Title: PDGF Engages an E2F-USP1 Signaling Pathway to Support ID2-mediated Survival of Proneural Glioma Cells

Running title: A PDGF-E2F-USP1-ID2 axis is required for proneural glioma

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Abstract:

Glioblastoma (GBM) is the most aggressive primary brain tumor and responds poorly to currently available therapies. Transcriptomic characterization of GBM has identified distinct molecular subtypes of GBM. Gain-of-function alterations leading to enhanced platelet-derived growth factor (PDGF) signaling are commonly observed in the proneural subtype of GBM and can drive gliomagenesis. However, little is known about the downstream effectors of PDGF signaling in GBM. Using a mouse model of proneural glioma and comparative transcriptomics, we determined that PDGF signaling upregulated ubiquitin specific peptidase 1 (Usp1) to promote the survival of murine proneural glioma cells. Mechanistically, we found that PDGF signaling regulated the expression of the E2F transcription factors, which directly bound to and activated Usp1. Furthermore, PDGF-mediated expression of USP1 led to the stabilization of Inhibitor of DNA-binding 2 (ID2), which we found to be required for glioma cell survival. Genetic ablation of Id2 delayed tumor-induced mortality, and pharmacological inhibition of USP1, resulting in decreased ID2 levels, also delayed tumorigenesis in mice. Notably, decreased USP1 expression was associated with prolonged survival in patients with proneural GBM, but not with other subtypes of GBM. Collectively, our findings describe a signaling cascade downstream of PDGF that sustains proneural GBM cells, and suggest that inhibition of the PDGF-E2F-USP1-ID2 axis could serve as a therapeutic strategy for proneural GBM featuring increased PDGF signaling.
**Introduction:**

GBM is the most lethal and aggressive primary brain tumor. Despite expansive diagnostic efforts and aggressive combination therapy, including surgery, ionizing radiation, and chemotherapy, the median survival of patients with GBM remains a modest 12-15 months (1,2). Although described as a single histopathological entity, transcriptomic profiling of GBM has led to the identification of multiple subtypes (3,4), with the latest report identifying four subtypes: proneural, neural, classical, and mesenchymal GBM (5). Each of these subtypes has an associated profile of genetic alterations (5,6). Proneural GBM, which is resistant to current therapy, is characterized by frequent amplifications in the genomic region encoding the receptor tyrosine kinase (RTK), platelet-derived growth factor receptor α (PDGFRA) (5). Multiple studies indicate that increased PDGF signaling is sufficient to drive gliomagenesis in murine models, emphasizing the importance of PDGF signaling in GBM (7-12). Transcriptomic characterization of PDGF-induced high-grade glioma in mice revealed a proneural GBM molecular signature, indicating that increased PDGF signaling is a driver of proneural gliomagenesis (11,12). Little is known, however, about the precise molecular pathways downstream of PDGF signaling causing malignancy in proneural glioma.

Among the cellular processes that are critical for GBM pathology, protein degradation is emerging as an important regulator of tumorigenesis and a target for cancer treatment (13-15). Ubiquitination and its reverse, deubiquitination, are major regulatory pathways that control protein degradation and contribute to cell cycle regulation, stem cell maintenance, and cellular survival, as well as other biological activities of importance for tumorigenesis (16,17). Although it is known that the cellular localization, post-translational modification, and altered structure of proteins can regulate their degradation (18), the repertoire of molecules that regulates the
destruction of specific targets is incompletely known. Deubiquitinases (DUBs), which include over 100 genes classified into five subgroups, catalyze the removal of ubiquitin molecules that are covalently bound to a substrate, thus diminishing its proteasomal degradation (17). Recent work has identified a role for USP1, a member of the ubiquitin specific peptidase (USP) group of DUBs, in the regulation of tumor pathogenesis (19-21) including GBM (22). Inhibition of USP1 by multiple strategies has been suggested as a potential strategy to enhance the therapeutic efficacy of chemotherapy and radiation in treating cancer (22-24). USP1, upon forming a molecular complex with UAF1 (WDR48), deubiquitinates substrates including PCNA, FANCI, FANCD2, and the short-lived Inhibitor of DNA Binding (ID) proteins ID1, ID2, and ID3 that each have a half-life of about 10 minutes (19,20,25-28). Members of the Id gene family, including Id2, have been proposed as mediators of GBM pathology and are known to be highly expressed in a number of different tumor types (28-30).

While studying the molecular events underlying the critical role of PDGF signaling in proneural glioma, we confirmed the regulation of multiple gene groups by PDGF signaling and discovered a previously unknown role for PDGF signaling in the regulation of protein degradation. We found that PDGF regulates the transcription of molecules key for protein degradation including E3 ubiquitin ligases, ubiquitin and SUMO (small ubiquitin-like modifier) related genes, and DUBs, among which we found USP1 to be critical for survival of PDGF-driven glioma spheroid cells (referred to as PDGF-GSCs in this study). Additionally, we found that PDGF signaling drives the expression of USP1 by upregulating the expression of E2f1, E2f2, and E2f3 (E2f1-3), transcription factors that interact with the retinoblastoma protein (pRB), and thereby increases their interaction with the Usp1 promoter. Furthermore, we determined that ID2 is a critical USP1 substrate that is required for survival in PDGF-GSCs. Targeting the PDGF-E2F-USP1-ID2 axis
by deletion of the Id2 gene or pharmacologic inhibition of USP1 delayed tumorigenesis of PDGF-driven glioma. Importantly, data from The Cancer Genome Atlas (TCGA) suggest the presence of a PDGF-USP1 axis in proneural GBM, and that USP1 is a prognostic factor in proneural GBM, but not in other subtypes of GBM. These results suggest that targeting the PDGF-E2F-USP1-ID2 axis can be a beneficial treatment strategy for proneural glioma. These findings may be applicable to other gliomas in which alterations in PDGF signaling are common including pediatric high-grade glioma and diffuse intrinsic pontine glioma (DIPG) as well as to tumors that share the pathobiology of being driven by PDGF signaling though occurring at other sites (31,32).

