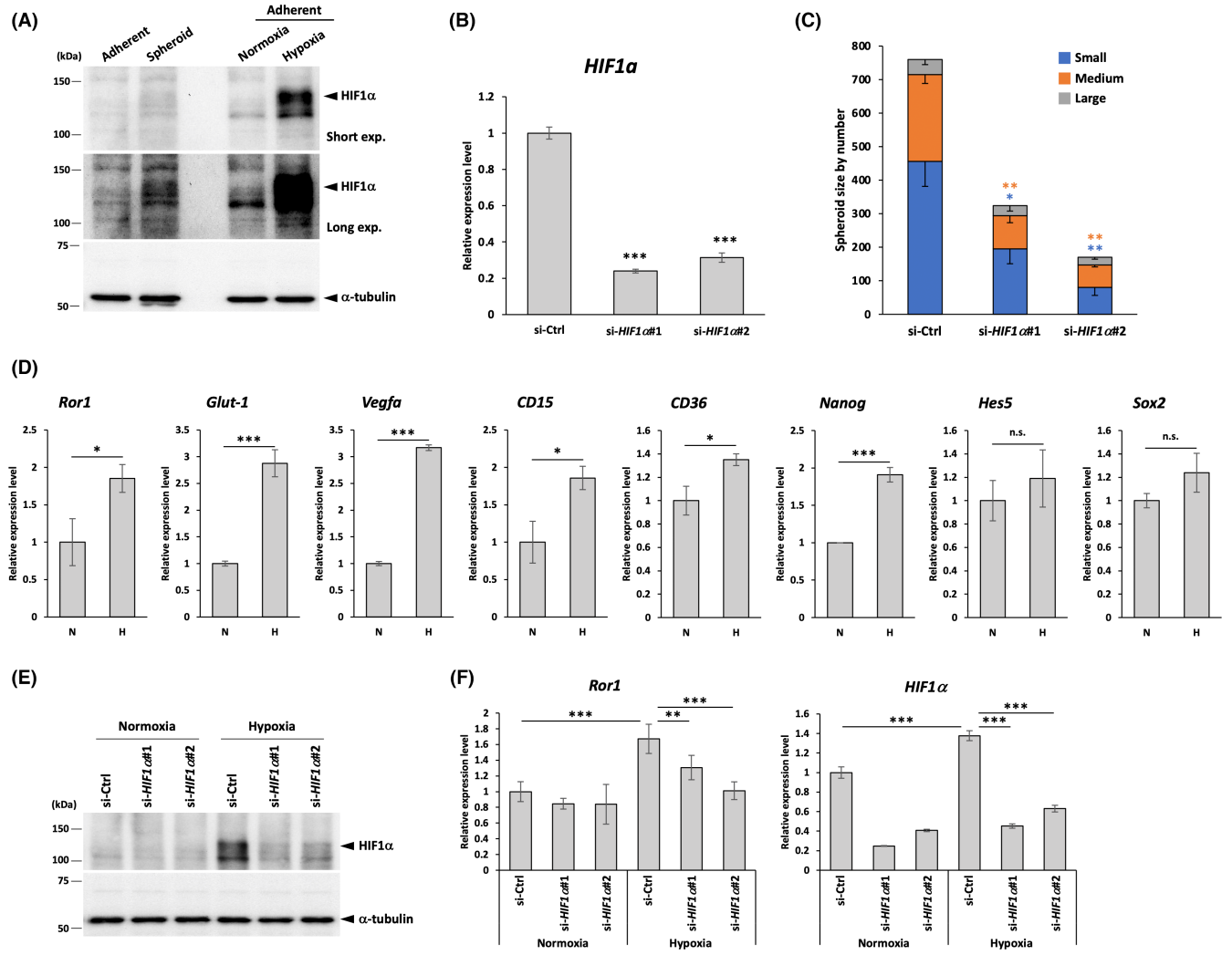

FIGURE 4 HIF1α is required for spheroid-forming capacities and expression of *Ror1* in T98G cells. (A) Cell lysates from T98G cells cultured under adherent or spheroid conditions and from adherent T98G cells cultured under normoxic or hypoxic conditions for 8 h were subjected to Western blotting with anti-HIF1α mAb. (B) The graph shows relative expression levels of *HIF1α* mRNA in T98G cells transfected with the indicated siRNAs as assessed by qRT-PCR. Data are expressed as mean ± SD. ***$P < 0.001$, Dunnett's post hoc test. (C) The graph shows the spheroid-forming capacities of T98G cells transfected with the indicated siRNAs. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, Dunnett's post hoc test. (D) Relative mRNA levels of the indicated genes in T98G cells cultured under either normoxic or hypoxic conditions for 8 h were determined by qRT-PCR. N, normoxia; H, hypoxia. Data are expressed as mean ± SD. *$P < 0.05$, ***$P < 0.001$, Student's *t*-test. n.s., not significant. (E, F) Adherent cultured T98G cells were transfected with the indicated siRNAs and cultured under either normoxic or hypoxic conditions for 8 h. Cellular proteins and mRNAs were isolated from these cells, and contents of HIF1α protein and mRNA levels of *Ror1* and *HIF1α* were determined by (E) Western blotting with the anti-HIF1α mAb or (F) qRT-PCR, respectively. Data are expressed as mean ± SD. **$P < 0.01$, ***$P < 0.001$, Tukey's post hoc test.

