Cytoplasmic cyclin D1 regulates glioblastoma dissemination

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**Abstract**

Glioblastoma (GBM) is a highly invasive brain neoplasia with an elevated recurrence rate after surgical resection. The cyclin D1 (Ccd1)/Cdk4–retinoblastoma 1 (RB1) axis is frequently altered in GBM, leading to overproliferation by RB1 deletion or by Ccd1–Cdk4 overactivation. High levels of Ccd1–Cdk4 also promote GBM cell invasion by mechanisms that are not so well understood. The purpose of this work is to elucidate the in vivo role of cytoplasmic Ccd1–Cdk4 activity in the dissemination of GBM. We show that Ccd1 activates the invasion of primary human GBM cells through cytoplasmic RB1-independent mechanisms. By using GBM mouse models, we observed that evaded GBM cells showed cytoplasmic Ccd1 colocalizing with regulators of cell invasion such as RalA and paxillin. Our genetic data strongly suggest that, in GBM cells, the Ccd1–Cdk4 complex is acting upstream of those regulators. Accordingly, expression of Ccd1 induces focal adhesion kinase, RalA and Rac1 activities. Finally, in vivo experiments demonstrated increased GBM dissemination after expression of membrane-targeted Ccd1. We conclude that Ccd1–Cdk4 activity promotes GBM dissemination through cytoplasmic and RB1-independent mechanisms. Therefore, inhibition of Ccd1–Cdk4 activity may be useful to hinder the dissemination of recurrent GBM.

Keywords: glioblastoma; cyclin D1; cytoplasm; migration; tumor dissemination

Revised 12 March 2019; Accepted 2 April 2019

Introduction

Diffuse gliomas are the most frequent tumors in adult brain and are characterized by extensive infiltration into brain parenchyma [1]. Among them, glioblastoma (GBM) is the most common and aggressive (grade IV) and shows a median survival of 15 months [2,3]. In recent years, to achieve a more accurate classification and to increase diagnostic and predictive outcomes, a broad experimental pursuit has been to elucidate genetic and epigenetic alterations involved in diffuse glioma etiology and development [4–10]. For example, we now know that a recurrent point mutation (R132H) in the isocitrate dehydrogenase 1 gene (IDH1) is mostly associated with low-grade diffuse gliomas and a better prognosis [4,9–11]. All of this genomic research has brought about a new classification of diffuse gliomas based on molecular fingerprints together with histological grading [12]. In this classification, GBMs are divided into IDH-wildtype and IDH-mutant. Most GBMs (90%) are IDH-wildtype, predominate in elderly patients and have a poor prognosis. The remaining GBMs are IDH-mutant, are less aggressive with a better prognosis, predominate in younger patients and evolved from a low-grade glioma (secondary GBMs). In addition to IDH status, there are other markers associated with specific subsets of patients. For example, p53, ATRX and H3K27M mutations, as well as the CpG island methylator phenotype, are also used as prognosis markers for GBM [8,10].
At present, regardless of the molecular characteristics, the standard treatment of patients with GBM includes surgical resection, radiotherapy and chemotherapy with temozolomide [13], an alkylating drug whose effects can be alleviated by DNA repair enzymes. A significant number of patients show intrinsic resistance to temozolomide due to expression of an alkyltransferase encoded by the MGMT gene [14]. When the MGMT promoter is unmethylated, expression of this DNA repair gene confers resistance to alkylating agents. Alternatively, patients with methylated MGMT promoter are sensitive to temozolomide [15,16].

After treatment, recurrences are most likely due to incomplete resection of the tumor. GBMs show specific histological patterns of invasion formed by the interaction of tumor cells with their immediate environment [17,18]. For example, cell migration along white matter tracts or blood vessels (perivascular) is frequently observed in biopsies.

Cdk4/6 are part of one of the core pathways frequently altered in GBM [5,8]. D-type cyclins (Ccnd) complexed with Cdk4/6 kinases phosphorylate and inhibit retinoblastoma protein (RB1) in the nucleus, thus promoting cell cycle entry and proliferation [19]. The activity of Ccnd-Cdk4/6 complexes can be hindered by the Cdk inhibitors p16 (CDKN2A), p15 (CDKN2B) and p18 (CDKN2C). The most frequent alterations leading to activation of the Cdk4/6 pathway in GBM are co-deletions of CDKN2A and CDKN2B loci (56%), amplification of CDK4 (14%) and deletion of RB1 (7.9%) [8]. The detection and analysis of these mutations are relevant for prognosis. For example, the presence of homozygous deletions in CDKN2A and CDKN2B loci drastically reduces the overall survival of patients with IDH-mutant gliomas irrespective of whether they are grade II astrocytic tumors or GBMs [20]. In xenograft models, GBM growth is significantly reduced after treatment with pharmacological inhibitors of Cdk4/6 activity [21,22]. However, RB1 nonexpressing GBMs are resistant to this treatment [23,24].

