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Phosphorylation Regulates Id2 Degradation and Mediates the Proliferation of Neural Precursor Cells

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Abstract

Inhibitor of DNA binding proteins (Id1-Id4) function to inhibit differentiation and promote proliferation of many different cell types. Among the Id family members, Id2 has been most extensively studied in the central nervous system (CNS). Id2 contributes to cultured neural precursor cell (NPC) proliferation as well as to the proliferation of CNS tumors such as glioblastoma that are likely to arise from NPC-like cells. We identified three phosphorylation sites near the N-terminus of Id2 in NPCs. To interrogate the importance of Id2 phosphorylation, *Id2*$^{-/-}$ NPCs were modified to express wild type (WT) Id2 or an Id2 mutant protein that could not be phosphorylated at the identified sites. We observed that NPCs expressing this mutant lacking phosphorylation near the N-terminus had higher steady-state levels of Id2 when compared to NPCs expressing WT Id2. This elevated level was the result of a longer half-life and reduced proteasome-mediated degradation. Moreover, NPCs expressing constitutively de-phosphorylated

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The authors indicate no potential conflicts of interest.

Id2 proliferated more rapidly than NPCs expressing WT Id2, a finding consistent with the well-characterized function of Id2 in driving proliferation. Observing that phosphorylation of Id2 modulates the degradation of this important cell-cycle regulator, we sought to identify a phosphatase that would stabilize Id2 enhancing its activity in NPCs and extended our analysis to include human glioblastoma-derived stem cells (GSCs). We found that expression of the phosphatase PP2A altered Id2 levels. Our findings suggest that inhibition of PP2A may be a novel strategy to regulate the proliferation of normal NPCs and malignant GSCs by decreasing Id2 levels.

Keywords

Neural stem cell; Glioma; Transcription factors; Cancer stem cells; Cellular proliferation; Neoplastic stem cell biology; Tissue-specific stem cells; Progenitor cells

Introduction

Basic helix-loop-helix (bHLH) transcription factors bind as dimers to consensus sequences in DNA known as E-boxes to regulate tissue-specific gene expression and promote differentiation of several different tissues [1–3]. In addition, they inhibit cell cycle progression by inducing the expression of key target genes including $p21^{cip1/waf1}$, $p16^{INK4A}$, $p15^{INK4B}$, and $p57^{Kip2}$ [4–7]. The four Id proteins; Id1, Id2, Id3, and Id4, can heterodimerize with bHLH proteins, but lacking a basic DNA-binding domain interrupt bHLH-DNA complexes and function as dominant-negative inhibitors of bHLH proteins [8]. Each of the Id proteins regulate cell-fate determination and proliferative pathways during embryogenesis and adult tissue maintenance in many tissue types, especially those of the central nervous system (CNS) [9–13]. Among these, Id2 has been extensively studied and found to inhibit bHLH-mediated transcription and downregulate the expression of cell cycle inhibitors [6, 7]. The result of this suppression is increased proliferation and an associated decrease in differentiation [14, 15]. Id2 can also promote the G_1-S transition by binding to the retinoblastoma family proteins; Rb, p107, and p130 [16, 17].

The abundance of Id proteins is a critical determinant of whether bHLH dimers can bind to E-boxes and mediate transcriptional regulation [8, 18]. Characterizing the molecular events that modulate Id2 levels in NPCs may provide insight into how Id2 contributes to important biologic processes. Although immediate-early induction of *Id2* messenger RNA (mRNA) as the result of mitogenic stimulation has been long recognized [19–22], previous studies indicate that proteasomal degradation of Id2 may also play an important role in maintaining physiologic Id2 protein concentrations [23–26]. Id2 contains a DEAD-box domain near its C terminus, which serves as the recognition site for the anaphase promoting complex/cyclosome (APC/C), a key mediator of proteasomal degradation [25]. Upon binding, APC/C, an E3 ubiquitin ligase, catalyzes the poly-ubiquitination of proteins targeting them for degradation by the 26S proteasome. In osteosarcoma cells, this process is antagonized by USP1, a serine protease that removes ubiquitin from Id2 thereby blocking Id2 degradation [26].

Phosphorylation regulates ubiquitin-mediated proteolysis of many proteins, including transcription factors [27–30]. In this study, we evaluated the role of phosphorylation in the regulation of Id2 degradation by the proteasome. Cyclin A/Cdk2 has been shown to phosphorylate Id2 at the serine 5 position which is located in a conserved amino acid sequence, SPVR [31–33], a modification likely to be involved in the regulation of cell cycle transit. The effect of serine 5 phosphorylation on Id2 function in cells of the CNS is largely unknown, however, it is thought that phosphorylation at this residue can effect several important activities of Id2 including its ability to bind bHLH proteins, localize to the nucleus, and promote proliferation [31–33].

