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**PID1 (NYGGF4), a New Growth-Inhibitory Gene in Embryonal Brain Tumors and Gliomas**

Anat Erdreich-Epstein¹,², Nathan Robison¹, Xiuhai Ren¹, Hong Zhou¹, Jingying Xu¹, Tom B. Davidson¹, Mathew Schur¹, Floyd H. Gilles², Lingyun Ji¹, Jemily Malvar¹, Gregory M. Shackleford¹,³,⁶, Ashley S. Margol¹, Richard Sposto¹,⁵, and Shahab Asgharazadeh¹,²

**Abstract**

**Purpose:** We present here the first report of PID1 (Phosphotyrosine Interaction Domain containing 1; NYGGF4) in cancer. PID1 was identified in 2006 as a gene that modulates insulin signaling and mitochondrial function in adipocytes and muscle cells.

**Experimental Design and Results:** Using four independent medulloblastoma datasets, we show that mean PID1 mRNA levels were lower in unfavorable medulloblastomas (groups 3 and 4, and anaplastic histology) compared with favorable medulloblastomas (SHH and WNT groups, and desmoplastic/nodular histology) and with fetal cerbellum. In two large independent glioma datasets, PID1 mRNA was lower in glioblastomas (GBM), the most malignant gliomas, compared with other astrocytomas, oligodendrogliomas and nontumor brains. Neural and proneural GBM subtypes had higher PID1 mRNA compared with classical and mesenchymal GBM. Importantly, overall survival and radiation-free progression-free survival were longer in medulloblastoma patients whose tumors had higher PID1 mRNA (univariate and multivariate analyses). Higher PID1 mRNA also correlated with longer overall survival in patients with glioma and GBM. In cell culture, overexpression of PID1 inhibited colony formation in medulloblastoma, atypical teratoid rhabdoid tumor (ATRT), and GBM cell lines. Increasing PID1 also increased cell death and apoptosis, inhibited proliferation, induced mitochondrial depolarization, and decreased serum-mediated phosphorylation of AKT and ERK in medulloblastoma, ATRT, and/or GBM cell lines, whereas siRNA to PID1 diminished mitochondrial depolarization.

**Conclusions:** These data are the first to link PID1 to cancer and suggest that PID1 may have a tumor inhibitory function in these pediatric and adult brain tumors. *Clin Cancer Res; 20(4); 827–36. ©2013 AACR.*

**Introduction**

Here, we report on PID1 (Phosphotyrosine Interaction Domain containing 1) in 2 important groups of brain tumors: embryonal brain tumors [medulloblastomas and atypical teratoid rhabdoid tumors (ATRT)] and gliomas. Medulloblastomas are the most common malignant brain tumors in children, where in high-risk disease prognosis remains poor. Moreover, in younger children, therapies that include whole brain irradiation are associated with serious long-term sequelae that hamper quality of life of survivors (1, 2). ATRT is another poor prognosis, highly malignant embryonal brain cancer of young children. Most ATRTs have lost expression and/or function of the INI1 (SMARCBI) tumor suppressor gene, which is part of the SWI/SNF chromatin-remodeling complex (3–5). Malignant gliomas, including glioblastoma multiforme (GBM), comprise the most common primary malignant brain tumors in adults and also carry poor prognosis (6, 7). Here, we provide novel clinical correlations of PID1 in medulloblastomas and gliomas, and demonstrate tumor-inhibitory effects of PID1 in cell lines of these 3 brain tumors.

The PID1 gene [also called NYGGF4 and phosphotyrosine binding (PTB)-containing, cubilin, and LRPI-interacting protein; PCL1; rhymes with "BID-1"] was identified in 2006 based on its differential expression in adipose tissue of obese compared with nonobese subjects (8). To date, PID1 has not been reported in the context of cancer. Although PID1 is known to contain a PTB domain/phosphotyrosine interaction domain (PID; ref. 8), the molecular mechanism
**Translational Relevance**

We present here the first report of PID1 (Phosphotyrosine Interaction Domain containing 1; NYGGF4) in cancer. PID1 was identified in 2006, and its role and molecular mechanism are still poorly understood. Using 6 independent datasets in 2 common brain tumors, medulloblastomas and gliomas, we show that: (i) PID1 mRNA is lowest in least favorable subgroups of medulloblastomas and gliomas; (ii) higher PID1 mRNA is directly correlated with longer patient survival; and (iii) PID1 causes increased cell death and decreased proliferation in medulloblastoma, glioma, and atypical rhabdoid tumor cell lines. These data in 2 brain tumor types suggest that it may be possible to incorporate PID1 into personalized molecular prognostic signatures that predict patient response and outcome. Ongoing work on the molecular function of PID1 intends to utilize this knowledge in the design of improved approaches to therapy.

(s) underlying its activities are poorly understood. PID1 mRNA increases during differentiation of 3T3-L1 preadipocytes to adipocytes (8), and is lower in brains of patients with Alzheimer disease compared with controls (9). PID1 overexpression increases proliferation of 3T3-L1 preadipocytes, but does not alter their adipogenic differentiation (8). In NIH-3T3 cells, however, overexpression of PID1 amino acids 84-230 (aa84-230), which includes its PTB domain, inhibits proliferation of 3T3-L1 preadipocytes to adipocytes (8), and is lower in brains of patients with Alzheimer disease compared with controls (9). PID1 overexpression increases proliferation of 3T3-L1 preadipocytes, but does not alter their adipogenic differentiation (8).

Materials and Methods

Details for additional methods can be found in Supplementary Materials.