The movement of invading cells along the extracellular matrix requires the coordination of different processes. Invading cells have to detach themselves partially from the matrix and, at the same time, extend protrusions such as lamellipodia to undergo migration [25]. Knockdown of Ccnd1 in macrophages and fibroblasts reduces adhesion and spreading on fibronectin plates and, at the same time, increases cell migration and invasion capacities [26,27]. Also, Ccnd1 down-regulation in GBM cell lines not only attenuates their proliferation but also their invasiveness [28,29]. Contrasting with the nuclear role of Ccnd1-Cdk4 in the regulation of proliferation, the control of cell invasiveness by Ccnd1-Cdk4 involves cytoplasmic mechanisms and targets [30,31]. In previous work, we described that cytoplasmic Ccnd1-Cdk4 promotes invasion in mouse fibroblasts and rat tumor cells through phosphorylation of the focal adhesion component paxillin (Pxn) and the Ral GTPase exchange factor (GEF) Rgl2 [30,32]. Ccnd1-Cdk4 induces the phosphorylation of Pxn at serine 83 [30], which is involved in focal adhesion kinase (FAK) and Rac1 activation [33]. On the other hand, Rgl2 activation promotes accumulation of Ral-GTP [34].

The purpose of this work is to reveal the importance of cytoplasm-associated Ccnd1 activity in the invasive properties of primary human tumor cells. We show that cytoplasmic Ccnd1-Cdk4 plays a role in the dissemination of GBM in vivo.

Materials and methods

Cell culture methods

GBM cell lines U251-MG and U87-MG were obtained from ATCC (Manassas, VA, USA). Cell line authentication was carried out by comparing the STR profile with the reference cell line in ATCC (Cell Line Authentication Service, StabVida, Caparica, Portugal). Cells were maintained in DMEM containing 10% FBS, penicillin/streptomycin at 37 °C and 5% CO₂ atmosphere. Palbociclib (Selleckchem, Houston, TX, USA) was used at a final concentration of 2 or 5 μM. Primary GBM cell cultures were isolated as previously described [35]. We managed to establish 12 primary cultures, 10 GBM and two astrocytoma (grade II), and characterized Ccnd1, Cdk4, RB1 and IDH1 status in all of them. For further studies, we used three strains with different RB1 and CDKN2A status: GBM65 (RB1-defective; CDKN2A-wildtype), GBM6 (RB1-wildtype; CDKN2A-defective) and GBM55 (RB1-wildtype; CDKN2A-wildtype). Proliferation, viability, spreading and invasion assays were adapted from previously described protocols [30] and are detailed in supplementary material, Supplementary materials and methods. For experiments using palbociclib, we treated the cells with the inhibitor for 12 h before the experiment.

Expression vectors

Human CCND1, mouse Pxn and human RGL2 were used to obtain N-terminal 3×HA fusions under Ubiquitin (UBI) or CMV promoters in a lentiviral vector derived from pDSL (Invitrogen, Carlsbad, CA, USA). Site-directed mutagenesis primers were used to obtain the different mutant alleles of the above genes, as described in supplementary material, Supplementary materials and methods.

The dominant-negative allele RalBS28N was obtained from Addgene (Watertown, MA, USA) (CJ Der). The plasmid pMMmCcnd1 was obtained from Source Bio Science (Nottingham, UK; Image ID 3155470).

Immunofluorescence and immunohistochemistry

 Cells were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature.
Fixed GBM cells were permeabilized with 0.2% Triton-X-100 for 3 min at room temperature and blocked with 3% BSA. For tissue immunofluorescence, fixed mouse brains were treated with 30% sucrose overnight, included in tissue freezing medium (72592; EMS, Hatfield, PA, USA) and kept at −80°C. Slides (20 μm) were prepared in a cryostat and stored at −80°C until use. Samples were transferred to room temperature and permeabilized with methanol-acetone at −20°C. Primary antibodies are described in supplementary material, Table S1. In both protocols, primary antibodies were combined with appropriate Alexa488- and/or Alexa594-labeled secondary antibodies (Molecular Probes, Eugene, OR, USA). Nuclei were stained with Hoescht (Molecular Probes). For GFP staining, we performed a sandwich with two secondary Alexa488 antibodies (goat anti-rabbit and rabbit anti-goat) to increase the signal. Images were acquired using 40 and 60× objectives in an Olympus FV1000 confocal system and EVOS microscope.

Details of immunohistochemistry in paraffin blocks can be found in supplementary material, Supplementary materials and methods. A tissue microarray (TMA) containing 20 samples of GBM and 20 of astrocytoma (TMA-324) was obtained from the Spanish National Cancer Research Center (CNIO) Biobank (Madrid, Spain).

Immunoblotting and Ral pulldown assay

For immunoblot analysis, protein samples were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore, Burlington, MA, USA) and incubated with primary antibodies (see supplementary material, Table S1). Appropriate peroxidase (HRP)-linked secondary antibodies (GE Healthcare, Chicago, IL, USA) were detected using the chemiluminescent HRP substrate Immobilon Western (Millipore). Chemiluminescence was recorded with a Chemidoc-MP imaging system (BioRad, Hercules, CA, USA). Ral activity was analyzed by measuring the GTP-bound form of Ral. The assays were performed using RalBP1 agarose (Upstate, Lake Placid NY, USA; cat# 14-415) according to the manufacturer’s instructions (see supplementary material, Supplementary materials and methods).