Dysregulated proliferation is a key characteristic of malignant cells. In addition to its well characterized role in normal NPCs, Id2 can regulate the proliferation of glioblastoma-derived stem cells (GSCs) and is expressed at a high level in human glial tumors, especially glioblastoma (GBM) [34]. GBM is the most common and most aggressive primary brain tumor of adults [35–37]. Recent studies from our lab have shown that p53, the tumor suppressor most commonly inactivated in GBM, suppresses Id2 [15] and that forced expression of Id2 in primary mouse NPCs is sufficient for the development of gliomas when cells are engrafted into a microenvironment with high levels of PDGF [38]. Whether or not Id2 is post-translationally modified in GSCs is unknown.

To understand better whether Id2 can be phosphorylated in NPCs and whether this post-translational modification regulates Id2 degradation by the proteasome we conducted a liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptide-mapping analysis of phosphorylation sites throughout the Id2 molecule. Our study identified phosphorylation of serine 5 in NPCs, a modification that was previously identified in myoblasts, fibroblasts, and osteosarcoma cells [31–33], and two additional, novel sites of phosphorylation (serine 14 and threonine 27) which have not been previously identified in any cell type. Phosphorylation of these three sites near the N-terminus enhances proteasomal degradation of Id2 in NPCs. When Id2 is not phosphorylated at these sites in NPCs, Id2 levels are elevated and the rate of NPC proliferation is increased. Further, we present evidence that in addition to being phosphorylated in NPCs, Id2 can also be phosphorylated at serine 5, serine 14, and threonine 27 in GSCs and that the glioma-associated phosphatase PP2A [39] is capable of dephosphorylating Id2 and regulating its steady-state level in both NPCs and GSCs.

Materials and Methods

Additional methodology is provided in Supporting Information.

Tissue Harvest and Culture

NPCs were isolated from the forebrains of neonatal *Id2*$^{-/-}$ mice as previously described [40] and cultured as adherent monolayers in Dulbecco's modification of Eagle's medium (DMEM)/F12 containing 10 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ, http://www.peprotech.com), 10 ng/ml fibroblast growth factor (FGF) (Peprotech, Rocky

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Hill, NJ, http://www.peprotech.com), 1× NEAA, 2× N2 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 2× B27 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 29mM glucose, L-glutamine, 4.5mM HEPES, BSA, 2-mercaptoethanol, laminin, 1% penicillin/streptomycin, and 0.1% gentamycin [41]. Primary human GBM-derived stem cells (GSCs) were generously provided by Dr. Steven Pollard (University of Edinburgh, Edinburgh, U.K.). GBM cell lines were purchased from ATCC.

Proliferation and Apoptosis Assays

Growth curves were prepared from 20,000 NPCs plated in 24-well plates in triplicate and live cells were counted using trypan blue exclusion. To determine cell cycle distribution, NPCs were fixed in 90% ethanol, stained with propidium iodide (PI), and analyzed on a MACSQuant flow cytometer. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling was performed according to manufacturer's instructions (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Apoptosis was quantified by live cell flow cytometry using PI/Annexin V.

Cell Cycle Exit and Cell Cycle Synchronization Assays

NPC cell cycle exit was induced by removal of the mitogens EGF and FGF for 24 hours in DMEM/F12 supplemented with B27 and 4% fetal bovine serum. For cell cycle synchronization experiments, 100nM nocodazole was added to cells for 16 hours to arrest cells in mitosis. Nocodazole was washed out and cells re-entered the cell cycle. Cell cycle distribution was determined using PI staining and flow cytometry.

Mass Spectrometry Analysis

Coomassie-stained SDS-PAGE gel separated Id2 immunoprecipitations were excised, completely destained in 50mM ammonium bicarbonate (SIGMA)/50% acetonitrile (ACN) (Honeywell Burdick & Jackson), dehydrated in ACN, rehydrated with trypsin (Promega, Madison, WI, http://www.promega.com) in 50mM ammonium bicarbonate (SIGMA), and digested overnight. Peptides were extracted from the gel with 50% ACN/5% formic acid (Honeywell Burdick & Jackson), dried, resuspended in 5% ACN/1% formic acid, and analyzed by nanoscale microcapillary LC-MS/MS essentially as described [42, 43] on a LTQ-Orbitrap (Thermo Electron) for data-dependent shotgun sequencing experiments.

Site-Directed Mutagenesis

The QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies #210519) was used to produce the Id2 alanine phospho-ablation mutant plasmids following the manufacturer's instructions.

Electroporation

NPCs and GSCs were electroporated (Lonza Nucleofector, Program A-033) to introduce siRNAs (Dharmacon) against various PP2A genes.