Patients, samples, and mRNA expression data

Medulloblastoma specimens and clinical records from 81 children diagnosed at Children’s Hospital Los Angeles (CHLA) between 1989 and 2008 were obtained according to a protocol approved by the local Institutional Review Board. Patient and sample characteristics are described in Supplementary Table S1. The Heidelberg microarray dataset includes 446 medulloblastomas, profiled on Affymetrix U133 plus2.0 arrays, and is a combination of published data on 230 patients obtained from the Gene Expression Omnibus (GSE10327, GSE12992, and GSE37418; refs. 11–13) and unpublished data on 216 additional patients from Heidelberg (M. Kool and S. Pfister; unpublished data). The published Toronto microarray dataset includes 103 medulloblastomas profiled on Affymetrix Human Exon Array data (GSE21140), and the published Boston microarray dataset includes 194 medulloblastomas profiled on Affymetrix U133A (14, 15). Molecular subgroups of medulloblastoma were identified either by using available published data, or by cluster analyses to assign molecular subgroup for the unpublished datasets (11, 12, 14), or for the CHLA analysis, by quantitative reverse transcription PCR (qRT-PCR) and a medulloblastoma gene signature derived from prior microarray studies (15, 16) and a CHLA study (manuscript in preparation). Glioma PID1 mRNA microarray data in REMBRANDT (Repository for Molecular BRain Neoplasia DaTa) were from Affymetrix U133 plus2.0 arrays (17). Glioblastoma PID1 mRNA data in TCGA (The Cancer Genome Atlas; The Cancer Genome Atlas Research Network, National Cancer Institute and National Human Genome Research Institute, Bethesda, MD) were from Agilent G5402A_07 microarrays; (18). For GBM clinical and PID1 correlations, publicly available data for 196 patients was obtained from TCGA and Verhaak and colleagues (19), and was accessed at https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/files:unifiedScaled.txt and TCGA_unified_CORE_ClinNC840.txt and https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm?mode=ApplyFilter&diseaseType=GBM (files: clinical_patient_gbm.txt and clinical_follow_up_v1.0_gbm.txt) on February 18, 2013.

RNA from CHLA medulloblastoma samples was extracted and processed using a previously published method (20). PID1 mRNA levels were obtained by qRT-PCR (PID1: forward primer: 5'-GATTGCTGGCAACCACCTGA-3', reverse primer: 5'-AAATGTAAAGGTTGGCAGGGC-3', probe: 5'-CTCAGGACATGTCCTCTCCAGACGT-3') and after normalization to 3 housekeeping genes (GAPDH/SDHA/HPRT1).

**Annexin V, cell proliferation, and mitochondrial depolarization**

Assays were performed on green fluorescent protein (GFP)-positive cells 24 hours after transfections. Annexin V staining was done by flow cytometry using the APC Annexin V Kit (Cat. No. 550474; BD Pharmingen) according to manufacturer’s instructions. Cell proliferation and viability were assessed by flow cytometry using the APC BrdUrd Flow Kit (Cat. No. 552598; BD Pharmingen). Mitochondrial depolarization was measured by flow cytometry using the MitoProbe DiIC1; (5) Assay Kit for flow cytometry (Cat. No. M34151; Invitrogen).
Cell culture

The cell lines used were: GBM (U87, U251, LN18, LN229, CHLA-07-BSCGBM), medulloblastoma (D283MED, D425MED, UW-228-2, CHLA-259, CHLA-01-MED, CHLA-01R-MED), and ATRT (BT-12, CHLA-05-ATRT, CHLA-06-ATRT). Details for their culture are in Supplementary Materials.

Plasmids

PiD1 variant 1 (NM_017933.4; PiD1-1) and variant 2 (NM_00100818.1; PiD1-2) human cDNA ORFs with a turbo-GFP (tGFP) C-terminal tag in pCMV6-AC-tGFP (pCMV6-AC-PiD1-tGFP) were from OriGene (Cat. No. PS100010, RG212451, RG212505). PiD1 in the pCLS-2A-tGFP plasmid (ref. 21; from Dr. M.A. Lawlor, University of Michigan, Ann Arbor, MI) was expressed 5’ to the foot and mouth disease virus–derived 2A self-cleaving peptide sequence. In pCIENS (a pcDNA3.1-based CMV promoter-driven expression vector that also expresses eGFP via an EMCV IRES), PiD1 (variant 1) was expressed from the CMV promoter.

Statistical analysis

Details of the statistical analysis of patient-related information can be found in the Supplementary Materials.

In vitro experiments were analyzed using GraphPad Prism version 5.0 for MAC (GraphPad Software; www.graphpad.com). Results are depicted as mean ± SEM from at least 3 independent experiments unless stated otherwise. P values represent unpaired 2-sided Student t-test unless stated differently.

Results

**PiD1 mRNA is higher in favorable medulloblastomas and correlates with longer rf-PFS and OS in patients with medulloblastoma**

qRT-PCR of 81 pediatric medulloblastoma tumors showed that mean PiD1 mRNA levels were significantly higher in medulloblastomas with desmoplastic/nodular histology compared with those with anaplastic histology (P < 0.001; Fig. 1A; patient characteristics in Supplementary Table S1). Supporting this, analysis of PiD1 in the 4 medulloblastoma core molecular subgroups (11, 22, 23) showed that SHH group medulloblastomas had higher mean PiD1 mRNA compared with the less favorable subgroup, groups 3 and 4 (Fig. 1B). Microarray data from 3 other independent medulloblastoma datasets similarly revealed higher mean PiD1 mRNA in the more favorable subgroups (SHH and WNT) compared with medulloblastomas in groups 3 and 4 (Fig. 1C and Supplementary Fig. S1A; refs. 11–13). Mean expression of PiD1 mRNA was higher in fetal cerebellum compared with groups 3 and 4 medulloblastomas, and was similar to PiD1 mRNA in the WNT and SHH groups (Fig. 1C).