Materials and Methods:

Cell Culture

PDGF-GSCs were cultured in petri dishes in GSC media (DMEM/F12 without L-Glutamine (Corning) supplemented with 1% Penicillin/Streptomycin and 1X B27 (Gibco) without vitamin A). PDGF-GSCs were dissociated following incubation in Accutase (Millipore) for 5 minutes at 37°C. Dox (100 ng/ml) (Sigma), Pimozide (5 µM) (Sigma), Palbociclib Isethionate (5 µM, PD0332991, PD) (Selleckchem), and AG1295 (10 µM) (Santa Cruz Biotechnology) were added to cultures for times indicated in the figure legends. MG132 (1 µM) (Sigma) was added to cultures for 1 hour or 30 minutes when treating in combination with Dox or AG1295 respectively. Primary astrocytes and primary MEF cells were grown on tissue culture dishes in growth media (GM) (DMEM with L-glutamine (Corning) supplemented with 1% Penicillin/Streptomycin (Corning) and 10% Fetal Bovine Serum (Corning)).

Transfections

PDGF-GSCs were transfected using LipoD293 (Signagen) as per product guidelines.
AnnexinV/PI Flow Cytometry Analysis

AnnexinV/PI staining was performed using the annexinV/PI Apoptosis Detection Kit II (BD Pharmingen) following the manufacturer’s guidelines. The FL1 channel was used to detect annexinV-FITC and the FL3 channel was used to detect PI.

Western Blot Analysis

Western Blot Analysis was done as previously described (33). The antibodies used and the working dilutions are shown in Supplementary Table 1. Antibodies that recognize ID proteins were validated in a previous report from our laboratory (33).

RNA Extraction and qRT-PCR

For microarray analysis, RNA was purified using the Trizol method. For qRT-PCR, RNA was extracted using the RNeasy kit as per product guidelines (Qiagen). Data was quantified relative to untreated control in each GSC culture, and normalized either only to 18S Ct values, or 18S Ct values followed by another normalization to \(Id2\) Ct values (Fig. S6), using the \(2^{-\Delta\Delta Ct}\) method. Primers are provided in Supplementary Table 2.

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP was performed as previously described (33). Data were analyzed using the \(2^{-\Delta\Delta Ct}\) method with normalization to the IgG isotype control ChIP of cells that were treated in a manner identical to the experimental cells. Primer sequences are provided in Supplementary Table 2.

Lentiviral Infection

Lentiviruses were prepared using 293FT cells using an optimized Signagen protocol. A three vector second generation system using pCMV-Delta8.9, pVSVG, and the pLKO.1 transfer vector was used. 293FT cells were transfected with the following ratio of plasmid DNA: 6.5:4.5:10 (pCMV-Delta8.9, VSV-G, pLKO.1 transfer vector respectively). After overnight incubation, the
transfected cells were washed gently with 1X PBS without calcium or magnesium and incubated in GSC media for 48 hours to prepare a lentiviral supernatant. Conditioned media was collected, spun down at 210g for 5 minutes, filtered through a 0.45µm filter (Millipore), and then aliquoted and frozen at -80ºC. Mission pLKO.1-puro vectors encoding shRNA sequences were purchased from Sigma. ShRNA sequences are available in Supplementary Table 3. Mission SHC002 was used as a mammalian non-target shRNA control (ShControl) (Sigma).

Statistics

GraphPad Prism 6 was used for statistical analysis. Two-tailed student T-test was used to evaluate differences between two groups. For experiments involving multiple comparisons, we performed one-way analysis of variance (ANOVA) with the Tukey post-hoc test to evaluate differences. We evaluated bivariate correlations using the Pearson correlation test. 2-way ANOVA test with Sidak’s multiple comparison was performed to evaluate significant differences in the mouse Pimozide treatment experiment (Fig. 5F). Log-rank (Mantel-Cox) was used to perform significance analysis on survival curves with 2 groups. We examined survival curves with more than 2 groups using the Log-rank (Mantel-cox) and the log-rank test for trend.

Complete methods can be found in the SI Materials and Methods.
Results:

**PDGFB-driven gliomas in mice belong to the proneural subtype and are addicted to PDGF signaling.**

To study the molecular determinants of malignancy downstream of PDGF signaling in proneural glioma, we used a previously characterized model from our laboratory (*Gfap*-tTa/Tre-*PDGFB*). In this model, human PDGFβ (PDGFB) was expressed under the control of a Tet-off system driven by the glial fibrillary acidic protein (*Gfap*) promoter. High-grade glioma (glioma grade III-IV) developed in these mice upon doxycycline hyclate (Dox) removal (Fig. S1) (8). In characterizing these mice, we performed transcriptomic analysis of glioma tissues and PDGF-GSCs derived from this model. Our analysis revealed that these mouse glioma and PDGF-GSCs belong to the proneural subtype. A heatmap of Spearman r correlation scores demonstrates this correlation (Fig. 1A). Further analysis using hierarchical clustering of Spearman correlation scores (Fig. S2A), as well as a heatmap of expression values of the GBM subtyping signature gene set (11) (Fig. S2B), and clustering of these expression values (Fig. S2C) confirmed this interpretation. We evaluated PDGF-GSCs derived from tumors arising in three different mice and referred to them as GSC1, GSC2, and GSC3 in this study. We first tested if the expression of PDGFB in PDGF-GSCs was still responsive to Dox. Dox addition to the media of PDGF-GSCs suppressed PDGFB mRNA (Fig. 1B). Consistent with this finding, the level of phosphorylated Platelet-derived Growth Factor Receptor (pPDGFR) was reduced when PDGF-GSCs were treated with Dox (Fig. 1C). We found decreased cellular proliferation (Fig. 1D,E) and decreased sphere size (Fig. 1F) following treatment of PDGF-GSCs with Dox or AG1295, a chemical inhibitor of pPDGFR. To determine if the decrease in cell number in these cultures was
associated with cellular death, we performed annexinV/PI staining, and found that both Dox and AG1295 treatment induced cellular death (Fig. 1G,H).

**PDGF signaling regulates Usp1 expression to promote survival of PDGF-GSCs.**

To discover the molecular determinants of malignancy downstream of PDGF signaling in proneural glioma, we performed comparative transcriptomic analysis on untreated and Dox treated PDGF-GSCs. We detected 1706 genes that were significantly regulated by PDGF signaling (Fig. 2A). Of these, 885 genes were upregulated and 821 were downregulated. As expected, gene ontology annotation of these genes revealed that cell cycle and proliferation related genes constituted the major downregulated gene group in cells following PDGF signaling inhibition (Fig. 2B). Interestingly, a small percentage of genes regulating ubiquitination and protein degradation were downregulated in cells in which PDGF signaling had been inhibited. We sought to explore this previously unknown finding that suggests a role for PDGF signaling in the regulation of protein degradation. Of the genes we found in the ubiquitination and protein degradation group, *Usp1*, a negative regulator of ubiquitination, was the gene in the USP family most significantly regulated by PDGF (data not shown) and had approximately a 3-fold decrease following the inhibition of PDGF signaling in PDGF-GSCs. Further, *USP1* was highly expressed in human GBM relative to normal brain tissue (Fig. S3A), and was variably expressed in the different GBM subtypes (Fig. S3B). Importantly, USP1 can be therapeutically targeted by Pimozide (23,27), an antipsychotic drug of the diphenylbutylpiperidine class that has limited toxicities, has been approved for use in humans, and can cross the blood-brain barrier (34). These characteristics make USP1 a possible therapeutic target in GBM. Hence, we focused our study on USP1.

We first validated the results of our microarray. Inhibition of PDGF signaling by Dox
significantly decreased *Usp1* mRNA (Fig. 2C) and protein (Fig. 2D) expression in PDGF-GSCs. Importantly, exposure of neural stem cells (NSCs) to recombinant PDGF-B increases USP1 mRNA (Fig. S4A) and protein (Fig. S4B) expression. Further, *Usp1* promoter activity, which was high in PDGF-GSCs, was significantly decreased when PDGF signaling was blocked by AG1295 (Fig. 2E). These findings show that PDGF signaling can upregulate *Usp1* expression. Inhibition of USP1 has been previously shown to decrease proliferation of leukemia cells and spheroid formation of human GBM cells (22,27). To examine if *Usp1* affects the survival of PDGF-GSCs, we infected these cells with lentiviruses encoding *Usp1* targeting shRNAs (short hairpin RNAs) or a non-target control (ShControl) and performed annexinV/PI staining. We found that decreased USP1 expression led to cell death of PDGF-GSCs (Fig. 2F,G).

**PDGF signaling drives USP1 expression by regulating the expression of E2F1-3 and their binding to the USP1 promoter.**

To explore the mechanism by which PDGF signaling drives the expression of *Usp1*, we first analyzed the 1KB DNA sequence 5’ to the first exon of *Usp1* using the decipherment of DNA elements (DECODE) algorithm (http://www.sabiosciences.com/chipqpcrsearch.php). We found multiple transcription factor binding motifs in this promoter region including those corresponding to the PAX-6, NKX5-1, LUN-1, NF-Y, and the E2F factors. Among these transcription factors, only the E2F transcription factors E2F1-3 were regulated by PDGF signaling in our microarray analysis (Fig. 2A-B, Fig. 3A). The E2F transcription factor binding site motifs we identified in the mouse *Usp1* promoter are conserved in the human *USP1* promoter (data not shown). We validated our microarray results by determining that *E2f1-3* mRNA (Fig. 3B) and protein (Fig. 3C) levels were lower in PDGF-GSCs following treatment with Dox. Further, we found that E2F1-3 bind the *Usp1* promoter only during PDGF exposure...
(Fig. 3D). When we inhibited the activity of E2F1-3 by inducing active pRB using the cyclin dependent kinase 4/6 (CDK4/6) inhibitor Palbociclib (PD0332991, PD) in PDGF-GSCs, USP1 mRNA (Fig. 3E) and protein (Fig. 3F) levels decreased even in the presence of PDGF signaling. To determine the E2F binding motifs located in the Usp1 promoter at which E2F1-3 binding is specifically regulated by PDGF signaling, we sequentially deleted the 1KB Usp1 promoter used in our expression construct (Fig. 2E), 200bp at a time from the 5’ end. We then tested the activity of these constructs in the presence or absence of PDGF signaling. We found that the activity and responsiveness of the promoter reporter construct to PDGF inhibition by AG1295 was unchanged despite our trimming the promoter region to 0.8KB, 0.6KB, and 0.4KB. Unexpectedly, these promoter constructs had enhanced activity when compared to the 1KB promoter construct. Of particular note, however, was our finding that the 0.2KB promoter construct was still responsive to pPDGFR inhibition, although it had lower activity than the 0.4KB promoter (Fig. 3G). We concluded that the critical transcription factor binding motifs that mediate PDGF regulation of USP1 expression driven by PDGF signaling must be present in the 0.4KB promoter segment. Within this fragment were two E2F binding motifs, one of which was located in the 0.2KB promoter segment as well. A summary of the promoter segments we used and the location of the E2F binding motifs in them can be found in Fig. S5.