Studies in humans and animals statement

Human samples for primary cultures were provided by the Biobank of IRBLeida authorized by the Department of Health of Catalonia and registered in the National Register of Biobanks of the Carlos III Institute of Health (reference number B.0000682). The study was conducted following the principles of the Declaration of Helsinki (World Medical Association, 1964) and with the approval of the Ethics Committee for Scientific Research (CEIC) of HUAV-UdL.

Studies involving experiments with animals were subjected to approval by the Commission of Animal Experimentation (CEA), pursuant to Decree 214/1997 and Royal Decree 53/2013 from Spanish law on the use of animals for experimentation and other scientific purposes. The experiments were performed in the animal facility of the University of Lleida (L-registration number 9900005).

Mouse models

Immunodeficient SCID hr/hr male mice (12 weeks old; 20–25 g) were maintained in specific pathogen-free conditions and were injected with 5 × 10^5 U87 luciferase-expressing cells by intracranial injection in the subventricular zone of the right hemisphere (2 mm left of bregma, 1 mm anterior to coronal suture, 3 mm depth). Depending on the experiment, animals were euthanized 21 or 28 days after cell injection, brains were analyzed by bioluminescence and fluorescence, and finally fixed with paraformaldehyde; a sample was included in paraffin for H&E staining and immunohistochemistry. Luciferine (Goldbio, St. Louis, MO, USA) was injected into the animals and the signal was recorded with a PhotoneGerER (Biospace, Nesles la Vallée, France) and the software Photo-Acquisition. Image analysis was carried out in M3Vision™.

In the gliomagenesis model, tumors were generated by the injection of RCAS-hPDGFrα viruses in neonatal mice (Nestin/hv-a; Cdkn2ar−/−). Samples were obtained from mice as previously described [36].

Statistical analyses

Two-tailed t-tests allowing unequal variance or Mann–Whitney tests were used (*p < 0.05, **p < 0.01, ns, not significant). Comparisons among groups were made by one-way ANOVA and Tukey-HSD post-test. Throughout, error bars indicate SEM, except in Figure 4E (mean ± SD). In Figure 4D, mean values and confidence limits for a proportion were calculated. Analyses of results obtained from mice are detailed in supplementary material, Supplementary materials and methods.

Results

RB1-independent functions of Ccnd1 regulate the invasion efficiency of GBM cells

To address the importance of the Ccnd1-Cdk4-RB1 regulatory pathway in human GBM, first we analyzed the protein levels of Ccnd1, Cdk4 and RB1 in primary GBM cells, grade II astrocytoma cells as well as in U251-MG and U87-MG GBM cell lines (Figure 1A). All of the GBM samples showed expression of Ccnd1 and Cdk4, most displaying higher levels than the low-grade astrocytoma samples and GBM cell lines. In contrast, RB1 exhibited different levels among GBM samples and was not detected in two (GBM65 and GBM66). The Cdk4-specific inhibitor pabociclib impinges upon the proliferation rate of GBM cells only
Figure 1. Ccnd1 regulates the efficiency of cell invasion and adhesion independently of RB1. (A) Immunoblot to detect Ccnd1, Cdk4 and RB1 in primary GBMs, low-grade gliomas and U251-MG and U87-MG GBM cell lines. Actin was used as a loading control. (B and C) The same number of RB1-positive (GBM6) and RB1-negative (GBM65) cells were treated with the Ccnd1-Cdk4 inhibitor palbociclib (5 μM) (time 0) and the total cell number was determined after 36 and 72 h ($n=3$). (D) RB1-negative GBM65 cells were incubated in the presence of palbociclib (5 μM) in a transwell device coated with Matrigel. Invading cells were counted after 8 h ($n=3$). (E) The ratio of viable (Trypan blue negative) to total cells (D) ($n=3$). (F) Invasion efficiency tested as in (D) for GBM65 cells infected with scramble (scr) or shRNA anti-Ccnd1 (shD1) ($n=5$). The lower panel shows the levels of Ccnd1 and actin as a loading control. (G) Spread and numbers of RB1-negative GBM65 cells infected with scramble (scr) or shRNA anti-Ccnd1 (shD1) seeded on fibronectin plates and incubated for 30 min ($n=3$). Data are mean ± SEM, statistical significance was determined by Student’s $t$-test; $^*p \leq 0.05$, $^{**}p \leq 0.01$, ns, not significant.
when RB1 is present [22–24]. Hence, to confirm lack of RB1 in GBM65, we tested the growth rate of these cells after inhibition of Cdk4 activity with palbociclib compared with the RB1-positive strain GBM6. As expected, palbociclib abated the growth rate of GBM6 (Figure 1B) but had no effect on the growth of GBM65 (Figure 1C).

