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# 2 PAK3 is a key signature gene of the glioma proneural subtype 3 and affects its proliferation, differentiation and growth

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## 9 Abstract

**AQ1** **Purpose** Gliomas are the most lethal adult primary brain cancers. Recent advances in their molecular characterization have  
11 contributed to a better understanding of their pathophysiology, but there is still a need to identify key genes controlling  
12 glioma cell proliferation and differentiation. The p21-activated kinases PAK1 and PAK2 play essential roles in cell division  
13 and brain development and are well-known oncogenes. In contrast, the role of PAK3 in cancer is poorly understood. It is  
14 known, however that this gene is involved in brain ontogenesis and has been identified as a gene of the proneural subtype  
15 signature in glioblastomas.

16 **Methods** To better understand the role of PAK kinases in the pathophysiology of gliomas, we conducted expression analy-  
17 ses by querying multiple gene expression databases and analyzing primary human glioma samples. We next studied *PAK3*  
18 expression upon differentiation in patient-derived cell lines (PDCLs) and the effects of *PAK3* inhibition by lentiviral-mediated  
19 shRNA on glioma cell proliferation, differentiation and tumor growth.

20 **Results** We show that contrary to *PAK1* and *PAK2*, high *PAK3* expression positively correlates with a longer survival of  
21 glioma patients. We also found that *PAK3* displays differential expression patterns between glioma sub-groups with a higher  
22 expression in 1p/19q-codeleted oligodendrogliomas, and is highly expressed in tumors and PDCLs of the proneural subtype.  
23 In PDCLs, high *PAK3* expression negatively correlated with proliferation and positively correlated with neuronal differentia-  
24 tion. Inhibition of *PAK3* expression increased PDCL proliferation and glioma tumor growth in nude mice.

25 **Conclusions** Our results indicate that *PAK3* plays a unique role among PAKs in glioma development and may represent a  
26 potential therapeutic target.

27 **Keywords** Gliomas · Proneural signature · p21-activated kinase · Patient-derived cell line · Proliferation and neuronal  
28 differentiation · Glioma tumor growth

## 1 Introduction

Diffuse gliomas, including glioblastomas (GBMs), are the most common malignant primary brain tumors and contain diverse populations of cells ranging from highly tumorigenic stem-like cells to more differentiated cells [1, 2]. Molecular studies have related genetic alterations, gene expression profiles, and DNA methylation signatures to distinct glioma subtypes with prognostic value [3–7]. Those studies have also led to the deciphering of key signaling pathways involved in glioma biology, and enlightened the altered differentiation and multipotency of the tumor cells [8]. They also helped to characterize potential therapeutic targets relevant for each subtype of

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glioma. p21-activated kinases (PAKs) play important functional roles in neuronal differentiation and cancer biology. The three group I PAKs (*PAK1*, *PAK2* and *PAK3*) form a small gene family encoding the main effectors of the Rac and Cdc42 GTPases, which play crucial roles in cell proliferation and migration [9]. These kinases are expressed in the developing and adult brain where they have unique functions during brain development [10]. *PAK1* regulates cortical development via the control of progenitor cell proliferation and neuronal differentiation [11]. *PAK2* is an effector of the Rheb/TSC complexes in benign tuberous sclerosis in neuronal tissues [12].

Within this family, *PAK3* may have unique signaling properties since its mutations have been found to be responsible for several neurological disorders including intellectual disability [10]. *PAK3* is involved in brain development, regulating cell cycle exit of neuronal precursors during primary neurogenesis, and in neurite outgrowth and neuronal differentiation during telencephalon ontogenesis [13–15]. *PAK3* also regulates oligodendroglial lineage rate specification [16]. *PAK1* and *PAK2* are oncogenes often found to be overexpressed or activated in different cancers such as breast and colon cancer [17, 18]. Their over-activation and over-expression favour cell proliferation, inhibit apoptosis, enhance invasion and metastasis, and sustain angiogenesis, all cell functions that trigger oncogenesis [17]. In contrast, *PAK3* overexpression or mutation have only been associated with adrenocorticotrophic hormone-secreting tumors [19]. It has also been found, however, that this gene belongs to the proneural transcriptomic signature in glioblastomas (GBMs) [6, 20].

As yet, the role of *PAKs* in gliomas is scarcely described in the literature [21]. To investigate this further, we analyzed group *PAK1-3* expression according to histological and molecular criteria in a well-characterized patient cohort and in data deposited in public cancer databases. Contrary to *PAK1* and *PAK2*, high *PAK3* expression was found to be positively correlated with a longer patient survival. We also found that *PAK3* was differentially expressed between glioma subgroups, with a higher expression in 1p/19q-codeleted oligodendrogliomas. Comparison of *PAK* expression in GBM patient-derived cell lines (PDCLs) and in their parental tumors indicated that *PAK3* expression may serve as one of the more reliable markers of the proneural subtype. In PDCLs, increasing *PAK3* expression upon a set of differentiation paradigms was correlated with those of neuronal markers. Interestingly, short hairpin RNA-mediated inhibition of *PAK3* expression increased PDCL proliferation and astrocytic marker expression, and also accelerated in vivo tumor growth. Together, these data indicate that *PAK1-2* and *PAK3* play different roles in the pathophysiology of gliomas. *PAK3* exhibits an inhibitory role in glioma tumor growth and may be of potential interest for glioma therapy.

## 2 Materials and methods

### 2.1 In silico tumor database analysis

RNA-seq data were obtained from the transcriptomic database of Zhang et al. [22] and are expressed in FPKM (Fragments Per Kilobase of exon per Million reads mapped) values. RNAs were extracted from different cell types purified from the postnatal mouse cerebral cortex ([http://web.stanford.edu/group/barres\\_lab/brain\\_rnaseq.html](http://web.stanford.edu/group/barres_lab/brain_rnaseq.html)). Gene expression analyses from primary tumors were performed employing the Academic Medical Center (AMC) database, using the R2 genomics analysis and visualization platform (<http://r2.amc.nl>) and across different tumor datasets for each analyzed gene [23]. Kaplan–Meier survival curves of patients were derived from the Tumor Glioma—French—284 -dataset, composed of 276 glioma samples of all histologies and 8 control samples [24]. Samples were sorted according to the expression of each *PAK* and divided into two groups on the basis of a cutoff expression value giving the higher probability of confidence, using the log-rank test with Bonferroni correction for multiple comparisons. The clinical and molecular data of lower grade gliomas (LGGs) and GBM samples for this study were downloaded from The Cancer Genome Atlas database (<https://tcga-data.nci.nih.gov/docs/publications/tcga/>) generated by the TCGA Research Network (<http://cancergenome.nih.gov/>), firstly in 2014 and subsequently in February 2016 (cancer genome). mRNA expression levels of *PAK1* and *PAK3* were obtained from RNAseq analyses using Illumina TruSeq Kit Paired-end Sequencing on an Illumina HiSeq2000. Detection of somatic variants (IDH-mut) from TCGA whole-exome sequencing and RNAseq data was performed using RADIA [25]. The mRNA levels were represented as “normalized count” corresponding to a transformation of the “raw\_count”. For each gene of interest, all “raw\_count” values were divided by the 75th percentile of the column patient (after removing zeros) and multiplied by 1000.

### 2.2 Glioma sample analyses

Patient samples (from the OncoNeuroTek tissue bank) and informed consent, RNA extraction and gene expression analyses have been described before [26].

### 2.3 GBM cell culture, shRNA and lentiviral infection

Primary sphere cultures were initially derived from patient tumors as described before [27]. Glioblastoma-derived spheres were cultured in DMEM/F12 medium supplemented with penicillin/streptomycin and B27 (Life Technologies),

referred to as basal medium, supplemented with EGF (20 ng/ml; Peprotech) and FGF (20 ng/ml; Miltenyi), referred to as proliferative medium. Spheres were dissociated using Stem-Pro Accutase (Life technologies) when they reached a size of 100–200  $\mu\text{m}$ . For cell growth analysis, dissociated cells were seeded at  $5 \times 10^4$  cells/ml in triplicates, and on day 1, 4 and 7, live cells from dissociated spheres were counted. Viability was tested by Trypan blue exclusion. For differentiation experiments, single-cell suspensions were seeded on a laminin substrate (10  $\mu\text{g}/\text{ml}$ ; Life technologies) in the proliferative medium, as described before [28]. Twenty-four hours later, the medium was replaced with basal medium containing 0.5% fetal calf serum, or in some experiments, with basal medium containing Bone Morphological Protein-4 (BMP-4, 10 ng/ml, Peprotech) or all-*trans*-retinoic acid (ATRA, 10  $\mu\text{M}$ , Sigma). For *PAK3* expression inhibition, viral particles containing TRC human *PAK3* shRNA (Thermo Scientific) cloned in the pLKO.1 lentiviral vector were produced at the ICM Vectorology platform and used to transduce PDCLs. After puromycin selection, the efficiency of *PAK3* expression inhibition was analyzed in proliferative and differentiative conditions by qRT-PCR.