Deletion of the E2F binding motif in the 0.4KB proximal to the first exon of the Usp1 gene (Del-E2F(1)), but not deletion of the distal E2F binding motif (Del-E2F(2)), rendered this promoter construct inactive and it was not responsive to AG1295 (Fig. 3H) or PD (Fig. 3I). Collectively, these results indicate that regulation of Usp1 expression by PDGF signaling is driven by enhanced expression of E2f1-3 and the binding of these factors to an E2F binding motif within 200bp of the first Usp1 exon.
**PDGF signaling stabilizes ID2 by regulating USP1 to promote survival of PDGF-GSCs.**

We sought to identify molecules responsible for USP1 mediated survival in PDGF-GSCs. ID proteins are known post-translational targets of USP1 (26,27), hence we examined whether they were regulated by PDGF signaling in a manner that parallels USP1 regulation. Inhibition of PDGF signaling by Dox led to a decrease in ID2 protein, and an inconsistent decrease in ID1 protein levels (Fig. 4A). ID3 and ID4 proteins were not expressed at detectable levels in these cells. The mRNA expression of Id genes was not downregulated by Dox (Fig. 4B, Fig. S6). Since ID2 was consistently regulated in PDGF-GSCs and was expressed at a higher level than Id1 at the mRNA level (Fig. S6), we focused on ID2. We treated cells with the combination of a proteasome inhibitor, MG132, and either Dox or AG1295 to evaluate ID2 degradation in the absence of PDGF signaling. We found that the decrease in ID2 observed following inhibition of PDGF signaling was primarily a result of proteasomal degradation (Fig. 4C,D). Consistent with these data, we observed that ID2 was ubiquitinated in PDGF-GSCs when PDGF signaling was inhibited (Fig. 4E). To determine if ID2 was a target of USP1 stabilization in PDGF-GSCs, we infected these cells with lentiviruses encoding USP1 specific ShRNAs or a ShControl and compared the levels of ID2 following infection. We found that decreased USP1 expression led to a decrease in ID2 protein levels (Fig. 4F). USP1 has been shown to bind ID2 and cleave ubiquitin molecules covalently bound to ID2 (26). We evaluated whether USP1 interacts with ID2 in PDGF-GSCs and whether this interaction was regulated by PDGF signaling. We found that USP1 binds ID2 when PDGF-GSCs are stimulated by PDGF (Fig. 4G). We then checked whether ID2, like USP1 (Fig. 2F,G), is important for the survival for PDGF-GSCs. We infected PDGF-GSCs with lentiviruses encoding Id2 specific ShRNAs and determined that decreased ID2 levels led to cell death of PDGF-GSCs (Fig. 4H-J). Our results provide evidence that ID2 is post-
translationally stabilized by PDGF-mediated regulation of ID2 ubiquitination through USP1 to support tumor cell survival.

**Deletion of Id2 delays tumorigenesis in a mouse model of PDGF-driven glioma.**

We sought to determine if loss of Id2 has an impact on mortality induced by PDGF-driven glioma *in vivo*. We developed an *Id2<sup>fl/fl</sup>* mouse and crossed it to a previously reported transgenic mouse in which the *Gfap* promoter, the same promoter element driving gliomagenesis, regulated Cre recombinase expression (*Gfap-Cre*) (35). From this cross we obtained *Gfap-Cre/Id2<sup>fl/fl</sup>* mice that conditionally delete *Id2* in *Gfap* expressing cells such as postnatal NSCs (36) and astrocytes (37), and we verified that such a deletion occurs (Fig. S7). We then crossed the *Id2<sup>fl/fl</sup>* mouse or the *Gfap-Cre/Id2<sup>fl/fl</sup>* into the *Gfap-tTa/Tre-PDGFB* mouse model. From these crosses we obtained mice that carried the Dox regulatable *PDGFB* allele and *Id2* that was either expressed normally (designated *Gfap-tTa/Tre-PDGFB/Id2<sup>fl/fl</sup>*), or deleted in *Gfap* expressing cells (designated *Gfap-tTa/Tre-PDGFB/Id2<sup>fl/fl</sup>/Gfap-Cre*) (8). We maintained these mice on Dox throughout development *in utero* and postnatally until day 20 (Dox+/−). We found that deletion of *Id2* significantly enhanced median survival by ~100 days (Fig. 5A,B). Mice bearing the genotypes described above did not develop tumors when Dox was continuously provided in drinking water (Dox+/+) (Fig. 5A,C). Tumor incidence was very similar in the mice studied (Fig. 5C). These findings indicate that loss of *Id2* can significantly delay tumor induced mortality in mice engineered to develop PDGF-driven proneural glioma.