The original tumors of GBM65 (RB1-negative) and two RB1-positive samples, GBM55 and GBM6, were further characterized by immunohistochemistry to determine the IDH1 and CDKN2A (p16INKA) status. All of these samples belong to the IDH1-wildtype group of GBM (see supplementary material, Figure S1). Regarding the CDKN2A status, positive staining for p16INKA was present in GBM65 and GBM55 but was not observed in GBM6 (see supplementary material, Figure S1). Therefore, GBM6 cells could support higher Ccnd1-CDK4 activity than GBM55 and GBM65 due to the absence of this cell cycle inhibitor. Next, we also tested the MGMT promoter methylation status of GBM6 and GBM65 samples (see supplementary material, Figure S2A). In accordance with their methylation status, only GBM65 cells expressed MGMT and were consequently resistant to temozolomide (see supplementary material, Figure S2B,C).

Finally, to test the importance of Ccnd1-Cdk4 activity on GBM invasiveness, we determined the efficiency of invasion of RB1-negative (GBM65) and RB1-positive (GBM6 and GBM55) primary GBM cells. Interestingly, GBM cell invasion capacity was clearly reduced by palbociclib regardless of RB1 status (Figure 1D; see supplementary material, Figure S3A,C). These results reinforce the idea that cytoplasmic targets such as Pxn [30] and RalA [32] in GBM cells (such as perivascular cells) exhibited a sharp cytoplasmic signal for HA-Ccnd1 (Figure 3B, see supplementary material, Figure S2B,C). Moreover, Ccnd1 also colocalized with Rac1 activity associated to the membranes in the absence of Ccnd1 (Figure 3D). This was indicative of less Rac1 activity in human GBM cells in different Ccnd1 conditions. By pulldown assays, we observed an increase in active Ral (Ral-GTP) in cells expressing high levels of Ccnd1 (Figure 4E). Overall, our data indicate that Ccnd1-associated activity promotes the induction of the Pxn-FAK-Rac1 and Ral pathways in primary GBM cells. These observations suggest that this axis may be implicated in the cytoplasmic functions of Ccnd1 in GBM cell invasion.

Localization of the Ccnd1-Cdk4 activity in GBM cells

Using patient-derived glioma TMAs, we confirmed that Ccnd1 expression is higher in GBM than in grade I and II astrocytoma biopsies (Figure 2A,B) [37,38]. However, only a small proportion of cells showed clear cytoplasmic signal for Ccnd1 (Figure 2C). This result was not completely unexpected, as Ccnd1 is preferentially accumulated in the cytoplasm of cells located at the invasive fronts in other solid tumors [39]. To analyze better Ccnd1 localization during GBM invasion, we used a mouse model of gliomagenesis, which allows us to visualize the dissemination of tumor cells along the entire brain [36]. We infected neonatal Nestin-hv-a Cdkn2a+/− mice with an RCAS retroviral vector expressing human PDGFRα. At 4 months of age, these mice showed human-like proneural GBM (Figure 2D) [36]. We found a strong nuclear signal for Ccnd1 in these tumors, but interestingly we also detected GBM cells containing cytoplasmic Ccnd1 that were escaping outside the tumor (Figure 2E). These cells also showed positive PDGFRα staining, a marker of proneural-like tumor cells [36].

To characterize further the localization of Ccnd1 in the invasive GBM cells, we next used a xenograft model wherein human GBM cells (U87-MG) were injected into the brains of SCID mice. To determine the localization of Ccnd1, cells were infected with lentiviral particles harboring an HA-Ccnd1 construct (see supplementary material, Figure S4B). Three weeks after injection, mice developed intracranial tumors. We observed that a high proportion of cells in the tumor mass showed HA-Ccnd1 accumulated in the nucleus (Figure 3A,C). In contrast, most of the evaded cells (such as perivascular cells) exhibited a sharp cytoplasmic signal for HA-Ccnd1 (Figure 3B, see supplementary material, Figure S4A). Moreover, Ccnd1 also colocalized with cytoplasmic targets such as Pxn [30] and RalA [32] in the cytoplasm and membranes of primary GBM cells (Figure 3D,E and see supplementary material, Figure S4C). These results reinforce the idea that cytoplasmic Ccnd1 could promote invasion of human GBM cells through the regulation of Pxn and Ral GTP activity.

Ccnd1 promotes FAK, Rac1 and RalA activation in human GBM cells

To study the Ccnd1-Cdk4-Pxn-FAK-Rac1 axis in human GBM, we first determined the impact of Ccnd1 on FAK activation. Expression of Ccnd1 in GBM65 cells stimulated the phosphorylation of FAK at tyrosine 397 (Figure 4A,B), a key event in FAK activation. Next, we analyzed the levels of Rac1 activation in GBM cells with yellow fluorescent protein-p21 binding domain of PAK1 (YFP-PBD), a construct that is a fluorescent biosensor of Rac1 activity [40], because PBD specifically binds to Rac1-GTP forms. There were fewer cells with accumulation of YFP signal in the membrane or protrusion tips in the GBM sample knocked down for Ccnd1 (Figure 4C,D). This was indicative of lesser Rac1 activity associated with the membranes in the absence of Ccnd1. Finally, we determined RalA activity in GBM cells in different Ccnd1 conditions. By pulldown assays, we observed an increase in active Ral (Ral-GTP) in cells expressing high levels of Ccnd1 (Figure 4E). Overall, our data indicate that Ccnd1-associated activity promotes the induction of the Pxn-FAK-Rac1 and Ral pathways in primary GBM cells. These observations suggest that this axis may be implicated in the cytoplasmic functions of Ccnd1 in GBM cell invasion.
Ccn1 promotes invasion in human GBM cells through stimulation of Rac1, FAK and RalA activities