expressed highly in glioma tissues when compared with adjacent normal brain tissues.[33] Kaplan–Meier survival analysis using the TCGA database revealed that higher expression levels of *Wnt5a* were correlated with poorer prognosis in patients with gliomas (Figure S6A). Elevated expression levels of *Wnt5a* were also associated with severer tumor grades (Figure S6B). These findings indicate that Wnt5a derived from tumor-surrounding cells might promote the malignant characteristics of glioblastomas. To test whether Wnt5a can also contribute to regulate the stemness of glioblastoma cells through its binding to Ror1, we examined the effects of treatment with recombinant Wnt5a in the presence or absence of forced expression of *Ror1* in T98G and A172 cells on their spheroid-forming capacities. The number of spheroids, particularly those of small size, was increased by treatment with recombinant Wnt5a or forced expression of *Ror1*, respectively, and was further enhanced by their combination (Figures 6F,G and S4C,D). We also examined the phosphorylation status of Dvl2, a well-known surrogate marker of canonical and noncanonical Wnt signaling (including the Wnt5a–Ror1 axis), by Western blotting analysis to detect a phosphorylation-dependent electrophoretic mobility shift of Dvl2. Indeed, a phosphatase-sensitive electrophoretic mobility shift of Dvl2 was detected in spheroid-forming