## 2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. RNA quality analysis was performed using a Bio-Rad's Experion system and nanodrop, and cDNAs were generated from 0.5  $\mu\text{g}$  mRNAs using an iScript Reverse Transcription Supermix (Biorad). Real-time PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Biorad) using a SsoAdvanced Universal SYBRGreen Supermix (Biorad). Primers were designed by retrieving nucleotide sequences from the NCBI gene database using the Primer3Plus program (<http://www.bioinformatics.nl/cgi-bin/prime3plus/primer3plus.cgi/>). Gene-specific primers were synthesized by Eurofins Genomics and their sequences are listed in [Supplementary Materials](#). Samples were amplified in triplicate and relative copy numbers were determined using the comparative  $\Delta\Delta\text{Ct}$  method, with *PPIA* and *GAPDH* as normalization genes, and expression in a human embryonic brain mRNA sample as exogenous reference (Clinisciences).

## 2.5 Western blotting

For Western blot analysis, protein samples (10  $\mu\text{g}$ ) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore), as previously described [29]. Immunoblot analyses were performed using antibodies described in [Supplementary Materials](#). Immunodetection was performed using a Luminata Crescendo Western HRP

substrate (Merck Millipore). Quantification of chemiluminescence signals was performed using NIH ImageJ software.

## 2.6 Immunocytochemistry

Monolayer PDCL cultures were PFA-fixed and immunolabeled for native proteins using antibodies described in [Supplementary Materials](#). Images were acquired in a sequential mode, using suitable filter cubes on a Leica DMI6000 microscope controlled by SimplePCI Software (Compix-Hamamatsu).

## 2.7 Animal experimentation

All animal experiments were conducted in accordance with the European Communities Council Directive for animal care and experimentation (Directive 2010/63/UE), and following the guidelines of the animal facility in Orsay (France) approved by the Ethical Committee (France, CEEA 59) and the Direction of veterinary services (France, agreement D91-471-104).  $1 \times 10^6$  dissociated cells were subcutaneously injected into the left flank of 8-weeks-old female nude mice. Tumor growth was monitored regularly and measured twice a week using a caliper as soon as tumors reach 25  $\text{mm}^3$ .

## 2.8 Statistical analysis

Patient survival was defined as the time between histological diagnosis and either death or the last follow-up. Differences between Kaplan–Meier survival curves were assessed using the log-rank test. For samples from the Pitié-Salpêtrière Hospital's cohort, gene expression levels were measured by qRT-PCR and compared between different molecular subgroups of patients using a one-way analysis of variance (ANOVA) and Mann–Whitney test (two-tailed) included in GraphPad Prism software. In the TCGA datasets, gene expression was compared between glioma subgroups with ANOVA and Tukey's comparison tests using R software. For cellular and molecular assays, experiments were performed in triplicates and repeated independently at least three times. Whenever relevant, Kruskal–Wallis or Mann–Whitney Wilcoxon tests were performed using R software. Differences were considered significant below  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$  thresholds.

## 229 3 Results

### 230 3.1 A higher *PAK3* expression in gliomas 231 is associated with a longer patient survival

232 We first analyzed *PAK1-3* expressions in postnatal brain  
233 cell types by interrogating the database from the Barres  
234 group [22]. We found that *PAK1* is highly expressed in  
235 neurons, oligodendrocyte lineage cells, microglia and to a  
236 lesser extent in astrocytes (Fig. 1a). *PAK2* was expressed  
237 in all cell types and represented the main *PAK* isoform in  
238 astrocytes and endothelial cells. *PAK3* expression, overall  
239 lower than that of the two other *PAK* genes, was restricted  
240 to neurons and cells of the oligodendroglial lineage. We  
241 next compared the expression levels of each *PAK* isoform  
242 in tumors from the nervous system and other organs, by  
243 mining the Academic Medical Center (AMC) database  
244 (Fig. 1b) [23]. *PAK1* was almost equally expressed in all  
245 tumor types, but at a slightly lower level in gliomas com-  
246 pared with other tumors, and less than in normal brain  
247 tissue. *PAK2* was expressed at higher levels in all tumor  
248 types including gliomas, compared with normal brain tis-  
249 sue. Interestingly, *PAK3* levels were higher in gliomas  
250 and neuroblastomas compared with other cancers, with  
251 a heterogeneous distribution in gliomas. Interestingly, we  
252 found that *PAK3* displayed the same pattern of expression  
253 as *DCX* in neural cell types and also in tumors [19, 22]  
254 (Fig. 1a,b; Supplementary Fig. S1). The relatively lower  
255 expression of *PAK1* and *PAK3* in gliomas compared to  
256 normal brain tissue is likely due to the high proportion  
257 of astrocyte-like cells, which express the lowest levels of  
258 *PAK1* and *PAK3*.

259 We next investigated the correlation between *PAKs*  
260 expression and patient overall survival in a large cohort  
261 of glioma samples of diverse histological types and grades  
262 described by French and colleagues [24] (Fig. 1c). As we  
263 found that *PAK2* expression was not significantly corre-  
264 lated with survival ( $p = 0.084$ ), we decided to focus on  
265 *PAK1* and *PAK3*. Interestingly, high *PAK1* expression was  
266 found to be associated with a shorter survival and, con-  
267 versely, high *PAK3* expression with a longer survival. For  
268 *PAK3*, similar results were obtained from the Kawaguchi  
269 and REMBRANT glioma databases, underscoring a cor-  
270 relation between *PAK3* expression and patient survival  
271 (Supplementary Fig. S2). For *PAK1* expression, similar  
272 results were obtained from most databases but not all, sug-  
273 gesting that the relationship between *PAK1* expression and  
274 tumor progression is weaker, or may depend on the dif-  
275 ferent histological subtypes composing the cohort (data  
276 not shown). Using R2 software, we found that the *PAK1*  
277 and *PAK3* expression levels did not correlate with each  
278 other ( $r = 0.54$ ), supporting an independent expression