We used genetic approaches to dissect the role of Pxn and Ral GTPases as mediators of Ccn1 signaling in GBM cells. We first infected GBM65 cells with either an allele of Pxn (Pxn S83A S178A) that cannot be phosphorylated by Ccn1-Cdk4 or with a dominant negative allele of RalA (RalAS31N) (Figure 5B). GBM cells expressing these mutants were less invasive than wildtype cells, suggesting that active Pxn and Ral pathways are required for GBM invasion (Figure 5A). In a second experiment, we infected GBM65 cells with either a phosphomimetic allele of Pxn (S83E S178E) or with an Rgl2-CAAX construct, a RalGTPase
Figure 3. Ccnd1 localizes in the cytoplasm of GBM cells and colocalizes with Pxn and RalA. (A) Confocal microscopy of U87-MG cells infected with lentiviral particles containing 3HA-Ccnd1 3 weeks after injection into the brains of SCID mice. Anti-HA (green) and anti-Glut1 (red) detect HA-Ccnd1 and blood vessels, respectively. Nuclei were stained with Hoechst (blue). The white contour designates regions with tumor cells (scale bar 10 μm). (B) Representative image of evaded cells in (A). (C) The number of infected cells (green) showing nuclear accumulation of HA-Ccnd1. Data are mean ± SEM (n = 3 independent mouse brains). **p ≤ 0.01 Student’s t-test. (D and E) Confocal microscopy of anti-Ccnd1 (green) and anti-Pxn (red) in (D) and anti-RalA (red) in (E) of cells seeded on fibronectin plates for 2 h. Nuclei were stained with Hoechst (blue). Scale bar 10 μm.

activator targeted to cell membranes (Figure 5D). Then, we tested the invasion capacity of these cells after inhibition of Ccnd1-Cdk4 activity with palbociclib. In the presence of these hyperactive alleles, the inhibition of Ccnd1-dependent activity produced a more modest reduction in invasion capacity than wildtype cells (Figure 5C). This result is consistent with Ccnd1-Cdk4 regulating invasion of GBM65 cells through Pxn and Ral pathways.

We also tested the dependence of GBM cell adhesion on Ccnd1, Pxn and Ral activities. U251-MG cells stably expressing interfering RNA against human CCND1 (depleted of human Ccnd1) were transfected with mouse Ccnd1-GFP and either a nonphosphorylatable
Figure 4. Ccnd1 stimulates Fak, Rac1 and RalA activation in human GBM cells. (A) Immunoblot of FAK Y397, β-actin and Ccnd1 in CCND1-knockdown GBM65 cells (shD1) transfected with mouse Ccnd1 (MmD1, n = 9) or GFP (n = 6) maintained in low serum for 16 h. (B) Densitometry; β-actin was used as a loading control. Data are expressed as mean ± SEM. Significance was determined by t-test. (C) GBM65 cells were infected with interference RNA against CCND1 or scrambled RNA control. Once Ccnd1 was downregulated, cells were transfected with YFP-PBD (a sensor of Rac1 activity). Two days after transfection, cells were seeded on fibronectin for 2 h. Arrows indicate membrane regions with accumulation of YFP. (D) Mean values and confidence limits from three experiments (α = 0.05; n ≥ 100). (E) Immunoblotting of RalA-GTP and total RalA in Ccnd1-knockdown GBM65 cells (shD1) transfected with mouse Ccnd1 (MmD1) or GFP. Active RalA-GTP was affinity purified after 24 h with RalBP beads. RalBP beads are shown as the pulldown control. The experiment was repeated twice. Relative mean values ± SD for the RalA-GTP/total RalA ratios are shown.

Pxn allele (S83A S178A) or a dominant negative allele of RalB (RalBS28N). Cells expressing Ccnd1-GFP required more time to spread on fibronectin. However, this delay was not observed in the presence of inactive alleles of Pxn or Ral GTPase (Figure 5E), which suggests that Ccnd1 also regulates the adhesion of GBM cells through Pxn and Ral pathways.

To envisage the clinical relevance of cytoplasmic Ccnd1-associated activity, we analyzed the frequency of genetic alterations for the cytoplasmic targets of Ccnd1-Cdk4. We used a TCGA dataset of diffuse gliomas (GBM-LGG) from the GlioVis platform to obtain the data [41]. Interestingly, we observed that an increment in gene copy number of RAC1 and RALA was linked with IDH-wildtype (‘high-grade’) gliomas (see supplementary material, Figure S5A,B). Consequently, activation of cytoplasmic targets of Ccnd1-Cdk4, RAC1 and RALA appears to be associated with glioma aggressiveness. In accordance with the relevance of Ccnd1-Ck4 activity for glioma aggressiveness [20], we also observed that only an increment in CCND1 gene dosage reduced IDH-mutant (‘low-grade’) glioma survival (see supplementary material, Figure S5C).