In univariate analysis of the CHLA medulloblastoma patients (n = 81; Supplementary Table S1), rf-PFS was significantly longer in children whose tumor PiD1 mRNA was higher than the median of the cohort, compared with those with tumor PiD1 mRNA lower than median (rf-PFS 73% ± 11% vs. 11% ± 10%, respectively; P < 0.001; Fig. 1D). Difference in rf-PFS also remained significant when dividing the group into equal tertiles according to PiD1 mRNA (Supplementary Fig. S1B). In the non-CHLA medulloblastoma patients from Fig. 1C for whom survival data were available, overall survival (OS) was significantly longer in patients with higher tumor PiD1 mRNA compared with those with lower PiD1 mRNA (Fig. 1E), supporting the findings in the CHLA cohort (Fig. 1D). In multivariate analysis of the CHLA cohort, risk of radiation-free disease progression was significantly higher in patients with lower-than-median PiD1 mRNA compared with those with higher-than-median PiD1 mRNA (Table 1).

These analyses demonstrate that the clinically favorable medulloblastomas (desmoplastic/nodular histology, or WNT and SHH molecular subgroups) had higher mean PiD1 mRNA compared with clinically unfavorable medulloblastomas (anaplastic histology or groups 3 and 4), and that higher medulloblastoma PiD1 mRNA correlated with longer rf-PFS and OS.

**GBM have lower PiD1 mRNA compared with other gliomas and nontumor brains; higher PiD1 mRNA correlates with longer overall survival in patients with glioma**

Analysis of PiD1 mRNA data using the REMBRANDT glioma clinical genomics database (17) revealed that PiD1 mRNA levels were lower in the highest grade gliomas, GBMs, compared with nontumor brains, astrocytomas, and oligodendrogliomas (Fig. 2A). The TCGA glioma dataset (18) similarly showed that mean PiD1 mRNA in GBM was significantly lower than its level in nontumor brains [95% confidence interval (CI), 0.34–0.38; P < 0.0001; Fig. 2B]. For patients with GBM for whom tumor molecular subtype (classical, mesenchymal, neural, proneural) and clinical data were available and unified PiD1 mRNA expression values from 3 platforms in TCGA were analyzed (see Materials and Methods), there were significant differences in mean PiD1 mRNA among the molecular subtypes (P < 0.0001; Supplementary Fig. S2A and Table S2). Neural and proneural GBM subtypes showed higher PiD1 mRNA compared with classical and mesenchymal subtypes. All paired comparisons between GBM with known subtypes were also significantly different (after Bonferroni adjustment for multiple comparisons) with the exception of the neural versus proneural pairing (P = 0.88). Differences in PiD1 mRNA between GBM subtypes remained significant even after adjusting for age at diagnosis (P < 0.0001).

Survival analysis using the REMBRANDT dataset revealed significant differences in OS among patients who were stratified according to PiD1 mRNA. PiD1 mRNA was directly correlated to OS in patients with any diagnosis of glioma, or within the subset of astrocytomas that does not include GBMs (Fig. 2C and D, Supplementary Fig. 2B–E; using 2 available PiD1 probesets). For GBMs in the TCGA dataset, PiD1 mRNA was univariately significantly associated with survival (Cox regression analysis, P = 0.031; Fig. 2E), but the REMBRANDT dataset did not reveal such correlation (not shown). Although the PiD1 mRNA association with OS in GBM (TCGA) was no
longer significant after adjusting for molecular subtype \( (P = 0.15) \), the relative failure rate decrease per unit increase in \( \text{PID1} \) mRNA was only slightly attenuated in our analysis \( (0.83 \pm 0.071 \text{ univariate}, 0.85 \pm 0.094 \text{ multivariate}) \). In Cox multivariate analysis, with age at diagnosis, and \( \text{PID1} \) expression and subtype as variables (TCGA), \( \text{PID1} \) was marginally significant \( (P = 0.056) \). The relative failure rate decrease per unit increase in \( \text{PID1} \) mRNA was 0.81 \( \pm 0.091 \).

Thus, similar to our findings in medulloblastomas, the most malignant gliomas (i.e., GBMs) had lower \( \text{PID1} \) mRNA compared with other gliomas or with nontumor brains. In addition, higher tumor \( \text{PID1} \) mRNA correlated with longer OS in patients with glioma.

**\( \text{PID1} \) confers growth disadvantage in brain tumor cell lines**

We next asked if the clinical correlations we found for \( \text{PID1} \) mRNA pointed to a possible biological role for \( \text{PID1} \) in brain tumors. To evaluate a role in growth, we assessed the effect of \( \text{PID1} \) on colony formation in U251 GBM,
LN229 GBM, D283MED medulloblastoma, and CHLA-06-ATRT brain tumor cell lines as follows: cells were transfected with PID1-tGFP or tGFP control for 24 hours, tGFP-expressing cells were sorted by flow cytometry and plated at equal numbers, and colonies were allowed to form over 2 weeks without further selection. Each of the cell lines formed significantly fewer colonies when transfected with PID1-tGFP than did cells transfected with the control tGFP vector, despite an equal number of sorted tGFP+ cells plated for the tGFP control and PID1-tGFP transfected cells (Fig. 3A and B). Both variants of PID1 (variant 1: NM_017933, variant 2: NM_001100818) showed similar effects in this assay. Similar inhibition of colony formation was also observed using the self-cleaving bicistronic pCLS-PID1-2A-eGFP compared with its control vector, pCLS-2A-eGFP (21) as well as with the bicistronic pCIENS-PID1 compared with pCIENS control vector (Supplementary Fig. S3A and S3B). Expression of PID1 at the time of flow sorting and plating of the tGFP-expressing cells was verified by Western blotting (24 hours after transfection; Supplementary Fig. S3C). These data indicate that PID1 confers growth and/or survival disadvantage upon brain tumor cell lines ectopically expressing PID1.