**Pimozide inhibits USP1 mediated ID2 stabilization and delays tumorigenesis of PDGF-driven glioma.**
Our results provide strong evidence that a PDGF-E2F-USP1-ID2 axis is important for PDGF-driven glioma. We next examined whether pharmacological inhibition of this axis, achieved by inhibition of USP1 by Pimozide, can affect PDGF-driven glioma. We first treated PDGF-GSCs with Pimozide and examined ID2 protein levels in these cells. Inhibition of USP1 by Pimozide led to decreased ID2 protein levels (Fig. 6A) and induced cell death in PDGF-GSCs (Fig. 6B,C). We compared the sensitivity to Pimozide of PDGF-GSCs, GSCs derived from a v-erb-B/Trp53-/− glioma model (38), and astrocytes and mouse embryo fibroblasts (MEFs) from wildtype mice. We found that PDGF-GSCs are significantly more sensitive to Pimozide than the other cell types including EGF-driven glioma cells which represent other subtypes of GBM (Fig. 6D,E). We then determined whether Pimozide could inhibit PDGF-driven glioma in vivo. We implanted syngeneic grafts of PDGF-GSCs subcutaneously in C57BL/6J mice and, upon palpable tumor detection, gavaged the mice with Pimozide (60 mg/kg) or a vehicle control three times a week. Treatment with Pimozide significantly delayed the growth of PDGF-driven gliomas in these mice (Fig. 6F-I). We measured the total body weight of mice that were treated with Pimozide and the vehicle control and did not observe a decrease in the weight of mice receiving Pimozide during the course of treatment (Fig. 6J). To determine if Pimozide administration decreased ID2 in PDGF-driven gliomas in vivo, we performed a western blot analysis of ID2 in lysates from eight independent tumors derived from animals treated with seven treatments of Pimozide or drug vehicle. Our results show that Pimozide administration decreased ID2 protein levels in PDGF-driven glioma (Fig. 6K). These data indicate that the PDGF-E2F-USP1-ID2 pathway is critical for PDGF-driven glioma and raises the possibility that Pimozide could contribute to the clinical management of PDGF-driven glioma.
**USP1** mRNA expression correlates with **PDGFRA** mRNA expression and is associated with survival of patients with proneural GBM.

To examine if expression levels of **PDGFRA** and **USP1** are correlated in GBM tissues and if **USP1** expression is associated with the survival of patients with GBM, we analyzed gene expression data from TCGA. In a cohort of 206 patients (39), we found that mRNA expression of **PDGFRA** and **USP1** correlated directly in all GBM patients analyzed (Fig. 7A). When we analyzed this correlation in the four GBM subtypes, we found that **USP1** expression correlated with **PDGFRA** expression only in the proneural subtype (Fig. 7A). Correlation analysis of **EGFR** and other RTKs (Supplementary Table 6) with **USP1** showed no direct correlation. We then sought to determine if **USP1** expression was associated with the overall survival of human GBM patients. We found that patients in the proneural subtype whose tumors had low **USP1** expression had a significantly increased overall survival when compared to the remaining proneural GBM patients (Fig. 7B). This association was not observed for other GBM subtypes (Fig. 7B). Taken together, these data demonstrate the correlation of **PDGFRA** and **USP1** mRNA levels in GBM patients, particularly in proneural GBM, and reveal that low **USP1** mRNA expression is associated with better overall survival of GBM patients.

**Discussion:**

The promise of precision medicine for oncology is that therapeutic interventions tailored to target pathways functionally required by individual tumors will provide more effective and less toxic cancer treatments. We found a therapeutically targetable signaling axis critical for survival of PDGF-GSCs in a mouse model of PDGF-driven proneural glioma and evidence of this axis in human proneural GBM. The **Gfap**-tTa/Tre-**PDGFB** mouse we studied, like other mouse models of PDGF-driven glioma, displays a molecular signature similar to human proneural GBM (Fig. 1
and Fig. S2) (11,12). Importantly, GSCs derived from our model required PDGF signaling for survival, providing a unique reagent for use in dissecting the critical role of PDGF in these gliomas (Fig. 1). In evaluating gene networks regulated by PDGF signaling, we found several gene groups involved in metabolism, proliferation and cell cycle, signal transduction, apoptosis, and protein degradation that were differentially regulated by PDGF. While the importance of growth factors for metabolism and proliferation has been widely recognized (40), few reports link growth factor signaling to regulation of protein degradation (41,42). The novelty of this finding stimulated our interest in studying these genes. We found and characterized a previously unknown role for PDGF signaling in the regulation of protein degradation and in supporting tumor cell survival, which had not been previously described (Fig. 2). We found that USP1, a therapeutically targetable DUB, is regulated by PDGF signaling (Fig. 2). Importantly, this pathway is also active in NSCs, which are known to be glioma initiating cells, and suggests a possible mechanism by which PDGF signaling can drive gliomagenesis (Fig. S4). PDGF stimulation led to an increase in Usp1 expression to promote tumor cell survival. These findings are consistent with recent work of others who reported a role for USP1 in the maintenance of human GBM-derived GSCs (22), and extend those findings by defining a mechanism by which survival is mediated and a recognizable glioma subgroup in which USP1 is likely to be an efficacious therapeutic target.