Accumulation of Ccnd1 in cell membranes promotes GBM dissemination in vivo

In previous work, we fused the farnesylation site of K-Ras protein (CAAX) to the C-terminus of Ccnd1, creating Ccnd1 that associates to cell membranes and promotes Ral activation and cell invasion in prostate and endometrium tumor cell lines [39]. In this work, we expressed Ccnd1-CAAX in GBM cells and observed that treatment with palbociclib drastically reduced the invasion capacity (see supplementary material, Figure S3I). This result suggests that inhibiting cytoplasmic
Figure 5. Ccnd1 promotes invasion of human GBM cells through Pxn phosphorylation and Ral activation. (A) GBM65 cells infected with wild-type Pxn, a nonphosphorylatable Pxn allele or the dominant negative allele RalAS31N. Cells were seeded 48 h later on to Matrigel-coated 24-well transwell filters and allowed to invade for 7 h. Values are expressed as mean ± SEM (n = 3). *p < 0.05, one-way ANOVA and Tukey-HSD post-test. (B) Immunoblots showing Pxn and Ral (anti-HA) in the infected cells in (A). Actin was used as a loading control. (C) Invasion of GBM65 cells infected with a phosphomimetic Pxn allele or with Rgl2-CAAX tested as in (A) but cells were also treated with palbociclib (5 μM) or DMSO as the control. (D) Immunoblots showing Pxn and Ral (anti-HA) in the infected cells in (C). Actin was used as a loading control. (E) Percentage of spreading of U251 cells stably expressing shD1 transfected with GFP (vector) or with Ccnd1-GFP and co-transfected with nonphosphorylatable Pxn (S83A S178A) or the dominant negative allele RalBS28N. Mean ± SEM (n = 3). **p < 0.01, one-way ANOVA and Tukey-HSD post-test.

Ccnd1 would reverse the invasive phenotype of GBM cells.

To test whether membrane-associated Ccnd1 promotes GBM dissemination in vivo, luciferase-expressing U87-MG cells were co-infected with lentiviral vectors expressing GFP and either Ccnd1-CAAX or wildtype Ccnd1. Cells were intracranially injected and mice were euthanized 4 weeks later. The localization and size of the tumors were analyzed by bioluminescence (Figure 6A). Tumors harboring Ccnd1-CAAX exhibited distant nodes from the original tumor (Figure 6B,C) and showed disseminated groups of tumor cells (cell foci) in the surrounding brain tissue (Figure 6F,G). Conversely, control tumors remained for the most part encapsulated.
Figure 6. Membrane-targeted Ccnd1 induces GBM dissemination in vivo. Human U87-MG cells stably expressing luciferase were co-infected with lentiviruses harboring GFP and Ccnd1-CAAX, Ccnd1 or an empty vector (control). Infected cells were inoculated intracranially in immunodeficient SCID male mice (n = 27, nine for each condition). Mice were euthanized 4 weeks after intracranial injection. To detect tumor cells (green), serial sections of brain were immunostained with Alexa Fluor-488-rabbit anti-GFP. The mouse brain (red) was contrasted with AlexaFluor-594-anti-mouse. (A) Tumor location and size were observed by luciferine-mediated luminescence. (B) Representative image of a tumor expressing Ccnd1-CAAX showing disseminated nodules (arrows). (C) Luminescence and GFP showing a contralateral nodule in sample 2.2. (D and E) Representative pictures and percentage of Ki67-positive cells (mean ± SEM, n = 5 for each condition). (F and G) Images from a tumor expressing Ccnd1-CAAX showing evaded tumor cells (arrow). (H and I) Images from an encapsulated control tumor. (J) The number of disseminated versus nondisseminated tumors (n = 9 for each condition). Statistical significance was calculated with Fisher’s exact test (*p < 0.05; **p < 0.01). (K) Diagram of the proposed Ccnd1-Cdk4 regulatory network in GBM cells.
Interestingly, Pxn seems to behave as an oncogene in the stream target of Ccnd1-Cdk4 in human GBM cells. Only those tumors that showed intermediate and distal cell foci were considered to be disseminated tumors. By these criteria, the total number of dispersed tumors was increased in Ccnd1-CAAX samples (Figure 6J). Furthermore, we confirmed that Ccnd1-CAAX was localized in the cell membrane of evaded tumor cells (see supplementary material, Figure S7). Overall, these results indicate that the accumulation of Ccnd1 in the membrane of GBM cells promotes GBM dissemination in vivo.