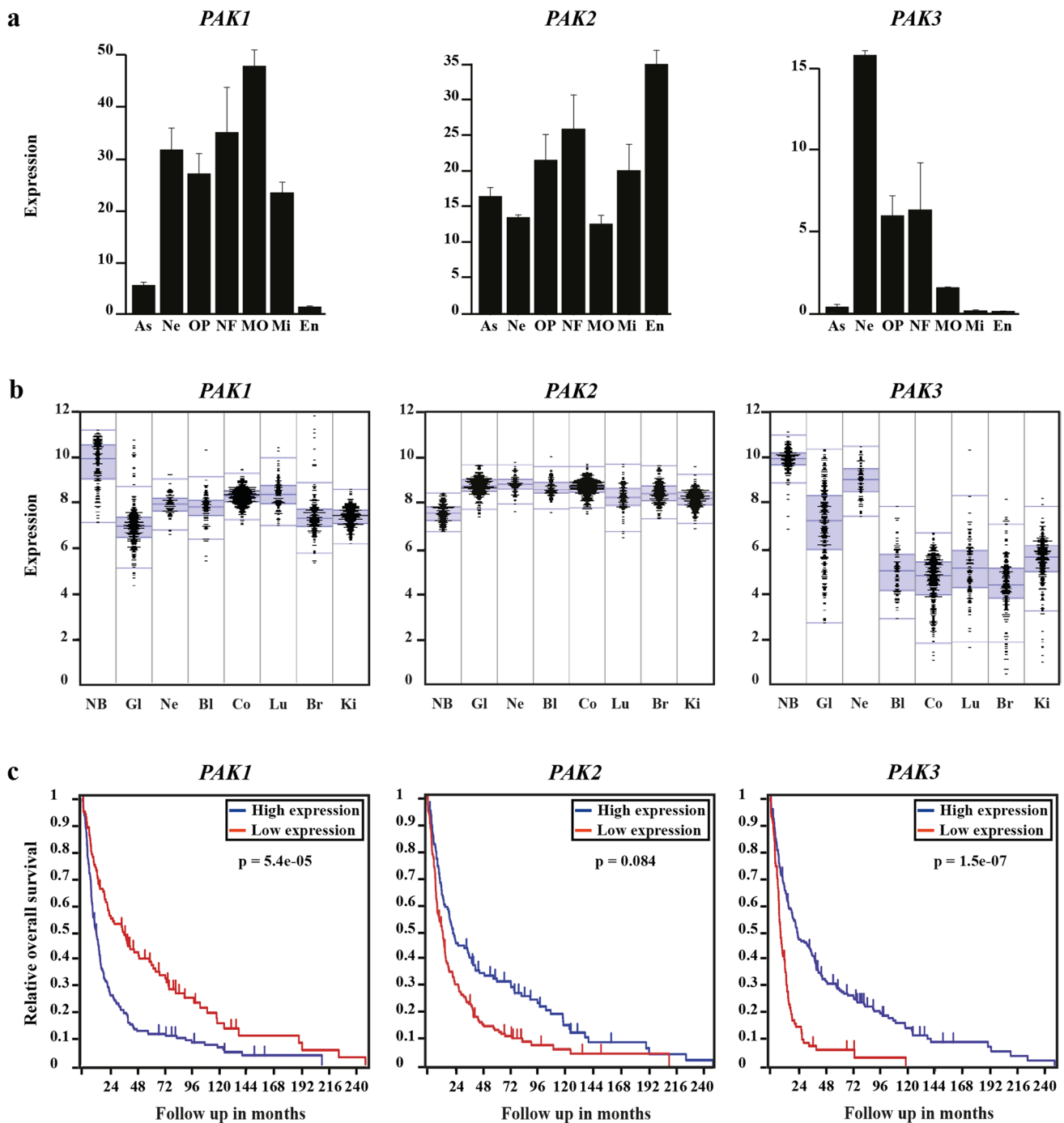
for each gene during cancer progression. Together, these  
data point to distinct roles for *PAK1* and *PAK3* in glioma  
development.

### 282 3.2 *PAK3* is highly expressed in *IDH*-mutant 283 and 1p/19q-codeleted oligodendrogliomas

284 To better understand the roles of *PAK1* and *PAK3* in the  
285 pathophysiology of gliomas, we next set out to investigate  
286 more precisely their expression levels in glioma subtypes,  
287 using qRT-PCR in 223 tumor samples from the OncoNeu-  
288 roTek bank (Fig. 2 a-c). By doing so, we did not observe  
289 any significant difference in *PAK1* expression between  
290 grade II/III gliomas (lower grade gliomas: LGGs) and  
291 GBMs, whereas *PAK3* was significantly higher expressed  
292 in LGGs than in GBMs (Fig. 2a). More specifically, *PAK3*  
293 expression was higher in oligodendrogliomas compared  
294 with astrocytomas or oligoastrocytomas. In contrast, *PAK1*  
295 expression was not significantly different among the LGG  
296 subtypes (Fig. 2b). In view of the new molecular classifi-  
297 cation for gliomas, we examined *PAK1* and *PAK3* expres-  
298 sion according to the major alterations *IDH1/2* and 1/19q  
299 codeletion. Analysis of *PAK* expression in *IDH*-WT and  
300 *IDH*-mutant LGGs revealed opposite results for each iso-  
301 form, with a tendency towards increased *PAK1* expression  
302 in *IDH*-WT-LGGs and a significantly higher expression of  
303 *PAK3* in *IDH*-mutant LGGs (Fig. 2c). Moreover, in contrast  
304 to *PAK1*, *PAK3* expression was significantly higher in gli-  
305 omas harboring the 1p/19q codeletion compared with non-  
306 codeleted tumors. To validate these results, we performed a  
307 meta-analysis of data from the TCGA including 582 human  
308 glioma samples (Fig. 2 d-f). For *PAK3*, we detected a lower  
309 expression in GBMs than in LGGs, a higher expression in  
310 oligodendrogliomas than in other histological types, and  
311 a higher expression in *IDH*-mutant LGGs compared with  
312 non-mutant LGGs. In line with the PSH data, the TCGA  
313 analysis revealed a higher expression of *PAK1* in oligoden-  
314 drogliomas and a lower expression in *IDH*-mutant LGGs.  
315 Together, these results indicate a higher *PAK3* expression  
316 in less aggressive gliomas.

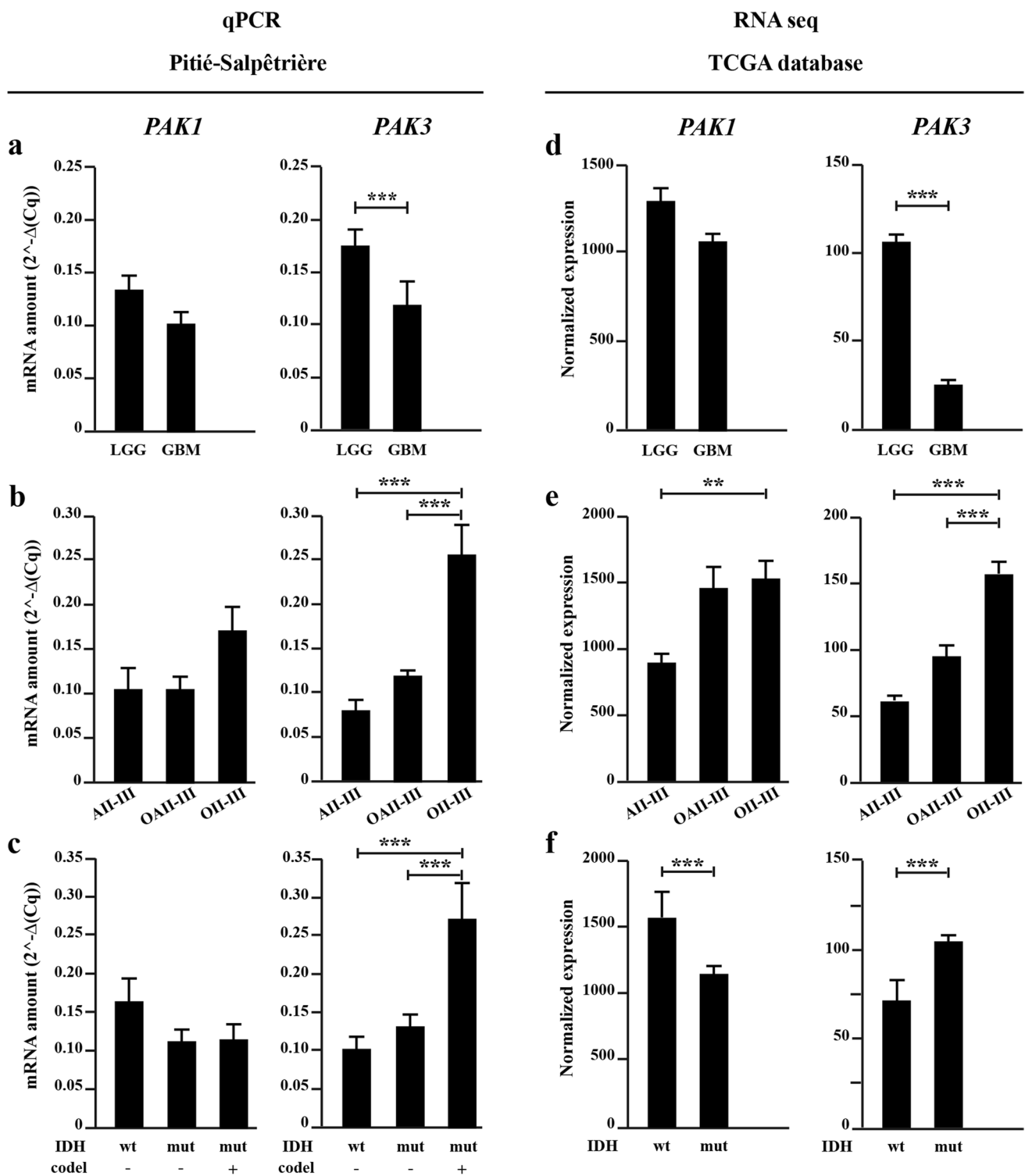
### 317 3.3 High *PAK3* expression is a reliable marker 318 for proneural subtypes of tumors and PDCLs