The E2F family of transcription factors encodes activators and repressors. E2f1-3 are transcriptional activators that control cell cycle progression (43). We found that PDGF signaling drives the expression of E2f1-3 leading to enhanced binding to the USP1 promoter and causing increased Usp1 expression. These results link transcription driven by E2F activator proteins to
regulation of protein ubiquitination and to tumor cell survival in the context of PDGF-driven proneural glioma.

Our findings indicating that USP1 is required for tumor cell survival suggested that substrates of USP1 could be important for maintaining the survival of PDGF-GSCs. The ID family of transcriptional regulators has been implicated in several aspects of tumorigenesis and in the pathology of glioma (28,29). Given that ID1, ID2, and ID3 are known to be deubiquitinated and stabilized by USP1 (26), we hypothesized that these USP1 substrates could be important for maintaining PDGF-GSC survival. While previous studies have described regulation of Id gene mRNA expression by PDGF signaling in human and mouse fibroblasts (44,45), we found that PDGF signaling did not upregulate *Id* gene mRNA expression in PDGF-GSCs (Fig. S6). PDGF signaling, however, did increase protein levels of ID2 by regulating *Usp1* to decrease ID2 ubiquitination and subsequent proteasomal degradation (Fig. 4). Consistent with the role of USP1 in mediating PDGF-GSC survival (Fig. 2), inhibition of *Id2* expression by shRNA indicated that ID2 also was required for survival of PDGF-GSCs (Fig. 4). This observation suggests strongly that other molecules in the same pathway as ID2 and USP1 could be important for PDGF-GSC survival, and may have potential as therapeutic targets. Interestingly, CDC20, an E3 ubiquitin ligase, a component of the anaphase-promoting complex (APC), which is known to ubiquitinate ID2 (46), was downregulated when PDGF signaling was inhibited suggesting that other ubiquitin ligases may ubiquitinate ID2 in PDGF-driven glioma.

With the importance of ID2 for the survival of PDGF-GSCs *in vitro* being clear (Fig. 4), we sought to examine the *in vivo* role of *Id2* in PDGF-driven glioma. Deletion of *Id2* in tumor cells led to a significant increase in the duration of survival of mice who develop glioma without expression of *Id2* (Fig. 5), but did not fully eliminate tumor formation in our model. We have not
yet pursued this finding, but several explanations are worthy of consideration. Some, such as incomplete Cre recombination \textit{in vivo}, seem less important than others. Other may be more important, such as detection of redundancy in ID activity. In cells without \textit{Id2}, the presence of dysregulated PDGF during tumorigenesis may facilitate the selection of cells expressing other \textit{Id} genes, such as \textit{Id1}. Such a finding would be consistent with the observations of others that in a transgenic PDGF-driven glioma mouse model loss of \textit{Id1} led to increased overall survival (47). Furthermore, the development of tumors in all animals suggests that \textit{ID2} may be able to contribute to different stages of tumor development (48). We interpret the finding of delayed death in animals deleting \textit{Id2} in \textit{Gfap} expressing cells as indicating an important role for \textit{Id2} in tumor progression.

Our findings indicating that a PDGF-E2F-USP1-ID2 axis is required for the survival of PDGF-GSCs raises the possibility of a therapeutic benefit from inhibition of USP1 in patients with PDGF-driven glioma. We used Pimozide, a drug that is known to inhibit USP1 (23), is active in the CNS (34), and approved for human use as an anti-psychotic drug (34) to examine pharmacological targeting of the PDGF-E2F-USP1-ID2 axis in established glioma. \textit{In vitro}, PDGF-GSCs displayed a much higher sensitivity to Pimozide than normal cells or GSCs derived from a mouse brain tumor model not driven by PDGF. \textit{In vivo}, inhibition of USP1, by Pimozide, led to decreased ID2 levels and delayed the growth of PDGF-driven glioma in these tumors (Fig. 6). Using a subcutaneous syngeneic model to monitor critically the effect of USP1 inhibition \textit{in vivo}, we found that tumor growth decreased suggesting the potential of using Pimozide in treating brain tumor patients, especially since Pimozide is known to cross the blood-brain barrier (34). Future experimentation using orthotopic models of PDGF driven glioma will provide additional opportunities to examine such an effect in further detail. Our data are consistent with
the significant correlation between PDGFRA and USP1 expression in human GBM specimens from the TCGA database, and with our finding that USP1 expression may be of prognostic value for patients with proneural GBM (Fig. 7). In patients with proneural GBM, we posit that low expression of USP1 could lead to low levels of ID2 protein; decreased tumor cell survival; and as shown by others, increased tumor cell sensitivity to radiation therapy (22). Parallel in vitro studies using PDGF-responsive cultures derived from human proneural PDGF-driven GBM will allow further exploration of this signaling cascade. Importantly, the molecular pathway described here also might be implicated in other tumors in which enhanced PDGF signaling has been characterized. Such tumor types include pediatric high-grade glioma (32), diffuse intrinsic pontine glioma (32), myeloid tumors (49), GIST (50), and others (31). Pimozide administration might have therapeutic activity in patients with these tumor types. Our data provide strong preclinical evidence that Pimozide administration may have therapeutic activity in patients with proneural glioma through inhibition of the PDGF-E2F-USP1-ID2 axis.