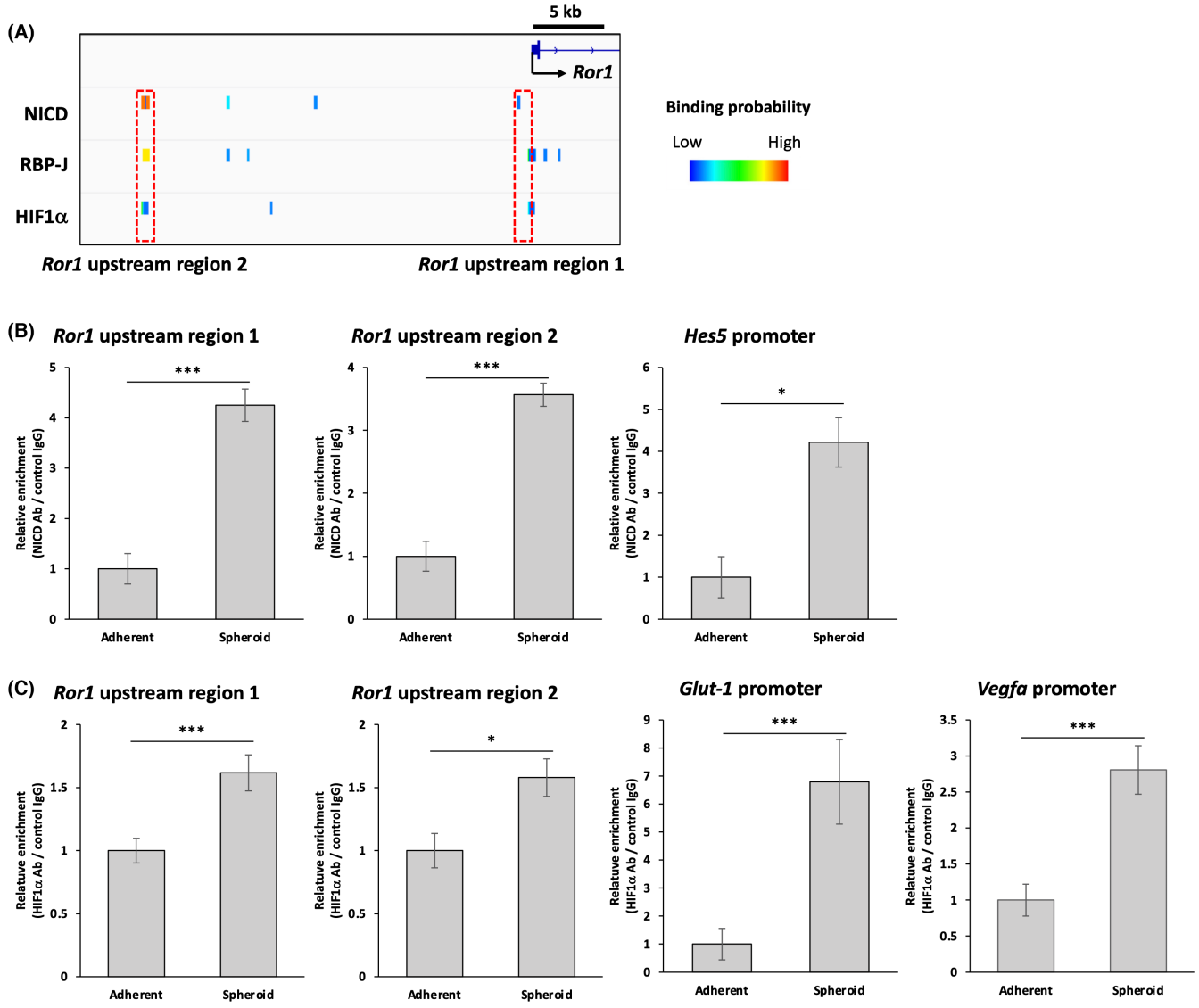

FIGURE 5 Induced expression of *Ror1* in T98G cells under spheroid culture conditions can be mediated by binding of NICD and HIF1α to the upstream regions of the *Ror1* gene. (A) A schematic representation of publicly available ChIP data obtained with various cell types around the *Ror1* gene. The data were retrieved from ChIP-Atlas and are mapped to the reference human genome (hg19) using the Integrative Genomics Viewer. The red dotted line boxes indicate the positions of primer sets for ChIP analysis. (B, C) ChIP analysis was performed using T98G cells cultured under adherent and spheroid conditions, and subjected to co-immunoprecipitation with either (B) rabbit anti-cleaved Notch1 antibody (NICD Ab) or control rabbit IgG (control IgG), or (C) rabbit anti-HIF1α antibody (HIF1α Ab) or control rabbit IgG (control IgG). Subsequently, qPCR analysis was performed using a primer set specific for the indicated gene regions. Values for the respective conditions are presented as fold enrichment relative to ChIPs with control rabbit IgG. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test.

T98G cells (Figure 6H), and was somewhat enhanced by Wnt5a stimulation and/or forced expression of *Ror1* (Figure 6I). When we suppressed expression of *Dvl2* in T98G cells by transfecting siRNAs against *Dvl2* (si-*Dvl2*#1 or si-*Dvl2*#2), the number of spheroids was decreased, and marginally but not significantly increased by Wnt5a stimulation (Figure 6J,K). These findings suggest that Dvl2 mediates Wnt5a-dependent spheroid formation of T98G cells. Collectively, these results indicate that the Wnt5a–Ror1 axis might play an important role in regulating the spheroid-forming capacities of glioblastoma cells at least partly depending on Dvl2.

4 | DISCUSSION

Although increased expression of Ror1 and subsequent Ror1-mediated signaling in cancer cells have been shown to promote cancer progression by regulating their proliferation and migration,[12,13] little is known about how expression levels of *Ror1* are upregulated markedly in various types of cancer cells. In this study, we show for the first time that expression of *Ror1* is upregulated by Notch and hypoxia signaling pathways in glioblastoma cells, especially those with stem cell-like characteristics.