319 GBMs can be classified in molecular subtypes according to  
320 their gene expression profiles. Interestingly, *PAK3* was found  
321 to be enriched in the proneural glioma subtype signature  
322 (Fig. S3) [6, 20]. We next examined this relationship using  
323 the transcriptional data obtained from tumors and matched  
324 cell lines described by Rosenberg [30]. We focused on a  
325 panel of selected genes that define the proneural signature,  
326 as established by Crisman [20]. We were able to confirm  
327 the higher expression of *PAK3* in samples classified as



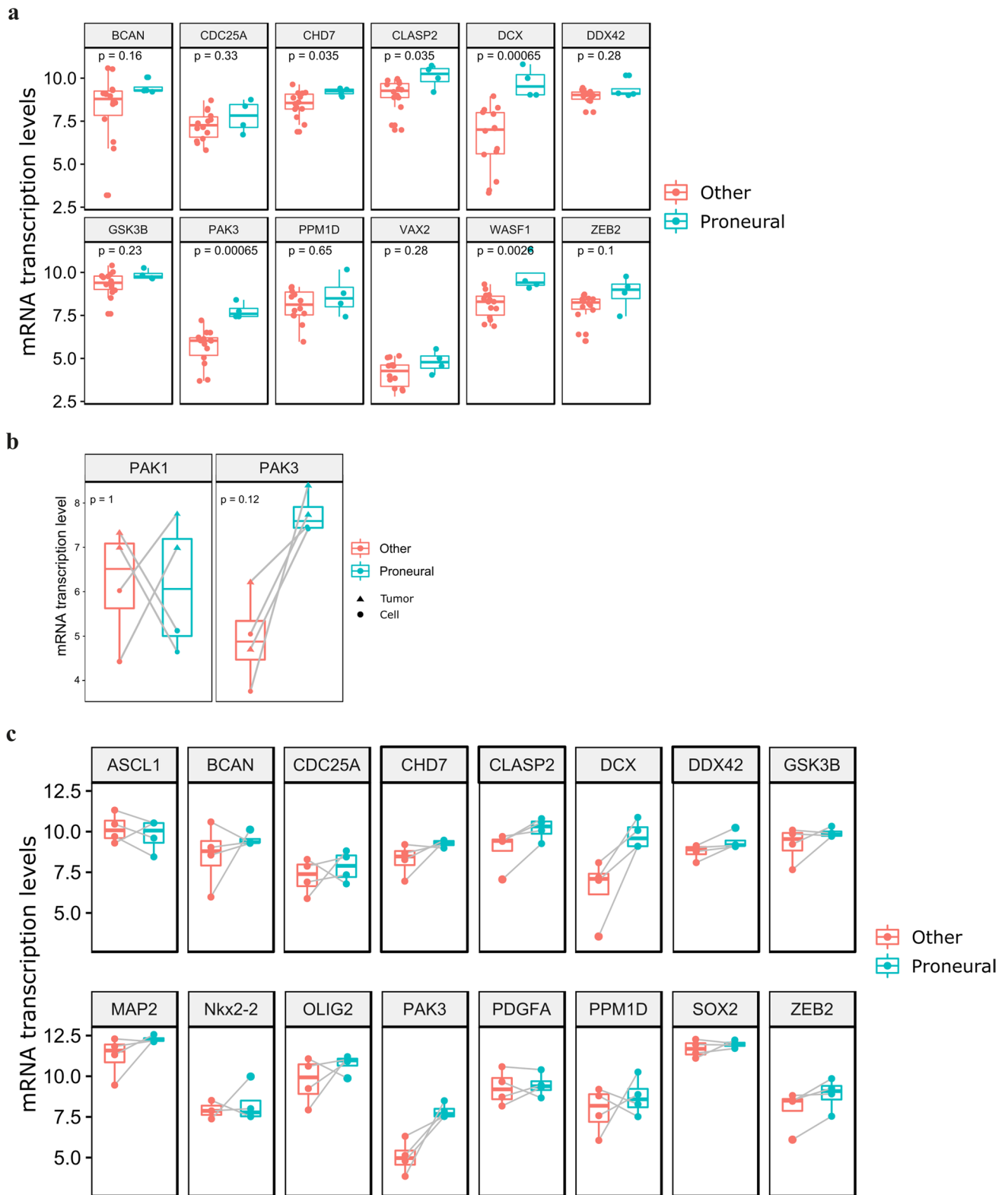
**Fig. 1** *PAK expression in CNS cellular population and correlation with survival of glioma patients.* **a**, RNA-seq analysis of PAK expression in the postnatal mouse cerebral cortex shows specific patterns in different cell types. Data are mined from the transcriptomic database of Zhang et al. [22] and expressed in FPKM (Fragments Per Kilobase of exon per Million reads mapped) values. Astrocyte: As; neuron: Ne; oligodendrocyte progenitor cell: OP; newly formed oligodendrocyte: NF; myelinating oligodendrocyte: MO; microglia: Mi; endothelial cell: En. **b**, *PAK1* is similarly expressed in all types of tumors, whereas *PAK3* is highly expressed in gliomas and neuroblastomas. Box plots show log<sub>2</sub> gene expression levels in tumors from the Affymetrix u133p2 microarray, and from R2 platform analysis-

Academic Medical Center (AMC). The data are derived from the following cohorts: normal brain (NB, Berchtold, n=172); glioma (Gl, French, n=284); neuroblastoma (Ne, Versteeg, n=88); bladder (Bl, Riester, n=93); colon (Co, Sieber Smith, n=355); lung (Lu, Expo, n=114); breast (Br, Bos, n=204); kidney (Ki, Expo, n=261). **c**, Contrary to *PAK1*, high *PAK3* expression in gliomas is associated with a longer patient survival. Kaplan-Meier survival plot splitted according to *PAK* expression level, AMC, French database. For *PAK1*, high expression n=185, low expression n=88; for *PAK2*, high expression n=113, low expression n=160; for *PAK3*, high expression n=186, low expression n=87



**Fig. 2** *PAK3* is highly expressed in IDH-mutant and 1p/19q-codeleted glioma subtypes. **a-c**: *PAK1* and *PAK3* gene expression levels were analyzed by qRT-PCR in glioma samples from the OncoNeuroTek bank. *PAK1* and *PAK3* mRNA expression levels are presented as  $\Delta\Delta Cq$ ; **a**, in lower grade gliomas (LGGs, n=122) versus glioblastomas (GBMs, n=101); **b**, in the various histological classes of LGG (A: astrocytoma, n=15; O: oligodendroglioma, n=54; OA: oligoastrocytoma, n=53) and including II and III tumor grades; **c**, accord-

ing to IDH status and to 1p/19q-codeletion (IDH wild type: WT, n=28; IDH mutant: mut; no codeletion 1p/19q: -, n=34; codeletion 1p/19q: +, n=47). **d-f**, *PAK1* and *PAK3* gene expression analyzed from the TCGA database; **d**, in lower grade gliomas (LGGs, n=417) versus glioblastomas (GBMs, n=165); **e**, in the various histological classes of LGG (A, n=149; O, n=159; OA, n=109) and including II and III tumor grades; **f**, according to IDH status (wild type: WT, n=98; mutant: mut, n=434)



**Fig. 3 High PAK3 expression in primary tumors and their PDCLs correlates with proneural subtypes.** Data were extracted from a transcriptome analysis performed by Rosenberg et al. [30]. **a**, Expression of Crisman proneural signature genes in proneural or

non proneural subtypes (16 samples corresponding to 8 tumors and 8 PDCLs); **b**, *PAK1* and *PAK3* expression levels in tumors and their corresponding PDCLs. **c**, expression levels of proneural signature genes in paired tumors and corresponding PDCLs



proneural, regardless of sample type (tumors or PDCLs) (Fig. 3a). PDCLs have been developed both for analysing the biology of human tumors and as preclinical models for screening potential therapeutic agents. The analysis of phenotype transition may be relevant with regard to tumor evolution towards a more aggressive mesenchymal phenotype [3]. Although in most cases PDCLs maintain the molecular subgroup signature of the parental tumor, some PDCLs acquire characteristics of other phenotypic groups [30]. We asked whether the *PAK3* expression level may represent a marker for the proneural subtype during tumor/PDCL phenotypic transition. Previously, GBM subtypes were found to be maintained in 5/9 of the paired tumors/PDCLs, whereas 4/9 cases exhibited subtype changes [30]. Interestingly, when we analysed these cases, we consistently observed higher *PAK3* levels in the proneural samples, regardless of the kind of change and tissue (Fig. 3b,c). Note that *PAK1* expression was always lower in PDCLs compared to tumors, without subtype correlation (Fig. 3b). In case the subtype changes during cell line establishment, increased expression of *PAK3* was observed in PDCLs which acquire this proneural subtype and, conversely, its expression was found to decrease when the PDCLs lost this proneural subtype. Expression comparisons of the other proneural genes, such as *ASCL1*, *DCX*, *MAP2*, *NKX2.2*, *OLIG2*, *PDGFRA* and *SOX2* selected from the Verhaak list, and *BCAN*, *CDC25A*, *CHD7*, *CLAPS2*, *DCX*, *DDX42*, *GSK3*, *PPM1D*, *WASF1* and *ZEB2* selected from the shortlist defined by Crisman, we found that tumors and cell samples attributed to the proneural subtype compared to other subtypes clearly indicated that among the genes of the proneural signature, only *PAK3* and *DCX* displayed a higher expression in proneural tumors and PDCLs (Fig. 3c). Thus, these data indicate that *PAK3* serves as a reliable marker for the proneural characterization of tumors and PDCLs.