**PID1 inhibits proliferation and promotes cell death of cultured brain tumor cell lines**

To characterize the PID1-induced growth disadvantage, we analyzed proliferation and cell death, measuring BrdUrd/7AAD uptake in tGFP-positive cells 24 hours after transient transfection with pCMV6-PID1-tGFP or pCMV6-tGFP control. GBM (U251) and medulloblastoma (UW-228-2) cell lines as well as primary medulloblastoma cells (CHA-259) expressing PID1-tGFP had significantly more cells in the sub-G0–G1 phase compared with cells expressing tGFP+ control vector (Fig. 4A and B and Supplementary Fig. S4), indicating that PID1 increased cell death. PID1-tGFP also decreased proliferation, as indicated by the decreased proportion of cells in S-phase (Fig. 4A and B). Apoptosis accounted for at least some of the cell death, as annexin V binding was higher in PID1-tGFP–expressing cells compared with tGFP controls (Fig. 4C). There were very few 7AAD-positive/annexin V–negative cells, indicating that necrosis was only minimal. As mitochondria are involved in metabolism and several forms of cell death and PID1 has been reported to induce mitochondrial dysfunction in adipocytes and myocytes (24–28), we examined the effect of PID1 on mitochondrial depolarization. U251 GBM, D283MED medulloblastoma, and CHLA-06-ATRT cell lines transfected with PID1-tGFP showed increased depolarization of mitochondrial membrane potential compared with tGFP-transfected cells 24 hours after transfection (Fig. 4D). Conversely, siRNA knockdown of PID1 in U87 GBM (U87 express PID1 protein; Supplementary Fig. S5) diminished baseline depolarization of the mitochondrial membrane potential compared with nonsilencing siRNA control (Fig. 4E). siPID1 knockdown similarly diminished baseline depolarization of mitochondrial membrane potential.

**Table 1. Cox proportional hazards model for progression-free survival (patients who received irradiation as part of the primary treatment were censored at time of irradiation)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analyses</th>
<th>Multivariate analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HRa 95% CIa P</td>
<td>HRb 95% Cib P</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative risk increase per 1 year</td>
<td>1.2 (1.05, 1.4) 0.017</td>
<td>1.0 (0.84, 1.3) 0.78</td>
</tr>
<tr>
<td>≥3 years vs. &lt;3 years</td>
<td>3.5 (1.3, 9.1)</td>
<td>1.2 (0.36, 3.8)</td>
</tr>
<tr>
<td>Staged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>1.0 — 0.006</td>
<td>1.0 — 0.004</td>
</tr>
<tr>
<td>M+</td>
<td>4.7 (1.4, 15)</td>
<td>16 (2.0, 128)</td>
</tr>
<tr>
<td>Histologyd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td>1.0 — 0.21</td>
<td>1.0 — 0.27</td>
</tr>
<tr>
<td>Desmoplastic/nodular</td>
<td>0.42 (0.09, 2.0)</td>
<td>3.8 (0.29, 49)</td>
</tr>
<tr>
<td>Anaplastic/large cell</td>
<td>2.0 (0.54, 7.6)</td>
<td>0.35 (0.06, 1.9)</td>
</tr>
<tr>
<td>Molecular subgroupd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHH/WNT</td>
<td>1.0 — 0.048</td>
<td>1.0 — 0.75</td>
</tr>
<tr>
<td>Group 3</td>
<td>6.2 (1.5, 26)</td>
<td>2.7 (0.17, 44)</td>
</tr>
<tr>
<td>Group 4</td>
<td>3.2 (0.58, 18)</td>
<td>2.1 (0.12, 37)</td>
</tr>
<tr>
<td>PID1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1.0 — &lt;0.001</td>
<td>1.0 — 0.005</td>
</tr>
<tr>
<td>Low</td>
<td>6.8 (2.2, 21)</td>
<td>21 (1.9, 221)</td>
</tr>
</tbody>
</table>

aHR and CI from univariate Cox models. 
bHR, CI, and P-values from the Cox model that included age, stage, histology, molecular subgroup, and PID1 expression. 
cHR, CI, and P-values from the Cox model that included stage and PID1 expression. 
dMissing data for these variables were included in the models as a “missing” category.
mitochondrial depolarization in D283MED medulloblastoma cells (Fig. 4E). These data demonstrate that the inhibitory effect of PID1 on brain tumor cell line growth is the result of both inhibition of proliferation and increased cell death, and is associated with depolarization of mitochondrial membrane potential.

Finally, to begin investigating the molecular mechanism of PID1, we examined its effect on serum-mediated phosphorylation of AKT and ERK, 2 effectors that are central to a number of proliferation and survival signaling pathways. LN229 GBM cells transiently transfected with PID1, serum starved overnight and acutely stimulated with 20% FBS showed decrease in the serum-induced phosphorylation of AKT compared with empty vector-transfected cells (Fig. 4F). In addition, transient transfection of PID1 into D283MED medulloblastoma cells grown in FBS-containing medium caused decrease in phosphorylation of both AKT and ERK (Fig. 4G and Supplementary Fig. S6). This indicates that...
PID1 inhibits serum-mediated signaling pathways, which involve AKT and ERK.

In summary, using 6 independent datasets, we have shown that PID1 mRNA is lower in the more aggressive medulloblastomas and gliomas compared with their relatively more favorable counterparts, and that higher PID1 mRNA correlates with longer rf-PFS and OS in patients with medulloblastoma and longer OS in patients with glioma and GBM. We also showed that PID1 conferred a growth-inhibitory effect on cell lines of 3 types of brain tumors (medulloblastoma, GBM, and ATRT), which manifested as decreased proliferation and/or increased cell death. Finally, PID1 induced mitochondrial depolarization and inhibition of serum-mediated phosphorylation of AKT and ERK. Taken together, these findings suggest that PID1 is a novel growth inhibitor in gliomas and embryonal brain tumors.