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References

Figure Legends:

Figure 1. PDGFB-driven gliomas in mice belong to the proneural subtype and are addicted to PDGF signaling. (A) Heatmap of correlation (Spearman) r values of GBM subtyping signature genes in mouse and human samples (TCGA) with human GBM subtype specific centroid values. Full details of analysis are available in SI Materials and Methods (dataset in Supplementary Table 4). (B) PDGFB mRNA levels and (C) western blot analysis of PDGFRA and pPDGFR in lysates of PDGF-GSCs treated with Dox (48h). (D) Growth curves of GSCs treated with Dox or (E) with AG1295 compared to mock treated cells. (F) Phase contrast images of PDGF-GSCs treated with DMSO, Dox, or AG1295 for four days. (G) Pseudo-colored flow cytometry evaluation of annexinV/PI stained GSC1 treated with DMSO, Dox, or AG1295 as indicated and (H) quantification of live cells (annexinV⁻/PI⁻) in these cultures. Mean (B,D,E) ± 1 standard deviation (SD) or mean (H) ± 95% confidence interval (CI) of 3 independent experiments are shown. *** p <0.0005.

Figure 2. PDGF signaling regulates USP1 expression to promote survival of PDGF-GSCs. (A) Heatmap representation of unsupervised hierarchical clustering of Z-score values of all significantly regulated genes, as defined in SI Materials and Methods, in GSC1 following Dox treatment for 48 hours (n=3 independent replicates in each treatment arm, dataset in Supplementary Table 5). (B) Pie charts describing the general overview of Gene Ontology classes upregulated or downregulated in Dox treated GSC1 cells. The top four downregulated genes in the ubiquitination and protein degradation class are shown below the displaced red fraction. (C) USP1 mRNA and (D) protein levels determined by western blot analysis of lysates of PDGF-GSCs treated with Dox (48h). (E) Evaluation of Usp1 promoter activity in PDGF-GSCs treated with AG1295 as indicated in SI Materials and Methods. (F) Pseudo-colored flow
cytometry evaluation of annexinV/PI stained PDGF-GSCs transduced with lentiviruses encoding ShControl or Usp1 targeting ShRNAs (ShUSP1A and B) and (G) quantification of live cells (annexinV-/PI-) in these analyses. Mean (C) ± 1 SD or mean (E,G) ± 95% CI of 3 independent experiments are shown. *** p<0.0005

Figure 3. PDGF signaling drives USP1 expression by regulating the expression of E2F1-3 and their binding to the USP1 promoter. (A) Heatmap representation of Z-score values for E2F1-3 genes in GSC1 following Dox treatment for 48 hours. Data are from the microarray shown in Fig. 2A. (B) E2F1-3 mRNA and (C) protein levels in PDGF-GSCs treated with Dox (48h). (D) ChIP analysis using antibodies specific for E2F1, E2F2, and E2F3 to determine binding of E2F1-3 to the USP1 promoter in PDGF-GSCs following Dox treatment for 48 hours. (E,F) Effect of palbociclib (+PD, 5µM, 24h) treatment on (E) USP1 mRNA and (F) protein levels of USP1, pPDGFR, pRB, and p-pRB. (G) Evaluation of the activity of truncated Usp1 promoter constructs in PDGF-GSCs treated with AG1295 (10µM) as indicated in SI Materials and Methods. (H) Evaluation of the activity of the 0.4KB Usp1 promoter construct (WT) or this same construct in which either the first or second E2F binding site was deleted in PDGF-GSCs treated with AG1295 (10µM) or (I) palbociclib (PD) (5µM) as indicated in SI Materials and Methods. Mean (B,D,E,G,H,I) ± 1 SD of 3 independent experiments is shown. N.S. non-specific. *** p<0.0005

Figure 4. PDGF signaling stabilizes ID2 by regulating USP1 to promote survival of PDGF-GSCs. (A) Western blot analysis of ID family members and (B) evaluation of Id2 mRNA levels in lysates of PDGF-GSCs treated with Dox (48h). (C) Western blot analysis of pPDGFR and ID2 and (D) ID2 in lysates of PDGF-GSCs treated with indicated drugs. Dox and AG1295 were added for 24 hours. (E) Western blot analysis of ubiquitin in anti-ID2 or control IgG immunoprecipitates from lysates of GSC1 that were treated with indicated drugs. Dox was added
for 24 hours. Duration of MG132 treatment is indicated in Materials and Methods. (F) Western blot analysis of USP1 and ID2 in GSC1 infected with ShControl and ShUSP1A and B lentiviruses. (G) Western blot analysis of USP1 in anti-ID2 or control IgG immunoprecipitates of lysates from GSC1 that were treated with Dox for 48 hours. (H) Western blot analysis of ID2 in lysates of GSC1 cells infected with lentiviruses encoding ShControl or Id2 targeting ShRNAs (ShId2A, ShId2B). (I) Pseudo-colored flow cytometry evaluation of annexinV/PI stained GSC1 transduced with ShControl and ShId2A/B lentiviruses and (J) quantification of live cells (annexinV/PI−) in these cultures. Mean (B) ± 1 SD or mean (J) ± 95% CI of 3 independent experiments are shown. *** p<0.0005.

Figure 5. Deletion of Id2 delays tumorigenesis in a mouse model of PDGF-driven glioma. (A) Kaplan Meier survival curve of the mice with indicated genotypes treated from the time of conception throughout their life with Dox (Dox+/+) or treated with Dox from the time of conception until weaning, postnatal day 20 (Dox+/-). p values reflect comparison of mice on different Dox treatment regimens to Gfap-tTa/Tre-PDGFB/Id2fl/fl mice (Dox+/-). (B) Median survival and (C) Tumor incidence at the end of experiment of mice shown in panel (A).