Discussion

Cytoplasmic Ccnd1-Cdk4 plays a role in the dissemination of GBM in vivo. Taking advantage of xenograft and RCAS-Tva models, we found that Ccnd1 is cytoplasmic mainly in evaded GBM cells but not within the tumor masses, and that imposed membrane accumulation of Ccnd1 increases the number of evaded cells away from the tumor mass. These findings are in line with a number of reports that Ccnd1 functionally interacts with cytoplasmic targets involved in the regulation of cell adhesion and invasion, such as filamin A, pacsin, Rgl2 and Pxn [30–32,42]. Also, cytoplasmic Ccnd1 has been found at the invasive fronts of solid tumors [39] and the invasive blastoid variant of mantle cell lymphoma [43], suggesting an important role of cytoplasmic Ccnd1 in tumor invasion. Moreover, accumulation of membrane Ccnd1 increases the metastatic potential of endometrial tumor cells in a lung metastasis mouse model [39]. These findings may have clinical implications. Upon Ccnd1-Cdk4 inhibition we observed a similar reduction in invasiveness in IDH-wildtype cells with different CDKN2A, MGMT-methylation and RB1 backgrounds, highlighting that inhibition of Cdk4/6 activity may be a useful approach to reduce dissemination in a broad spectrum of gliomas. Of note, as GBM cell invasion is RB1-independent, invasion is sensitive to palbociclib in the absence of RB1. This is in accordance with previous findings showing that cell motility and invasion by Ccnd1 in mouse fibroblasts are also RB1-independent [27]. In our xenograft experiments, most tumors with membrane-associated Ccnd1 showed dissemination; yet their proliferation rate was similar to tumors with wild-type Ccnd1, emphasizing that the regulation of GBM cell invasion by Ccnd1 is unconnected to proliferative status.

Our genetic data also indicate that Pxn is a downstream target of Ccnd1-Cdk4 in human GBM cells. Interestingly, Pxn seems to behave as an oncogene in glioma progression and its overexpression is associated with high-grade gliomas [44]. Also, a positive correlation between GBM invasiveness and Pxn phosphorylation at tyrosine 118 has been reported [45]. Here, we showed that phosphorylation of Pxn at S83 and S178 is required for efficient invasion and inefficient spreading of GBM cells. Conceivably, these phosphorylations triggered by Ccnd1 could constitute an initial step in the induction of GBM invasion. Although specific phosphoPxn S83 (S85 in humans) and S178 antibodies for human cells are not available, we observed that expression of Ccnd1 activates downstream targets of Pxn, such as FAK and Rac1. Of note, the importance of these downstream targets in GBM invasion has also been reported by different authors [1,46,47]. For example, in GBM cell lines, loss of FAK inhibits EGF-induced invasion [48], whereas Rac1 hyperactivation correlates with an elevated invasive phenotype [49,50]. Furthermore, expression of a RalA dominant-negative allele reduced the invasion efficiency of primary GBM cells. Ral GTPases are regulators of exocyst formation and the secretory pathway, and are involved in the control of cell growth and invasion [34]. Ral activation also contributes to Ras-driven transformation and it is increased in many cancer types [51]. Surprisingly, very few data exist about the importance of Ral GTPases in GBM. Our results are consistent with a previous report showing that the knockdown of RalB decreases the invasiveness of human GBM cell lines [52].

We propose a model in which the cytoplasmic accumulation of Ccnd1-Cdk4 activity causes cell evasion in GBM through the activation of the Pxn-Fak-Rac1 axis and Ral GTPases (Figure 6K). Interestingly, cytoplasmic Ccnd1 has also been detected in human GBM recurrences after surgery and therapy [53]. Because palbociclib inhibits both the nuclear and the cytoplasmic activities of Ccnd1-Cdk4, we propose that it not only hinders cell proliferation in the presence of RB1, but also interferes with tumor dissemination independently of RB1 by precluding the cytoplasmic Ccnd1-Cdk4 activity. Therefore, it is conceivable that pharmacological inactivation of Ccnd1-Cdk4 could be used as treatment, not only against tumor cell proliferation, but also against their invasive capacity. Although additional work is required to address specifically the relevance of the cytoplasmic functions of Ccnd1-Cdk4 in glioma classification and outcome predictions, our results are in agreement with those works that sustain a clinical relevance of Ccnd and Cdk4/6 in the diagnosis and therapy of glioma [10,20].

Acknowledgements

We thank Sónia Rius, Marta Rafel, Laura Colàs, Ana Velasco and María Ruiz (IRBLleida-Biobank) for technical assistance, and María Jesús Artiga and Patricia González from Biobank and Histopathology Core Unit, CNIO for technical support. We especially...
thank Massimo Squatrito for providing the samples of the gliomagenesis model. This work was funded by the Spanish Ministry of Education and Science (BFU2013-42895-P; BFU2016-78826-P) and the Instituto de Salud Carlos III/FEDER (P13/01980), and supported by the Xarxa de Bancs de Tumors de Catalunya sponsored by Pla Director d’Oncología de Catalunya (XBTC), IRBLeida Biobank (B.0000682) and PLATAFORMA BIOBANCOS PT17/0015/0027. T Cemeli (FPU13/06590) and I Felip (FPU14/02674) were supported by a predoctoral fellowship from Ministerio de Educación, Cultura y Deportes and from Diputació de Lleida. M Guasch and M Nager were supported by a predoctoral fellowship from UdL. AJ Schuhmacher was supported by a research contract from the Ramón y Cajal program (RYC-2015-17622) by the Spanish Ministry for the Economy, Industry and Competitiveness (MINECO).