Discussion

The small number of articles reporting on PID1 to date have mostly been in the context of obesity and diabetes in adipocytes and muscle cells (8, 28–30) and in Alzheimer’s brains (9). Our work here is the first to report on PID1 in cancer.

Our clinical findings for PID1 using 6 independent datasets comprising 2 different types of brain cancers emphasize the validity of the association between lower PID1 mRNA and less favorable tumor subgroups. The in vitro experiments suggest that PID1 confers growth suppression because of combined inhibition of proliferation and increase in cell death. Regulation of PID1 expression in embryonal brain tumors and gliomas is currently unknown.

The molecular mechanism by which PID1 affects growth of brain tumor cells in culture is also unknown at this time. Our finding on depolarization of mitochondrial membrane potential by PID1 in brain tumor cell lines is consistent with reports that PID1 disrupts mitochondrial function in adipocytes and muscle cells (24–28). In adipocytes and muscle cells, PID1 also inhibits insulin-mediated phosphorylation of IRS-1 and AKT, and insulin-mediated translocation of the GLUT-4 glucose transporter to the membrane, resulting in decreased glucose uptake (28–30). Our experiments showing that PID1 inhibits phosphorylation of AKT and ERK suggest that PID1 may modulate signaling pathways involved in cell proliferation and survival. Although insulin signaling is less likely to be a target of PID1 modulation in brain tumor cell lines because of their low and/or infrequent expression of the insulin receptor, it will be interesting to examine if PID1 modulates signaling through the insulin-like growth factor receptor IGF1R, which is important in many brain tumors (31–40). The inhibition of insulin receptor signaling in adipocytes was hypothesized to be mediated via interaction of the PID1 PTB domain with the NPXY motif in the cytoplasmic tail of the insulin receptor (29); however, co-immunoprecipitation of the 2 proteins has not been reported yet. Using yeast 2 hybrid screens and pull-down experiments, several groups reported direct interaction between the PTB domain of overexpressed PID1 and the NPXY motif of low-density lipoprotein receptor-like-1 (LRP1; refs. 9 and 10). However, it is unlikely that LRP1 is a major player in the PID1 induced cell-cycle arrest in NIH-3T3, because the arrest could not be overcome by overexpression of LRP1 (10).

Cellular effects of PID1 may differ between cell types. In 3T3-L1 preadipocytes, PID1 increased proliferation (8), but in NIH3T3 cells a PID1 fragment that includes the PTB domain (aa84-230) caused cell-cycle arrest (10). Our findings that PID1 has a growth-suppressive effect in multiple brain tumor cell lines are consistent with the latter.
Examination of the Catalogue of Somatic Mutations in Cancer (COSMIC; ref. 41) revealed that among 7,055 tumors reported to date (July 18, 2013), there were only 27 mutations, of which 15 were nonsynonymous. No mutations or copy number variations of PID1 were found in the 510 brain tumors reported. The lack of PID1 copy number variations and PID1 mutations in brain tumors suggests that PID1 does not function as a classical tumor suppressor gene. The direct correlation of PID1 mRNA and survival in medulloblastoma and gliomas suggests that PID1 may render these brain tumors more susceptible to therapy, accounting for the better outcome in patients whose tumors expressed higher PID1 mRNA. This possibility is currently under investigation in our laboratory.

In summary, this is the first report to link PID1 to cancer and to brain tumors, to demonstrate correlation between PID1 mRNA level and survival in medulloblastomas and gliomas, and to show growth-inhibitory effect of PID1 in cultured medulloblastoma, GBM, and ATRT cell lines. Our data therefore suggest that PID1 may have a growth-modulating function in brain tumors. It will now be important to further investigate the molecular mechanism(s) of PID1’s effects in pediatric and adult malignant brain tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Erdreich-Epstein, J. Xu, G.M. Shackleford
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Erdreich-Epstein, N. Robison, X. Ren, H. Zhou, J. Xu, T.B. Davidson, M. Schur, F.H. Gilles, A. Margol, M.D. Krieger, D.T.W. Jones, S.M. Pfister, M. Kool, S. Asgharzadeh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Erdreich-Epstein, X. Ren, H. Zhou,
References


SUPPLEMENTAL METHODS:

CHLA Patients (additional details)

Patients were identified from institutional pathology databases and clinical patient lists. Of 128 patients identified with embryonal tumors of the posterior fossa, 90 had frozen tumor samples adequate for RNA extraction. Sections of each frozen specimen embedded in OCT were used for histology and RNA preparation. Specimens with less than 50% viable tumor on a representative section were excluded. Original histologic specimens for all samples were reviewed when available. Histopathologic subclass was assigned according to 2007 World Health Organization criteria by reviewers blinded to outcome. Medulloblastomas were assigned to molecular subgroups based on non-negative matrix factorization (NMF) clustering as previously described (1) and using the available HuEx array data on 66 tumor samples. Histopathology was classified as unknown in two specimens where sections adequate for histologic classification from the same block as the RNA preparation were not available. INI-1 immunostaining on all specimens with available paraffin-embedded specimens caused reclassification of five specimens as atypical teratoid rhabdoid tumor (ATRT) and their exclusion, four by INI-1 immunohistochemistry (IHC) and one by histopathologic criteria. Total RNA was extracted from frozen specimens using RNA STAT-60 isolation reagent (Tel-Test, Inc.) and purified using RNeasy Mini Kit (Qiagen, Valencia, CA). Clinical records of all patients were accessed. Clinical covariates obtained included age at diagnosis, presence/site of metastatic disease, date and extent of resection, therapy received, date of first chemotherapy and first radiotherapy, date of most recent follow-up, and, where applicable, date of disease progression and/or death. Date of resection was defined as date of final resection before beginning radiation or chemotherapy. Incomplete resection was defined as >1.5 cm² residual disease on first post-operative imaging. Disease stage was assigned according to the Chang staging system. When exact date of first radiotherapy was not available, time of radiation was
estimated from available clinical data and treatment protocol. Disease progression was defined as appearance of one or more new lesions, 40% increase in the product of the diameters of an existing lesion compared to the smallest measurement taken after start of treatment, or (when precise measurements were not available) unequivocal progression of an existing lesion. Date of progression was defined as date of first formally reviewed imaging study showing disease progression. Tumor growth or a new lesion occurring before the first adjuvant therapy was not classified as progression. Last follow-up was defined as the date of most recent formally reviewed magnetic resonance imaging of the brain. Two patients who had disease progression after having radiation only (without chemotherapy) were excluded from the study. For two of the 83 patients eligible for analysis, there was insufficient RNA. Therefore, 81 patients were included in the analysis.