#### 3.4 High *PAK3* expression negatively relates to PDCL proliferation

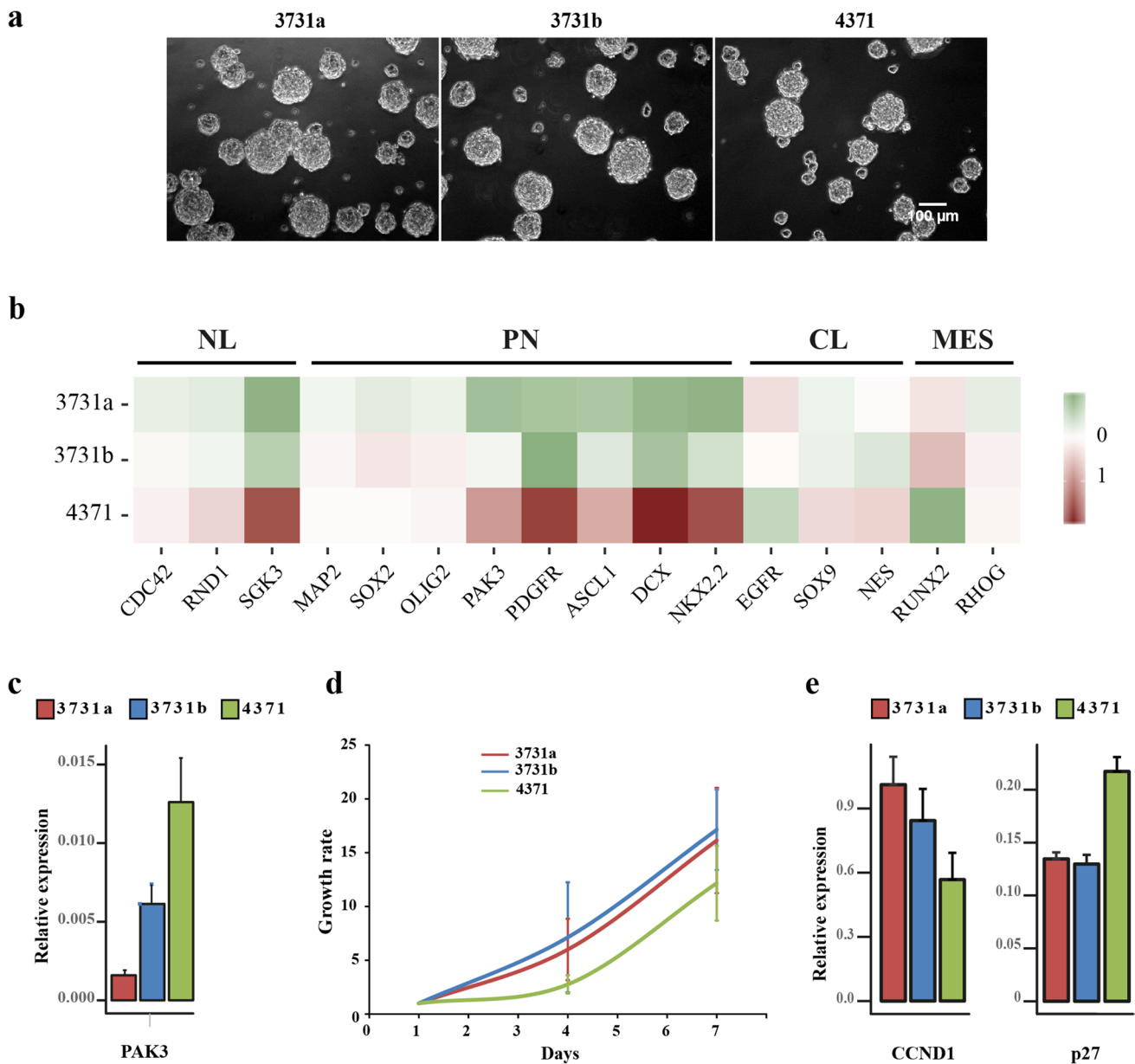
In order to better characterize the association between *PAK3* expression and glioma cell biology, we analyzed *PAK3* expression in 3 PDCL cultures named 3731a, 3731b and 4371 [27] that are growing as spheroids in EGF-FGF defined medium (Fig. 4a). These PDCLs harbor *EGFR* amplifications and 3731a and b, derived from the same initial 3731 cell line, express the mutated oncogene *EGFRvIII* [27]. We found that the two 3731 PDCLs displayed a transcriptomic profil with a more pronounced mesenchymal/classical signature compared to the 4371 PDCL, which displays a proneural expression phenotype, as established by qRT-PCR analysis of genes representative of the four Verhaak GBM subtypes (heatmap: Fig. 4b, Fig. S4).

In cultured spheroids, *PAK3* expression was significantly higher in 4371 cells compared to the two other cell lines, as measured by qRT-PCR (Fig. 4c). Cell growth analysis showed that 3731a and 3731b exhibit faster proliferation rates than 4371, with fold increases after one week, of 16, 17 and 12, respectively (Fig. 4d). Accordingly, the expression levels of *CCND1* and of *CDKN1B* were the lowest and highest, respectively, in the slow proliferative 4371 cells compared with the 3731a/b cells (Fig. 4e). We conclude that *PAK3* expression is higher in slower growing PDCLs.

#### 3.5 High *PAK3* expression positively associates with neuronal differentiation

We next analyzed *PAK3* expression during PDCL differentiation induced after growth factor withdrawal in monolayer cultures as previously described [31]. In these conditions, no cell death was observed in the three PDCL cultures (data not shown). *PAK3* expression was analyzed after 3 days (t3) and 8 days (t8), compared with proliferative conditions (t0). We found that in the two 3731 PDCL cultures *PAK3* expression was strongly induced at both the mRNA and protein levels, in contrast to that in 4371 cells (Fig. 5a-b, Fig. S5a). In proliferative conditions (t0), the proportion of KI67<sup>+</sup> cycling cells varied between PDCL cultures, from 90% (3731b) to 60% (4371) (Fig. 5c and S5b, first column), in accordance with the rates observed in sphere cultures (Fig. 4b). After growth factor withdrawal, the proportion of KI67<sup>+</sup> cells was largely reduced for 3731a and 4371 (t8, 22%), and to a lesser extent for 3731b, which remained highly proliferative (t8, 65%). Accordingly, *p27-KIP1* expression increased after induction of growth arrest in the three PDCL lines (Fig. 5d). We next analyzed GFAP and DCX expression in monolayer cultures by immunolabeling and mRNA and protein quantification (Fig. S5c, Fig. 5e-f). For 3731b cells, growth factor withdrawal had little effect on the expression of the cell differentiation markers *DCX* and *GFAP*. In contrast, we found that the 3731a and 4371 cell lines displayed a reduced proliferation with an increased expression of both GFAP and DCX.