Notch and hypoxia signaling pathways play crucial roles in the malignant characteristics of glioblastomas by promoting the cell proliferation, invasiveness, and stemness of glioblastoma cells both in vitro and in vivo, and are activated markedly in glioblastoma cells with stem cell-like characteristics.[27–29,31,34–36] Consistent with these findings, we observed that both signaling pathways are activated

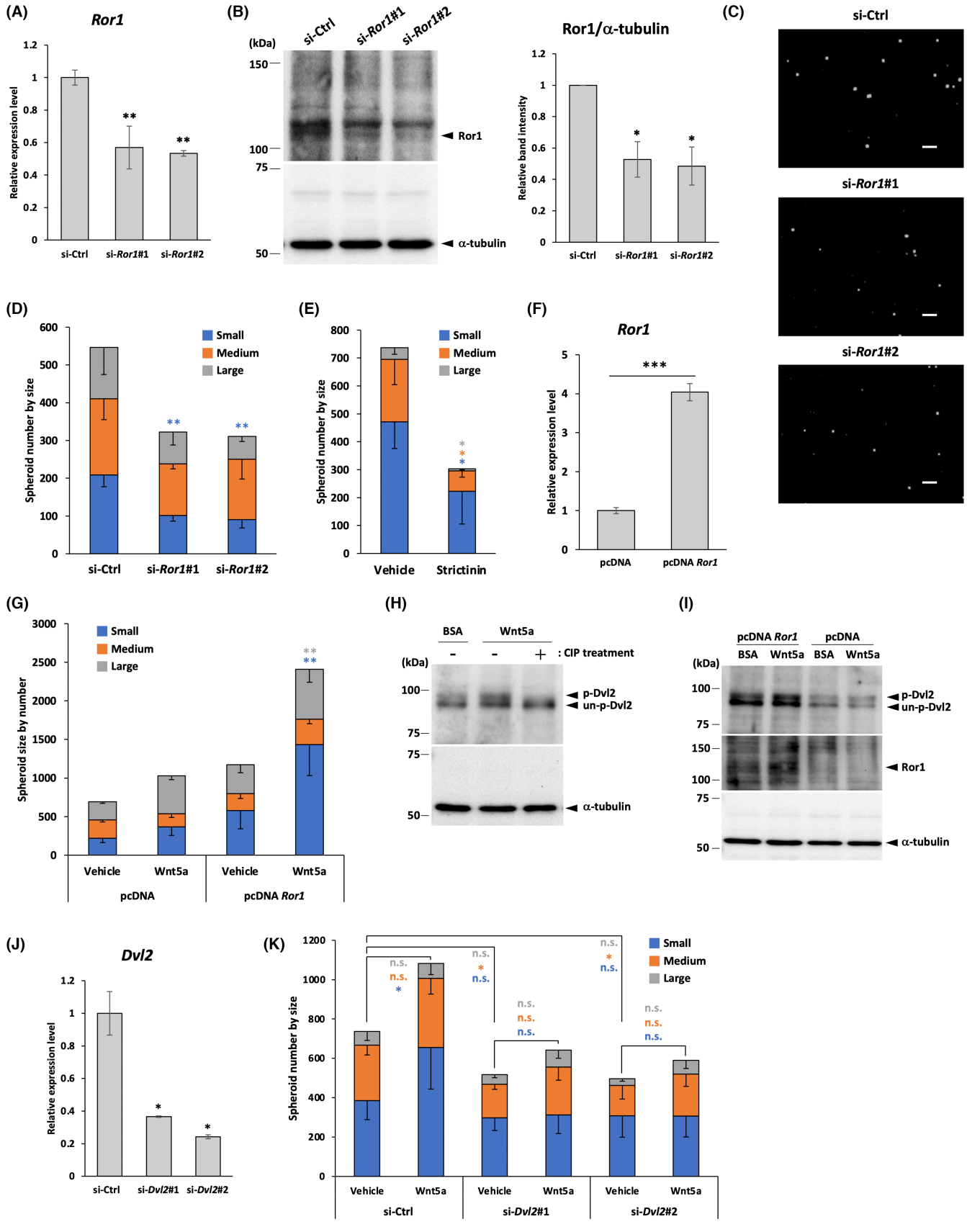

FIGURE 6 Wnt5a–Ror1–Dvl2 axis promotes spheroid-forming capacities of T98G cells. (A, F) mRNA levels of *Ror1* in T98G cells transfected with the indicated siRNAs or expression plasmids were determined by qRT-PCR. Data are expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, Dunnett's post hoc test or Student's *t*-test. (B) Cell lysates from T98G cells transfected with the indicated siRNAs were subjected to Western blotting with the anti-Ror1 mAb. The graph shows the relative band intensities of Ror1 normalized by those of α-tubulin. $^{*}P < 0.05$, Dunnett's post hoc test. (C) Representative images of spheroid formation assay with T98G cells treated with the indicated siRNAs. Scale bars, 200 μm. (D) The spheroid-forming capacities of T98G cells transfected with the indicated siRNAs. Data are expressed as mean ± SD. $^{**}P < 0.01$, Dunnett's post hoc test. (E) The spheroid-forming capacities of T98G cells treated with either Strictinin (100 μM; a Ror1 inhibitor) or vehicle (DMSO) alone. Data are expressed as mean ± SD. $^{*}P < 0.05$, Student's *t*-test. (G) The spheroid-forming capacities of T98G cells transfected with either plasmid encoding *Ror1* (pcDNA *Ror1*) or empty plasmid (pcDNA), and further cultured in the presence of either vehicle (0.1% BSA) or recombinant Wnt5a (200 ng/ml) for 6 days. $^{**}P < 0.01$, Dunnett's post hoc test. (H) Spheroid-forming T98G cells treated with either vehicle (0.1% BSA) or recombinant Wnt5a (200 ng/ml) for 3 h were solubilized, and the resultant lysates of Wnt5a-treated cells were incubated with or without 10 U of calf intestine alkaline phosphatase (CIP) for 1 h at 37°C. Cell lysates were subjected to Western blotting with the anti-Dvl2 mAb. Arrowheads indicate phosphorylated Dvl2 (p-Dvl2) and unphosphorylated Dvl2 (un-p-Dvl2), respectively. (I) Spheroid-forming T98G cells transfected with either plasmid encoding *Ror1* (pcDNA *Ror1*) or empty plasmid (pcDNA) were treated with either vehicle (0.1% BSA) or recombinant Wnt5a (200 ng/ml) for 3 h, and then solubilized and subjected to Western blotting with anti-Dvl2 and anti-Ror1 mAbs. (J) mRNA levels of *Dvl2* in T98G cells transfected with the indicated siRNAs were determined by qRT-PCR. Data are expressed as mean ± SD. $^{*}P < 0.05$, Dunnett's post hoc test. (K) The spheroid-forming capacities of T98G cells transfected with indicated siRNAs, and further cultured in the presence of either vehicle (0.1% BSA) or recombinant Wnt5a (200 ng/ml) for 6 days. $^{*}P < 0.05$, Tukey's post hoc test. n.s., not significant.