**Cell Culture**

Cell lines used were U251MG, U87, LN18 and LN229 GBM from ATCC, D283MED and D425MED medulloblastoma from Dr. Darrell Bigner (Duke University, Durham, NC), and UW228-2 medulloblastoma from Dr. John R. Silber (University of Washington, Seattle, WA). BT-12 ATRT were from Dr. Peter Houghton (Nationwide Children’s Hospital, Columbus, Ohio). CHLA-259 medulloblastoma (2), CHLA-01-MED and CHLA-01R-MED medulloblastoma, CHLA-05-ATRT and CHLA-06-ATRT (deposited at ATCC), and CHLA-07-BSGBM (GBM, from exophytic component of recurrent non-DPIG pediatric brain stem glioma) were generated at Children’s Hospital Los Angeles according to protocols approved by the local institutional Review Board. Cell lines were used no longer than six months after resuscitation from freezing. Cell lines were maintained as follows: U251, U87, LN18, LN229, D283MED and BT-12 in DMEM with 10% fetal bovine serum (FBS); UW-228-2 and D425MED in DMEM/F-12/10% FBS; CHLA-06-ATRT in IMDM/10% FBS/2mM glutamine, and CHLA-259 and CHLA-07-BSGBM in IMDM/20% FBS/2mM glutamine and ITS (1:1,000) (2). CHLA-01-MED, CHLA-01R-MED, and
CHLA-05-ATRT were maintained in DMEM/F12 1:1 with B-27 (Invitrogen, CA), EGF (20ng/ml, Invitrogen, CA) and bFGF (20ng/ml, Cell Sciences, MA). ECV304 bladder carcinoma were grown as described (3). Preadipocyte and adipocyte lysates were from Dr. Steve Mittleman (CHLA).

For transfections, cells were seeded in 6-well plates at 4x10^5 cells/well the day before transfection. Cells approaching 80-90% confluence were transfected with 2 µg of plasmid and 5 µl of Lipofectamine2000 (Invitrogen) in 1 ml serum-free medium for 5.5 hr according to manufacturer’s instructions. For colony formation assays tGFP+ cells were flow-sorted 24 hr after transfection, plated at various numbers (200–10,000 cells) in 6-well plates, and grown for 14 days in their usual FBS-containing growth medium without additional selection. Colonies were then stained with 1% crystal violet in methanol (room temperature, 10 min), washed in tap water and air-dried. Quantification was performed by scanning the plates seeded with 500 and 1000 cells and counting all colonies in each well by up to three observers, two of whom were blinded to sample identity, using the Photoshop counting tool. The number of colonies in control wells transfected with tGFP control varied between 50-250 colonies depending on the cell line, and was normalized to 1-fold.

Flow Cytometry

For assays analyzed by flow cytometry, a minimum of 5x10^5 of cells per sample were acquired for analysis using a SLR II flow cytometer (BD Biosciences). Cell clumps and sub-cellular debris were excluded using appropriate gating on forward and side light scatter. Data were analyzed using FACSDIVA software (BD Biosciences).
Intracellular staining of phospho-proteins

Fixation and permeabilization were according to the manufacturer’s instructions (BD Biosciences). Briefly, trypsinized cells were incubated with Cytofix buffer at 37°C for 10 min. Cells were then collected and pelleted by centrifugation and pellets were resuspended in Perm Buffer III and incubated on ice for 30 min. Cells were washed and stained with Alexa Fluor®647 mouse anti-Akt pS473 or Alexa Fluor®647 mouse Anti-ERK1/2 pT202/pY204 (BD Biosciences) for 30 min on ice. Cells were washed once with cold PBS and subjected to flow cytometry analysis.

Real time quantitative PCR

Real-time quantitative PCR was performed using the Applied Biosystems 7900HT sequence detection system (ABI, Foster City, CA). Template for PCR amplification was 10 ng cDNA (in 1 µl) generated from the clinical samples. PCR primers and probes were designed and synthesized using Primer Express software (ABI, Foster, CA) or SciTools (Integrated DNA Technologies, Inc.) and were checked for specificity against GeneBank. Variant 1 of PID1 was amplified using a unique primer pair. Amplification of the common region from both variants 1 and 2 was with a different set of primers (there are no primers unique to variant 2). Primers and probes: PID1 variant 1 (PID1-1; 93 bp, specific to PID1-1), forward primer: 5’-GATTGCTGGCAACCACCTGATGT-3’, reverse primer: 5’-AAATGTAAGGTGGCAGGGCCAC-3’, probe: 5’-TCGAGGACATGTTCCTTCTCCAGACGT-3’; GAPDH (127 bp), forward primer: 5’-CAACTACATGGTTTACATGTTCATAATG-3’, reverse primer: 5’-GGGATCTCCTCGCTCTCAGGAG-3’, probe: 5’-CGTTCTCAGCCTTGACGTTGCCA-3’. TaqMan real-time PCR data were analyzed using ABI Sequence Detector Software. PID1 mRNA was normalized to GAPDH mRNA, which was quantified in parallel in each sample.
**SDS-PAGE, western blotting**