Next, PDCL cultures were exposed to bone morphogenetic proteins 4 (BMP4) and *all-trans* retinoic acid (ATRA) in order to induce astroglial and neuronal differentiation, respectively [32, 33]. We found that BMP4 or ATRA treatment did not modify the proportion of proliferating cells compared to growth factor withdrawal alone (Fig. S6a). Cell lineage differentiation was assessed using qRT-PCR expression analysis of *DCX*, *GFAP*, *OLIG2*, *NG2*, *PDGFRA*, *SI00beta* and *TUBB3* (Fig. 5, Fig. S6b). BMP4 treatment had a relatively little effect on *PAK3* expression in the three PDCL cultures, except for a strong *GFAP* transcriptional activation in 3731b cells and moderate in 3731a cells, suggesting astroglial differentiation



**Fig. 4 High PAK3 expression negatively correlates with PDCL proliferation.** **a**, Representative images showing spheroids grown for 7 days. **b**, Heat map representation of subtype signature gene expression in 3731a, 3731b and 4371 cells. Expression is shown relatively to the mean (red: higher expression level; green: lower expression; white for the mean). Genes are organized according to gene expression signatures: Mesenchymal (MES), Classical (CL), Proneural

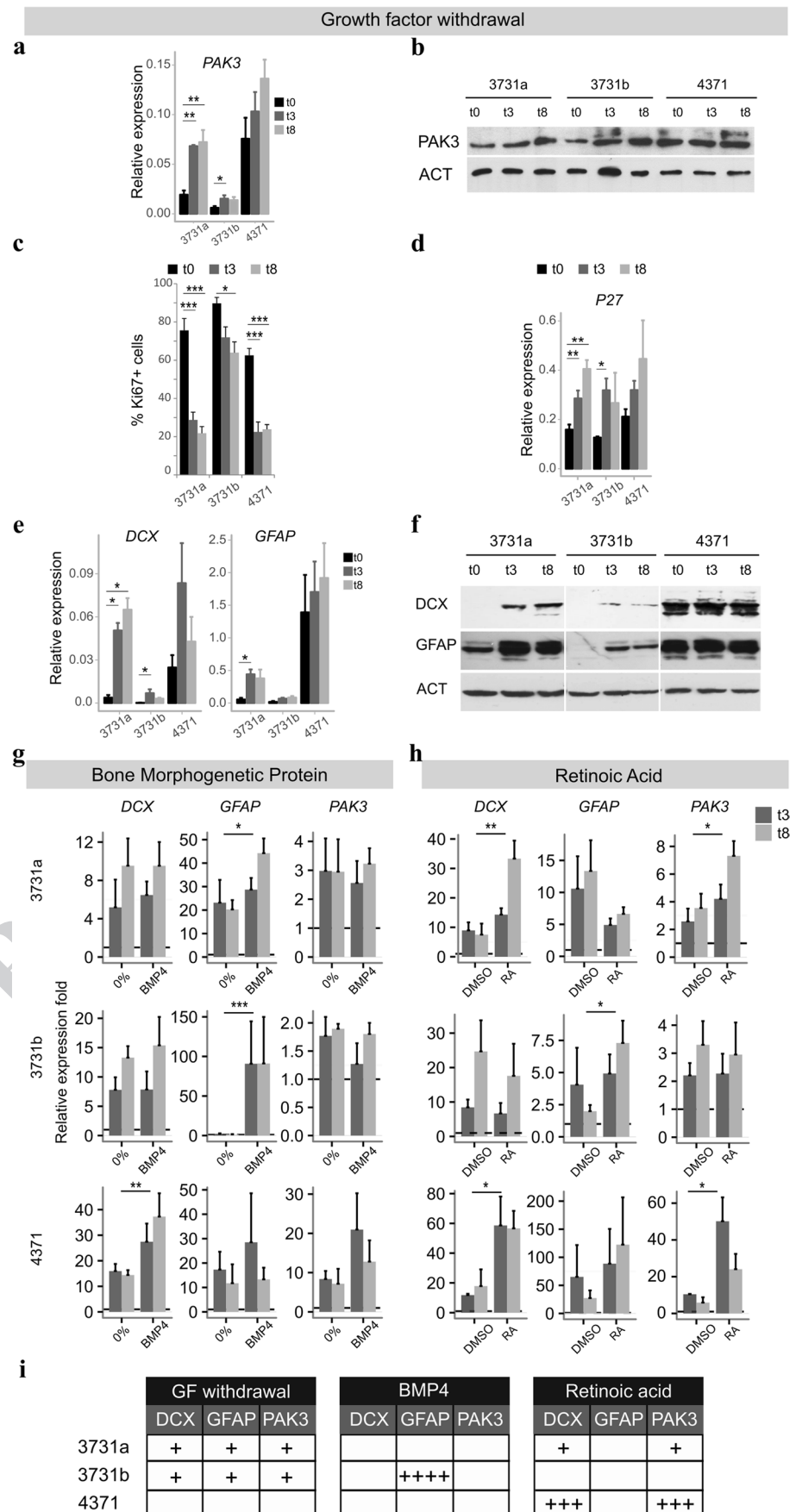
(PN) and Neural (NL). **c**, qRT-PCR analysis of *PAK3* expression in PDCL spheres. Expression levels are presented as  $\Delta\Delta Cq$ , relatively to human fetal brain as external reference. **d**, Fold increase in cell number over time, day1 to 7 (d1, d4 d7). **e**, qRT-PCR analysis of cell cycle genes. Expression levels are presented as  $\Delta\Delta Cq$ , relatively to human fetal brain as external reference. For all analyses, data represent at least three independent experiments

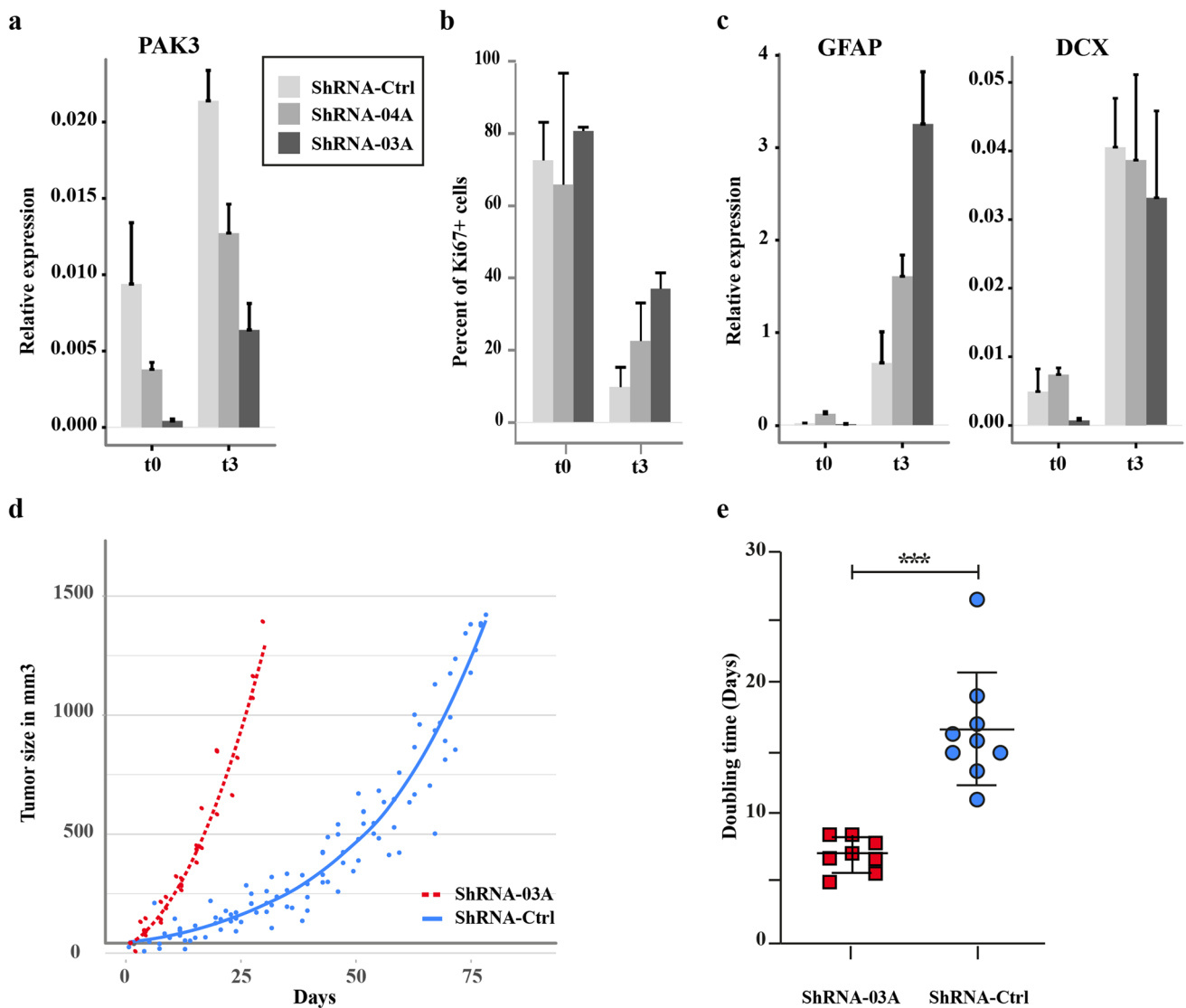
429 (Fig. 5g). ATRA treatment induced a strong transcriptional activation of *PAK3* in 4371 cells and, to a lesser extent, in 3731a cells, associated with expression of the neuronal markers *DCX* (Fig. 5h) or *TUBB3* (Fig. S6b). In summary, we found that growth factor withdrawal induced an increase in *PAK3* expression associated with *GFAP* and *DCX* expression in mesenchymal/classical 3731 cell