in spheroid-forming glioblastoma cells and are required for their spheroid-forming capacities. When Notch signaling in spheroid-forming T98G cells was inhibited by treatment with DAPT, the expression levels of *Ror1* were significantly decreased (Figure 3D), suggesting that *Ror1* is likely to be expressed in GSCs, which can be maintained through Notch signaling. Interestingly, forced expression of NICD in adherent cultured T98G cells is sufficient to induce expression of *Ror1* in the cells without affecting the expression levels of well-known GSC marker genes, i.e. *CD15*, *CD36*, and *Nanog* (Figure 3F). We also show that NICD binds efficiently to the proximal and distal upstream regions of the *Ror1* gene under spheroid culture conditions (Figure 5B). These findings indicate that *Ror1* might be a transcriptional target gene of Notch signaling in glioblastoma cells. In addition to Notch signaling, we demonstrated the involvement of hypoxic signaling in induced expression of *Ror1* in glioblastoma cells. Indeed, hypoxic culture conditions can induce expression of *Ror1* in a HIF1α-dependent manner (Figure 4D–F). It has been reported that HIF1α can activate Notch signaling by interacting with NICD in glioblastoma cells,[37] although we failed to detect apparent changes in expression levels of Notch target genes in T98G cells under hypoxic culture conditions (Figure 4D). In fact, HIF1α also binds to the upstream regions of the *Ror1* gene (Figure 5C), indicating that expression of *Ror1* might be regulated transcriptionally by HIF1α. Because NICD and HIF1α appear to bind to similar, if not identical, promoter regions of the *Ror1* gene, it is intriguing to investigate whether NICD and HIF1α can cooperatively upregulate expression of *Ror1* in glioblastoma cells, where both Notch and hypoxia signaling pathways are activated. GSCs have been shown to be located in specific microenvironments (e.g. hypoxic niches) within tumors.[29] In the brain, endothelial cells and nerve fibers associated with tumors have also been shown to act as stem cell niches for GSCs by providing Notch ligands, such as Jag1 and Dll4.[34,36] Future study will be required to clarify whether *Ror1* is indeed expressed in the GSCs associated with these stem cell niches in vivo.