**Author contributions statement**

TC performed constructions and experiments. TC, IF, SC and MG performed mice xenograft experiments. MN and JH obtained primary GBM cultures. AJS implemented the mouse gliomagenesis model and provided samples. MS and SG performed immunohistochemistry and analyzed pathology data. MN, FF, XD, JH, SC, AJS and NP provided cell culture support, contributed materials and helped with procedures. EG designed the project and wrote the manuscript. All authors contributed discussions and comments on the manuscript.

**References**

1. Louis DN. Molecular pathology of malignant gliomas. *Annu Rev Pathol Mech Dis* 2006; 1: 97–117.
2. Alifisier C, Trafalis DT. Glioblastoma multiforme: pathogenesis and treatment. *Pharmacol Ther* 2015; 152: 63–82.
3. Schwartzbaum JA, Fisher JL, Aldape KD, et al. Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol* 2006; 2: 494–503.
4. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* 2008; 321: 1807–1812.
5. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; 455: 1061–1068.
6. Verhaak RGW, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRα, IDH1, EGFR, and NF1. *Cancer Cell* 2010; 17: 98–110.
7. Noushmehr H, Weisenberger DJ, Diefes K, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 2010; 17: 510–522.
8. Brennan CW, Verhaak RGW, McKenna A, et al. The somatic genomic landscape of glioblastoma. *Cell* 2013; 155: 462–477.
9. Cancer Genome Atlas Research Network. The cancer genome atlas research network. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *Nat Engl J Med* 2015; 372: 2481–2498.
10. Cencarelli M, Barthel FP, Malta TM, et al. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell* 2016; 164: 550–563.
11. Balss J, Meyer J, Mueller W, et al. Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathol* 2008; 116: 597–602.
12. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol* 2016; 131: 803–820.
13. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 2009; 10: 459–466.
14. Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 2000; 343: 1350–1354.
15. Hegi ME, Diserens AC, Godard S, et al. Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. *Clin Cancer Res* 2004; 10: 1871–1874.
16. Paz MF, Yaya-Tur R, Rojas-Marcos I, et al. CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. *Clin Cancer Res* 2004; 10: 4933–4938.
17. Cuddapah VA, Robel S, Watkins S, et al. A neurocentric perspective on glioma invasion. *Nat Rev Neurosci* 2014; 15: 455–465.
18. Scherer HJ. Structural development in gliomas. *Am J Cancer* 1938; 34: 333–351.
19. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; 13: 1501–1512.
20. Shirahata M, Ono T, Stichel D, et al. Novel, improved grading system(s) for IDH-mutant astrocytic gliomas. *Acta Neuropathol* 2018; 136: 153–166.
21. Fry DW, Harvey PJ, Keller PR, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol Cancer Ther* 2004; 3: 1427–1438.
22. Michaud K, Solomon DA, Oermann E, et al. Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. *Cancer Res* 2010; 70: 3228–3238.
23. Wiedemeyer WR, Dunn IF, Quayle SN, et al. Pattern of retinoblastoma pathway inactivation dictates response to CDK4/6 inhibition in GBM. *Proc Natl Acad Sci U S A* 2010; 107: 11501–11506.
24. CenL, Carlson Bl, Schroeder MA, et al. p16-Cdk4-Rb axis controls sensitivity to a cyclin-dependent kinase inhibitor PD0332991 in glioblastoma xenograft cells. *Neuro Oncol* 2012; 14: 870–881.
25. Gardel ML, Schneider IC, Aratyn-Schaus Y, et al. Mechanical integration of adhesion and motility dynamics in cell migration. *Annu Rev Cell Dev Biol* 2010; 26: 315–333.
26. Neumeister P, Pixley FJ, Xiong Y, et al. Cyclin D1 governs adhesion and motility of macrophages. *Mol Biol Cell* 2004; 14: 2005–2015.
27. Li Z, Wang C, Jiao X, et al. Cyclin D1 regulates cellular migration through the inhibition of thrombospondin 1 and ROCK signaling. *Mol Cell Biol* 2006; 26: 4240–4256.
28. Arato-Ohshima T, Sawa H. Over-expression of cyclin D1 induces glioma invasion by increasing matrix metalloproteinase activity and cell motility. *Int J Cancer* 1999; 83: 387–392.
29. Wang J, Wang Q, Cui Y, et al. Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells. *Cancer Res* 2010; 70: 597–602.
30. Scher R, Gold J, Diederichs S, et al. Cyclin D1 governs adhesion and motility of macrophages. *Mol Biol Cell* 2004; 14: 2005–2015.
31. Li Z, Wang C, Jiao X, et al. Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells. *Cancer Res* 2010; 70: 597–602.
SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. IDH1 and CDKN2A status

Figure S2. MGMT promoter methylation status

Figure S3. Ccnd1-Cdk4 activity regulates the efficiency of cell invasion in GBM cells

Figure S4. Ccnd1 colocalizes with Pxn and RalA in the cytoplasm and membranes of GBM cells

Figure S5. Relevance of cytoplasmic Ccnd1-associated activity in glioma outcome

Figure S6. Quantification of GBM dissemination in vivo

Figure S7. Ccnd1-CAAX localizes in the membrane of human U87-MG cells in intracranial induced tumors

Table S1. Details of the used primary antibodies

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