Whole cell lysates were resolved by SDS-PAGE and proteins were identified by western blot as described (3). Anti-PID1 rabbit polyclonal antibody (Sigma-Aldrich cat# HPA036103) was used at 1:1,000. Specificity of this commercially available antibody was verified by comparing cells transfected with control vectors to cells transfected with tGFP-PID1 (Suppl Fig 3C). The identity of the PID1-antibody-reactive band was further confirmed by proteomic analysis of a silver-stained band of HA-immunoprecipitation of HEK293T cells overexpressing HA-PID1 (vs. HA) and migrating at the same position as a protein band reacting with the anti-PID1 band on an adjacent lane processed by western blotting (not shown). Anti-GAPDH mouse monoclonal (1:20,000) was from Santa Cruz Biotechnology, CA.

**siRNA transfections**

A mix of two PID1-specific siRNAs (GGGAGATCAATGATGACCTGT and GGAATTGGAATCCGATGATGG) designed using the siRNA Selection Program (4) at [http://sirna.wi.mit.edu/reference.php](http://sirna.wi.mit.edu/reference.php) were used at 1:1 ratio. Non-silencing negative control siRNA was from Qiagen, AllStars. FAM-labeled siRNA negative control was from Life Technologies. For transfection, control siControl (mix of 100nM non-silencing negative control siRNA + 10nM FAM-labeled siRNA negative control) or siPID1 (100nM of 1:1 mix of the two PID1-specific siRNAs + 10nM FAM-labeled siRNA negative control) were transfected using lipofectamine2000 for 5hrs. Assessment of mitochondrial potential and validation of PID1 knockdown by western blot and qRT-PCR were performed 24 hrs later.

**Statistical Analysis**

For the medulloblastoma samples from the CHLA cohort (Supplemental Table 1), we performed analysis of radiation-free progression-free survival (rf-PFS) to evaluate the
effect of PID1 on the outcome of patients who did not receive radiation (or prior to irradiation). rf-PFS in relation to patient and disease characteristics were based on the logrank test, product-limit estimator, and univariate and multivariate Cox regression analysis (5). rf-PFS was defined as the minimum interval from date of diagnosis to date of irradiation, tumor recurrence, tumor progression, or last follow-up; patients who received irradiation as part of their primary treatment were censored at time of irradiation (details in Supplemental Table 1). Overall survival (OS) was defined as the interval from date of diagnosis to date of death or last follow-up. For all analyses, patients who died of treatment-related complications unequivocally unrelated to disease progression were censored at time of death. Patient and disease characteristics evaluated by multivariate analyses included age, disease stage, histology, molecular subgrouping, and PID1 mRNA. Age was treated as a continuous variable in the CHLA medulloblastoma models. Hazard ratios for patients ≥3 years vs. <3 years were calculated for a patient with mean age of the ≥3 years group with reference to a patient with the mean age of the <3 years group. Estimates of the hazard ratios, 95% confidence intervals (CI), and p-values from the likelihood ratio tests are presented. All p-values reported were two-sided. For non-CHLA medulloblastoma data, analysis of OS was also based on the product limit estimate and logrank tests. P-values <0.05 were considered significant. Statistical computations were performed using Stata (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP). Survival analysis for glioma patients in REMBRANDT was analyzed using the REMBRANDT online tools (6). The TCGA clinical dataset comprised 202 GBM patients (ICDO-3 code 9441/3) with data on PID1 mRNA, 196 of which had outcome (EFS and
OS) data as mentioned under “Patient Samples”. Among the 196 patients with outcome data, 190 were dead at last follow-up. Median time to death was 51 weeks. Median age at diagnosis was 55 years, with range 14 to 86 years, and 90% of patients between the ages of 27 and 77. Unified, scaled \textit{PID1} mRNA among the three microarray platforms (Affymetrix HuEx array, Affymetrix U133A array and Agilent 244K array) were used, as computed by Verhaak et al (7), with higher values indicating higher mRNA levels.

REFERENCES

**SUPPLEMENTAL DATA**

**Supplemental Table 1: Characteristics of the CHLA Medulloblastoma Patients**

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$^1$ As of the last follow up date, 41 patients received upfront XRT, 6 patients received XRT after chemotherapy as part of the primary treatment, and 1 patient received XRT as part of the primary treatment but whether it was an upfront XRT or XRT after chemotherapy was unknown. Median time from date of diagnosis to date starting XRT for patients who received XRT after chemotherapy was 4.6 months (range: 2.6 months, 17 months).

$^2$ P values were from Pearson’s chi-square test, or from Fisher’s exact test when the number of cases was limited in some categories. Missing/Unknown categories were excluded from the tests.
Supplemental Table 2:

Neural and Proneural GBM subgroups have higher *PID1* mRNA compared to Classical and Mesenchymal

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\(^1\)Unified *PID1* mRNA among the three array platforms (7) was expressed on a log scale with unknown origin, with higher values indicating higher expression levels.