In this study, we show that suppressed expression of *Ror1* in T98G and A172 cells resulted in decreased numbers of spheroids, while forced expression of *Ror1* tended to increase the number of spheroids, which was further augmented by simulation with Wnt5a (Figures 6G and S4D). These results suggest that binding of Wnt5a to Ror1, which can be upregulated by Notch and hypoxia signaling pathways in glioblastoma cells, might promote their spheroid-forming capacities. On the other hand, suppressed expression of *Ror1* failed to affect the spheroid-forming capacities of patient-derived glioblastoma (PDG) cells (Figure S7). We think that difference in the effects of *Ror1* knockdown on spheroid-forming capacities might be attributable to differential cellular contexts because the expression profiles of stem cell marker genes and Notch signaling target genes were slightly different among the glioblastoma cell lines and PDG cells (Figure S8) used in this study. Future study will be required to clarify the molecular basis of this issue.

We also found that expression levels of *Wnt5a* in the spheroid-forming glioblastoma cells were regulated differentially in a cell-line dependent manner, and were indeed decreased in spheroid-forming T98G cells (Figure 2B). Because it has been reported that other Wnt ligands such as Wnt16 can bind to Ror1,[38,39] it is interesting to determine whether these Wnt ligands can elicit Ror1-mediated signaling in regulating the spheroid-forming capacities of glioblastoma cells, including T98G cells.

We also show that stimulation of spheroid-forming T98G cells with Wnt5a enhances the phosphorylation level of Dvl2, a surrogate marker of canonical and/or noncanonical Wnt signaling (Figure 6H), and that Dvl2 might mediate Wnt5a-enhanced spheroid-forming capacities of T98G cells (Figure 6K). Meanwhile, expression of canonical Wnt signaling marker genes (*Axin2* and *CyclinD1*) was increased in spheroid-forming T98G cells (Figure S9). Thus, it will be important to clarify how Wnt5a–Ror1 signaling affects canonical and/or noncanonical Wnt signaling in regulating the spheroid-forming capacities of glioblastoma cells.

Our findings reveal that *Ror1* is expressed highly in glioblastoma cells with stem cell-like characteristics through the activation of Notch and hypoxia signaling pathways, and that NICD- and HIF1α-induced expression of *Ror1* is required for the spheroid-forming capacities of GSCs. However, the signaling pathway(s) downstream of Ror1 remains unclear. Future study will be required to determine how Ror1 can regulate the stem cell-like characteristics of glioblastoma cells. It can be envisaged that expression of Ror1 might be a potential diagnostic and/or prognostic marker of glioblastomas. Considering an inhibitory effect of Strictinin on the spheroid-forming capacities of GSCs, further screening of Ror1 inhibitors might help to develop drugs that are more suitable for therapeutic approaches to glioblastomas.

AUTHOR CONTRIBUTIONS

Tomohiro Ishikawa, Mitsuharu Endo, and Yasuhiro Minami designed the research. Tomohiro Ishikawa and Yasuka Ogura performed the experiments. Tomohiro Ishikawa and Mitsuharu Endo analyzed the data. Kazuhiro Tanaka, Hiroaki Nagashima, and Takashi Sasayama provided the resources and supervision, and contributed to sample preparation. Tomohiro Ishikawa, Mitsuharu Endo, and Yasuhiro Minami wrote the manuscript in consultation with Kazuhiro Tanaka, Hiroaki Nagashima, and Takashi Sasayama.

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CONFLICT OF INTEREST

Yasuhiro Minami is an editorial board member of *Cancer Science*.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed consent: N/A.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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