436 lines, whereas ATRA treatment induced *PAK3* and *DCX* 437 up-regulation mainly in the proneural PDCLs (Fig. 5i). 438 In contrast, BPM4 induced glial differentiation in mesenchymal cell lines without affecting *PAK3* expression. This 439 highlights a link between *PAK3* expression and neuronal 440 differentiation. 441

**Fig. 5 High PAK3 expression is positively correlated to neuronal differentiation.** a-f:

PDCLs were grown as monolayers in proliferative medium (t0) or without growth factors in 0.5% FCS for 3 (t3) or 8 (t8) days. **a**, qRT-PCR analysis of *PAK3* expression during the time course after growth factor withdrawal, expressed relatively to that in normal human fetal brain as external reference. **b**, Western blot analysis of *PAK3* protein expression with actin as loading control. **c**, Percentage of Ki67-positive cells relative to DAPI staining. **d**, qRT-PCR analysis of p27 expression relative to that in normal human fetal brain as external control. **e**, qRT-PCR analysis of *DCX* and *GFAP* expression relative to that in normal human fetal brain as external reference. **f**, Western blot analysis of *DCX* and *GFAP* expression with actin as control, in PDCLs over time. **g-i**: Cells were grown as monolayers in proliferative medium and then without growth factors in the absence of FBS (0%), or in the presence of BMP4, DMSO or *all trans* retinoic acid (ATRA) for 3 (t3) or 8 (t8) days. **g, h**, qRT-PCR analysis of gene expression at t3 and t8, expressed relative to the t0 value of each PDCL culture, in the absence (0%) or presence of BMP4 (**g**), and in the presence of DMSO as a control or ATRA (**h**). **i**, Diagram showing the variations in gene expression levels after growth factor withdrawal, BMP4 or ATRA treatment. For all these qRT-PCR (expressed as  $\Delta\Delta Cq$ ) or Western blot analyses, data represent at least three independent experiments





**Fig. 6** ShRNA-mediated inhibition of PAK3 expression modifies glioma cell proliferation, differentiation and tumor growth. **a-c:** 3731a PDCLs stably transduced with Control-shRNA (Ctrl) or PAK3-shRNA (03A, 04A) lentiviral vectors were grown as monolayers in proliferative medium (t0) or three days after growth factor removal and in 0.5% FCS (t3). **a**, qRT-PCR analysis of PAK3 expression relative to that in normal human fetal brain (n=3). **b**, Percentage of Ki67<sup>+</sup> positive cells. **c**, qRT-PCR analysis of GFAP and DCX expression relatively to that in normal human fetal brain (n=3). **d, e:** Mice

were subcutaneously injected with PAK3-shRNA-03A-transduced 3731a cells (03A) (n=9) or the Control-shRNA-transduced 3731a cells as control (Ctrl) (n=8). **d**, Tumor growth over time analyzed after tumors reached 25 mm<sup>3</sup>; mean curves were obtained by LOESS method. **e**, Mean doubling time ±SD of Control and Sh03A tumor growth. The doubling time parameter was extracted after exponential growth curve modeling. Each individual data point represents one mouse

442 **3.6 PAK3 knock-down alters glioma cell**  
 443 **proliferation, differentiation and tumor growth**

444 To more precisely define the role of PAK3 in glioma physi-  
 445 opathology, we knocked down PAK3 expression using  
 446 PAK3-specific shRNAs. We selected two shRNAs (03A  
 447 and 04) expressed from lentiviral vectors in COS7-infected  
 448 cells, based on their efficiency in inducing PAK3 expres-  
 449 sion decrease. The greatest efficacy of PAK3 expression

inhibition was obtained in 3731a cells with Sh-03A, and 450  
 to a lesser extent with Sh-04A, in proliferative conditions 451  
 (t0) and after growth factor withdrawal (t3), compared to 452  
 the Control-shRNA (Fig. 6a). In proliferative conditions 453  
 (t0), inhibition of PAK3 expression by the two shRNAs had 454  
 no effect on cell proliferation, as revealed by Ki67 analysis 455  
 (Fig. 6b). However, after three days without growth factors 456  
 (t3), the proportion of Ki67-positive cells increased in 457  
 the PAK3-knock down compared to control cells. We next 458

459 evaluated *GFAP* and *DCX* expression in *PAK3*-shRNA-  
 460 3731a cells, in proliferative conditions (t0) and after growth  
 461 factor withdrawal (t3), compared to Control-shRNA express-  
 462 ing cells. Inhibition of *PAK3* expression had no effect on  
 463 *GFAP* expression in proliferative medium (t0) whereas the  
 464 *GFAP* level increased notably after growth factor withdrawal  
 465 (t3) (Fig. 6c). Furthermore, no significative effect was  
 466 observed on *DCX* expression under the same experimental  
 467 conditions. These results indicate that inhibition of *PAK3*  
 468 expression in GBM cells induces astroglial differentiation.

469 Finally, to analyze the effect of *PAK3* inhibition on  
 470 glioma cell tumorigenicity in vivo, we decided to choose  
 471 the 3731a PDCL that forms subcutaneous tumors in mice,  
 472 whereas 4371 does not (data not shown). Sh-03A or Control-  
 473 shRNA transduced 3731a cells were injected subcutaneously  
 474 in nude mice. Monitoring of tumor sizes clearly indicated  
 475 that tumors grew faster in the Sh-03A-cells injected mice  
 476 compared to the Control-shRNA injected mice (Fig. 6d).  
 477 Doubling times were extracted from the exponential growth  
 478 curves. Compared to the control ( $15.38 \pm 3.23$  days), the  
 479 doubling time for the Sh-03A-cells injected mice was shorter  
 480 ( $6.43 \pm 1.64$  days), indicating an increase in tumor growth  
 481 (Fig. 6e). *PAK3* expression levels were found to be lower  
 482 in tumors isolated from the four mice injected with *PAK3*-  
 483 shRNA transduced cells, compared with tumors isolated  
 484 from mice injected with Control-shRNA-cells, indicating  
 485 that Sh-mediated *PAK3* silencing is maintained over time  
 486 (Fig. S7). This allows us to confirm the correlation between  
 487 *PAK3* expression in GBM cells and tumour growth. How-  
 488 ever, we did not observe any increase in the expression of  
 489 glial markers, such as *GFAP* or *SOX9*, in tumors derived  
 490 from cells transduced with *PAK3*-shRNA, compared to Con-  
 491 trol-shRNA tumors (Fig. S7). These results strongly suggest  
 492 that inhibition of *PAK3* expression in GBM cells impacts  
 493 their differentiation fate towards an astroglial phenotype  
 494 in vitro and promotes their tumor growth in vivo.

## 495 4 Discussion

496 We here report correlations between *PAK* expression in gliomas  
 497 and patient survival that are specific of each isoform.  
 498 We found that *PAK1* expression in tumors is negatively  
 499 correlated to patient survival, suggesting that *PAK1* may  
 500 favor glioma tumor growth, in accordance with its reported  
 501 oncogenic functions in other cancers [17, 18]. We found  
 502 that the *PAK2* expression level is not related to survival but  
 503 that, in contrast, a high *PAK3* expression is associated with  
 504 a better survival. These results clearly indicate distinct and  
 505 unique roles for each *PAK* isoform in the pathophysiology of  
 506 gliomas. Interestingly, we found a higher *PAK3* expression  
 507 level in 1p/19q-codeleted tumors, which are tumors with  
 508 oligodendrocyte characteristics associated with a better

509 prognosis, raising the question whether *PAK3* acts as tumor  
 510 suppressor or simply as a marker of the differentiated status  
 511 of these tumors. Indeed, during brain development *PAK3*  
 512 possesses differentiating functions, being involved in neu-  
 513 ronal cell specification, neuroblasts differentiation and oligo-  
 514 dendrocyte differentiation [13–16]. These functions may act  
 515 during tumor development and favor neuronal differentiation  
 516 of glioma cells.