The table shows the number of patients with GBM, the mean and standard deviation of the log2*PID1* mRNA, and median survival within each glioblastoma subtype in the TCGA cohort analyzed by Verhaak et al (7). There was a significant difference in *PID1* mRNA between the five groups as well as between the four groups with known subtype \((p<0.0001)\). All paired comparisons between known subtypes were also significantly different (after Bonferroni adjustment for multiple comparisons) with the exception of Neural vs. Proneural \((p=0.88)\).
Supplemental Figure 1:

A) Analysis of PID1 mRNA in microarrays from two non-overlapping independent published medulloblastoma series (1, 8) shows that PID1 mRNA is higher in SHH and WNT groups compared to Groups 3 and 4, similar to our findings. The Boston series was profiled on Affymetrix U133A arrays (8), and the Toronto series (n=103) was profiled on Affymetrix Exon arrays (1). mRNA values are expressed in arbitrary units, using the MAS5.0 normalized Affymetrix values; B) Kaplan-Meier curve of rf-PFS as function of PID1 mRNA depicted according to equal tertiles using the samples analyzed in Fig 1A; n=27 per tertile; low PID1: 0% rf-PFS at 2 years, intermediate PID1: 47%±23% rf-PFS at 5 years, high PID1: 80%±11% rf-PFS at 5 years; p<0.001 by Trend test.

Supplemental Fig 1: PID1 mRNA is lower in less favorable medulloblastomas in two additional independent datasets; PID1 mRNA correlates with rf-PFS when analyzing CHLA medulloblastomas by tertiles. A) Analysis of PID1 mRNA in microarrays from two non-overlapping independent published medulloblastoma series (1, 8) shows that PID1 mRNA is higher in SHH and WNT groups compared to Groups 3 and 4, similar to our findings. The Boston series was profiled on Affymetrix U133A arrays (8), and the Toronto series (n=103) was profiled on Affymetrix Exon arrays (1). mRNA values are expressed in arbitrary units, using the MAS5.0 normalized Affymetrix values; B) Kaplan-Meier curve of rf-PFS as function of PID1 mRNA depicted according to equal tertiles using the samples analyzed in Fig 1A; n=27 per tertile; low PID1: 0% rf-PFS at 2 years, intermediate PID1: 47%±23% rf-PFS at 5 years, high PID1: 80%±11% rf-PFS at 5 years; p<0.001 by Trend test.
Supplemental Figure 2: Proneural and Neural GBMs have higher PID1 mRNA; higher PID1 mRNA correlates with higher OS in patients with gliomas.

A) Graphic depiction of the data in Table 2 showing that Neural and Proneural GBMs have higher PID1 mRNA than classical and mesenchymal GBMs (p<0.0001); B) Log rank p-values between the three groups in the K-M curves in Fig 2C-D were calculated by REMBRABDT and were significant for each comparison; C-E) Kaplan Meier analysis (REMBRANDT) of OS
according to \textit{PID1} mRNA in all glioma patients (C) or only in astrocytoma patients (D), using probeset 237867\_s\_at; \( \geq 2x \) - red; \( \leq 2X \) - green; 0.5x to 2x - orange, each relative to non-tumor brain; E) Log rank p-values between the groups in the K-M curves in panels C-D was calculated by REMBRANDT.
Supplemental Figure 3:

A) Colony formation assay in U251 and LN229 cells (normalized and combined) performed as in Figure 3A-B, except that the vector used was the bicistronic pCLS-2A-eGFP plasmid (9) vs. pCLS-PID1-2A-eGFP; n=3 independent experiments performed in triplicates for two of the three.  
B) Colony formation assay performed as in A in U251 cells, except cells were transfected with the bicistronic vector pCIENS vs. pCIENS-PID1; n=3;  
C) Lysates of U251 cells 24 hr after mock-transfection (lipofectamin only), transfection with pCIENS vector or transfection with pCIENS-PID1 variant 1 or pCIENS-PID1 variant 2. Cells were used for all experiment 24 hr after transfection. The western blot demonstrates PID1 expression and specificity of the antibody against PID1 (Sigma-Aldrich, cat#HPA036103).
Supplemental Fig 4:

Supplemental Fig 4: PID1 increases the proportion of cells in Sub G₀/G₁ in CHLA-259 medulloblastoma cells. Sub-G₀/G₁ phase based on BrdU/7AAD uptake was measured by flow cytometry in CHLA-259 primary medulloblastoma cells 24 hr after transfection with pCMV6-AC-tGFP vs. pCMV6-AC-PID1-tGFP performed as in Figure 4A-B; n=3.
Supplemental Fig 5: Expression of PID1 protein in human brain tumors and cell lines.

**A)** Lysates of brain tumor cell lines were resolved by SDS-PAGE (left panel: 4-15% gradient gel, BioRad; middle and right panels: 12.5% gel) and probed with anti-PID1 antibody or anti-GAPDH. GBM cell lines: U87, CHLA-07-BSGBM, D54, LN18, LN229, U251; Medulloblastoma cell lines: CHLA-01-MED, CHLA-01R-MED, UW-228-2, D425MED, CHLA-259, D283MED; ATRT cell lines: CHLA-05-ATRT, CHLA-06-ATRT, BT-12. Adipocytes and pre-adipocytes, known to express PID1, were included as positive controls. A lane with U87 lysate was included on each of the three blots (performed in different experiments) to allow comparison. ECV304 bladder carcinoma lysate was also included for comparison; **B)** Western blot of lysates from U87 cells used for Fig 4E showing 30% decrease in PID1 protein by siPID1 using densitometry. Quantitative RT-PCR showed similar decrease in *PID1* mRNA in siPID1 samples (not shown). Since FACS measurements gated on the cells that incorporated the FAM-label (~30%) whereas the western blot and qRT-PCR measured PID1 in the whole population of cells, including those that did not incorporate the siRNAs, the PID1 knockdown in the FAM-labeled cells was likely more profound than reflected in the measurements of the whole population.
Supplemental Fig 6: PID1 diminishes phosphorylation of AKT and ERK in D283MED medulloblastoma cells grown in presence of serum. Representative flow cytometry tracing of phospho-AKT and phospho-ERK in eGFP-expressing cells from the samples comprising Fig 4G. The gray tracings are of cells transfected with pCIENS control vector and the white tracings are of pCIENS-PID1 transfected cells and are shifted to the left.