Figure 6. Pimozide inhibits USP1 mediated ID2 stabilization and delays tumorigenesis of PDGF-driven glioma. (A) Western blot analysis of ID2 in lysates of PDGF-GSCs treated with DMSO or Pimozide (5µM) for 24 hours. (B) Pseudo-colored flow cytometry evaluation of annexinV/PI stained PDGF-GSCs treated with DMSO or Pimozide (48h) and (C) quantification of live cells (annexinV/PI−) in these cultures. (D) Survival response of indicated cell types to Pimozide. (E) IC50 derived from data in panel (D). (*** indicates significance observed when comparing any PDGF-GSC to any other cell type included in the graph. (F) Tumor volume of implanted PDGF-GSCs (n=14 in Vehicle group, n=9 in Pimozide group). (G) Appearance, (H) volume, and (I)
weight of tumors extracted from mice described in (F) following the last Pimozide treatment. (J) Total body weight of mice with syngeneic implants of glioma receiving Pimozide or vehicle treatment regimens. (K) Western blot analysis of ID2 and USP1 in lysates from extracted tumors. All data points, mean ± 1 standard error of the mean (SEM) are shown (H,I). Mean values ± 95%CI (C), or ±1SEM (D,E,F,J) of 3 independent experiments are shown. ** p<0.005, *** p<0.0001.

Figure 7. USP1 mRNA expression correlates with PDGFRA mRNA expression and is associated with survival of patients with proneural GBM. (A) Dot plot of USP1 and PDGFRA mRNA levels in human GBM tissues. Molecular subtype, Pearson r value and p value are indicated above each graph. All data points with trend line are shown in graph. (B) Kaplan Meier survival curve showing overall survival of GBM patients grouped by USP1 expression level. Molecular subtype is indicated above the graph and p values are indicated in the graphs.
**Figure 1**

(A) Correlation (r) plot for Human TCGA GBM Patients.

(B) Relative Expression of PDGFβ in GSCs.

(C) Western Blot analysis for PDGFRA and Actin.

(D) Graph showing cell number over time for GSC-1 GSC-2 GSC-3.

(E) Graph showing cell number over time for GSC-1 DMSO AG1295 DMSO Dox AG1295.

(F) Images showing the effect of Dox and AG1295 on GSCs.

(G) Flow cytometry analysis for DMSO Dox AG1295.

(H) Bar graph showing percent live (annexinV/PI-) cells for GSCs.

**Legend:**
- **Mes.** Mesenchymal
- **Neural** Neural
- **Classical** Classical
- **Proneural** Proneural

**Color Key:**
- **DMSO** DMSO
- **Dox** Dox
- **AG1295** AG1295

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Figure 2

885 Genes Upregulated in Dox Treated Cells
- 5.43% Biological Adhesion
- 12.82% Signal Transduction
- 9.72% Neuronal Functions
- 14.46% Other
- 3.16% Ion and Nutrient Transport
- 1.83% Secretion
- 7.83% Apoptosis
- 0.57% Cellular Response To stress
- 22.10% Metabolism
- 14.71% Protein Catabolism
- 3.79% Lipid Metabolism
- 3.60% Transcription

821 Genes Downregulated in Dox Treated Cells
- 52.55% Cell Cycle and Proliferation
- 2.44% Ubiquitination and Protein Degradation
- 6.40% Protein/Nucleic Acid Transport
- 6.54% Cellular Response to Stress
- 4.32% Apoptosis
- 12.38% Nucleic/Amino Acid Metabolism
- 2.94% Transcription
- 12.44% Other

(Log2 Change)

Cdca3 (-2.3) Uhrf1 (-2.3) Cdc20 (-2.1) Usp1 (-1.6)

C

Dox - + - + - +
Relative USP1 Expression

USP1
Actin

E

Relative Luciferase Activity

G

Percent Live
(annexinV-/PI-) Cells

annexinV
PI

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Figure 5

A) 
- \( \text{Gfap-tTa/Tre-PDGFB/Id2}^{\text{fl/fl}} \) (Dox+/-)
- \( \text{Gfap-Tta/Tre-PDGFB/Id2}^{\text{fl/fl}}/\text{Gfap-Cre} \) (Dox+/-) (p<0.0001)
- \( \text{Gfap-Tta/Tre-PDGFB/Id2}^{\text{fl/fl}} \) (Dox+/-) (p<0.0001)

B) 
- \( \text{Gfap-Tta/Tre-PDGFB/Id2}^{\text{fl/fl}} \) (Dox+/-)
- \( \text{Gfap-Tta/Tre-PDGFB/Id2}^{\text{fl/fl}}/\text{Gfap-Cre} \) (Dox+/-)

C) 
- Median Survival (Days)

- Tumor Bearing
- Normal

Dox +/-  
- \( \text{Id2}^{\text{fl/fl}} \)
- \( \text{Id2}^{\text{fl/fl}}/\text{Gfap-Cre} \)

Dox +/+  
- \( \text{Id2}^{\text{fl/fl}} \)
- \( \text{Id2}^{\text{fl/fl}}/\text{Gfap-Cre} \)
Figure 7
PDGF Engages an E2F-USP1 Signaling Pathway to Support ID2-mediated Survival of Proneural Glioma Cells

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