517 To address this question, we performed a comparative  
 518 study on several PDCLs. The different cell cultures used dis-  
 519 played distinct characteristics, particularly regarding *PAK3*  
 520 expression. The two 3731 PDCL clones grew fast, exhibited  
 521 classical/mesenchymal transcriptomic signatures, formed  
 522 tumors after subcutaneous engraftment and displayed a low  
 523 *PAK3* expression, compared to the 4371 PDCL. Following  
 524 growth factor withdrawal or ATRA treatment the 3731a  
 525 subclone differentiated towards an immature neuronal state  
 526 associated with an increase in *PAK3* expression, whereas the  
 527 3731b subclone showed upregulated *GFAP* expression upon  
 528 BMP4 treatment, without any change in *PAK3* expression.  
 529 This suggests that these cells undergo an astroglial differ-  
 530 entiation or acquire stem cell-like properties, independently  
 531 of *PAK3* expression. The 4371 PDCL culture possesses  
 532 opposite characteristics: it grew more slowly, expressed  
 533 a more proneural transcriptomic profile and did not form  
 534 tumors after xenografting. Interestingly, it expressed a rela-  
 535 tively higher level of *PAK3* and neuroglial differentiation  
 536 markers. Growth factor withdrawal and BMP4 treatment  
 537 induced only a small increase in neuroglial differentiation,  
 538 whereas treatment with ATRA induced a strong increase in  
 539 the expression of neuronal markers such as *DCX*, as well  
 540 as *PAK3* expression. These results are in agreement with  
 541 distinct differentiation characteristics of PDCLs upon dif-  
 542 ferent differentiation-inducing regimens [34]. However, our  
 543 data clearly show that an increased *PAK3* expression was  
 544 associated with neuronal differentiation in two PDCL cul-  
 545 tures, suggesting that *PAK3* favors neuronal differentiation  
 546 and decreased proliferation of glioma cells, as *PAK3* does  
 547 during normal brain development.

548 We found that *PAK3* expression knockdown resulted in an  
 549 increase in PDCL proliferation associated with an increase  
 550 in *GFAP* expression in vitro and an increase in tumor growth  
 551 rate in engrafted mice. These results strongly suggest that  
 552 *PAK3* can promote neuronal differentiation and/or inhibit  
 553 astroglial differentiation, leading to slow tumor growth. In  
 554 our setting, we did not observe any increase in astrocytic  
 555 markers in *PAK3*-shRNA tumors. Among several possible  
 556 explanations, the most likely is that the analysis of the cel-  
 557 lular phenotype was carried out relatively late, i.e., mice  
 558 were euthanized after having reached a tumor stage at which  
 559 cellular regulations and phenotypes are radically modified.  
 560 Some brain-specific factors present in culture media of  
 561 PDCLs, but absent in the xenograft environment, could be

necessary for inducing an astrocytic phenotype shift. Astroglial cell fate inhibition may be due to a direct inhibition of astrocyte differentiation or an indirect effect of the activation of neuronal differentiation triggered at the expense of astrocytic differentiation, as observed earlier during *Xenopus* embryogenesis [13].

The *PAK3* gene belongs to a group of genes characteristic of a proneural signature. This was established in the princeps study of Verhaak and collaborators and also in the shortlist of 48 genes for this same subtype, established by different algorithms in the study of Crisman and colleagues [6, 20]. More recently, scRNA-seq allowed a delineation of the transcriptomes of single glioblastoma cells, their intratumoral heterogeneity and their phenotypic plasticity. The main identified subgroups of cells are characteristic of neuronal and glial lineages or their progenitors. Interestingly, the *PAK3* gene belongs to the top 30 genes that define neural progenitor cells (NPCs) specifically involved in the neuronal lineage (NPC2) [35]. Here, we confirmed a key status of the *PAK3* gene for a proneural signature in tumors as well as in PDCLs. This underlines its weight as a subgroup indicator, but at the same time raises the question of its role in the biology of these tumors. In particular, it remains to be determined how *PAK3* could have opposite roles to those of *PAK1* in the biology of gliomas. A first possibility may be that *PAK3* has its own substrates. Currently, most PAK substrates involved in cancer have been identified for *PAK1*, but these have not been validated for other group I kinases [19, 36]. A hypothesis may be that *PAK3* uses one or more yet unknown substrates that are not phosphorylated by *PAK1* and are involved in the inhibition of cell division, or in the initiation of neuronal differentiation. Another hypothesis may be that *PAK3* belongs to a signaling pathway involved in a negative regulation of tumor growth. We have previously shown that *PAK3* signaling differs from that of *PAK1* at several points, *PAK3* being activated by the Rho GTPase Cdc42 and preferentially binding to the Nck2/Grb4 adapter [37, 38]. These pathways are known to act in tumor proliferation, differentiation and progression, but their role in the biology of gliomas is still poorly understood. Another possibility may be that *PAK3* can act on cell proliferation by inhibiting *PAK1*. Indeed, the PAK kinases have been reported to form dimers that allow their trans-inhibition [39, 40]. However, we have previously shown that the *PAK3* protein prefers to form heterodimers with *PAK1* rather than homodimers, leading to inhibition of *PAK1* kinase activity [29]. Thus, a transcriptional increase of *PAK3* in gliomas could lead to a decrease in *PAK1* activity.

Although *PAK1* and *PAK2* are well characterized oncogenes, our data strongly suggest that *PAK3* acts as a tumor suppressor gene [17, 36, 41]. *PAK* genes are rarely mutated in cancer and the analysis of mutation databases does not allow to discriminate mutational patterns between *PAK1*

and *PAK3* (CBioPortal) [42]. On the other hand, *PAK1/2* genes are often overexpressed in cancer, as recently reported for breast cancer, in contrast to *PAK3* [43]. *PAK1* is mainly involved in cancer development through functional activation, due to transcriptional or post-translational processes. Here, our transcriptomic analysis showed a strong correlation between *PAK3* expression and patient survival, and the functional experiments we performed indicate that *PAK3* may indeed be considered as a tumor suppressor. One consequence of our results relates to possible therapeutic approaches. They stress the need to analyze, in different tumor types, the expression and activation of oncogenic PAK kinases such as *PAK1* or *PAK2*, but also that of *PAK3*. Indeed, the use of non-selective PAK kinase inhibitors could have deleterious effects by inhibiting the *PAK3* tumor suppressor gene. To date, there are no specific PAK inhibitors that do not inhibit *PAK3* [44]. Our data do suggest, however, that differential therapies may be developed depending on *PAK3* expression level. A therapeutic strategy for tumors with a low *PAK3* level could be to target the transcription of *PAK3* in order to force post-mitotic cell differentiation by targeting the proneural Notch/Neurogenin/NeuroD pathway previously identified in *Xenopus* [8, 13, 45]. Differentiation strategies have already been considered for GBM cancer stem cells [8, 34, 46]. However, this approach is complex and requires a more precise description of the function of the gene in different cellular contexts. This was recently illustrated by data showing that the effects of overexpression of *ASCL1*, which acts as a proneural gene during embryonic development and belongs to the proneural signature gene set that includes *PAK3*, depends on the GBM subtype [47]. Increasing *PAK3* expression may be a therapeutic goal in poorly differentiated tumors, since several factors such as neurogenin positively regulate its transcription [48].

We have provided omics and experimental data suggesting a unique role of *PAK3* in the pathophysiology of gliomas. These data need to be consolidated by other approaches such as orthotopic grafts of different PDCLs, in which the expression of *PAK3* and its kinase activity can be experimentally modified, in order to analyze tumor growth within the brain environment. Our results strongly indicate that each group I-PAK plays a different role in cancer progression, in particular in breast and brain cancer, and that *PAK3* acts as a tumor suppressor in gliomas and, as such, may represent a potential therapeutic target.

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## 684 Declarations

685 **Conflict of interest** The authors declare no potential conflict of interest.  
686

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