Results

Id2 can be Phosphorylated at Three Sites Near the N-terminus in NPCs

To identify Id2 phosphorylation sites in rapidly proliferating NPCs, we immunoprecipitated Id2 and used LC-MS/MS to analyze protease-digested peptides present in this Id2-enriched fraction. We were able to determine the sequence of 82.1% of the amino acids in Id2, including 24 out of the 28 serines, threonines, and tyrosines present in the Id2 molecule (data not shown). We consistently identified phosphorylation at three residues, each near the N-terminus. In addition to validating phosphorylation at serine 5 in NPCs as reported by others in other cell types [31–33], we identified two previously unrecognized phosphorylation sites, serine 14 and threonine 27 (Fig. 1A).

Phosphorylation Regulates the Steady-State Level of Id2 in NPCs

To understand the role of phosphorylation of Id2 in NPCs, we prepared retroviral vectors expressing variants of human Id2 (hId2) that could not be phosphorylated (see Materials and Methods). Sequence verified recombinants encoding Id2 mutated at either serine 5, serine 14, threonine 27 or at all three sites, which was designated as $hId2^{TPA}$ (triple phosphoablated) (Fig. 1B), were used to prepare retroviral stocks. To ensure that endogenous and highly phosphorylated Id2 (Fig. 1A) did not confound our analysis, we infected NPCs derived from *Id2*$^{-/-}$ mice with these recombinant retroviruses and after antibiotic selection, cells expressing these mutant alleles were maintained as polyclonal cultures. The cultures containing unmodified wild type (WT) hId2, ($hId2^{WT}$), or $hId2^{TPA}$ were denoted as $NPC^{Id2-/-}(hId2^{WT})$ or $NPC^{Id2-/-}(hId2^{TPA})$, respectively.

We examined the steady-state levels of Id2 in the NPC cultures described above expressing either $hId2^{WT}$ or $hId2^{TPA}$. Stable isotope labeling of amino acids in cell culture (SILAC) combined with mass spectrometry can be used to determine the relative abundance of a protein in two populations of cells. Using SILAC we found that the amount of Id2 protein in $NPC^{Id2-/-}(hId2^{TPA})$ was approximately 7.3 times higher than in $NPC^{Id2-/-}(hId2^{WT})$ (Fig. 2A, 2B). We confirmed the mass spectrometry analysis by Western blotting (Fig. 2C, inset) and used densitometry to quantitate several independent analyses (Fig. 2C). Interestingly, 2 of the cultures expressing Id2 with a single alanine substitution, $NPC^{Id2-/-}(hId2^{S5A})$ and $NPC^{Id2-/-}(hId2^{S14A})$ also had a higher level of Id2 compared to $NPC^{Id2-/-}(hId2^{WT})$ (Fig. 2D). As predicted, treatment of WT NPC cell lysate with alkaline phosphatase (AP), which dephosphorylates most proteins [44], resulted in an increase in Id2 protein levels (Fig. 2E).

In addition to detecting a higher steady-state level of $hId2^{TPA}$ compared to $hId2^{WT}$, we also examined the effect of phosphorylation on the half-life of Id2. We treated $NPC^{Id2-/-}(-hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ cultures with cyclohexamide to inhibit translation and monitored Id2 degradation over time. In cos7/5 cells, $hId2^{WT}$ is known to be a very short-lived protein with a half-life of only 15 minutes [24]. In our NPC cultures, we found the half-life of Id2 to be about 15 minutes, but the half-life of $hId2^{TPA}$ in $NPC^{Id2-/-}(hId2^{TPA})$ was approximately 100 minutes, 6.7 times longer than $hId2^{WT}$ in $NPC^{Id2-/-}(hId2^{WT})$ (Fig. 2F).

Loss of Id2 Phosphorylation Enhances NPC Proliferation

Id2 has been determined previously in our laboratory and others to play a role in cellular proliferation [14, 15, 33], where it promotes cell cycle progression through interactions with either bHLH proteins or the tumor suppressor protein, Rb [8, 16, 45, 46]. Consistent with the role of Id2 in promoting proliferation, we have also recently found that Id2 is required to maintain normal proliferation of NPCs [15]. To evaluate the possibility that phosphorylation of Id2 may regulate NPC proliferation, we first compared the proliferation of $NPC^{Id2-/-}(hId2^{TPA})$ to $NPC^{Id2-/-}(hId2^{WT})$ and found that *Id2*$^{-/-}$ NPCs expressing $hId2^{TPA}$ grew faster (Fig. 3A). The $NPC^{Id2-/-}(hId2^{TPA})$ also had a significantly higher proliferation index than $NPC^{Id2-/-}(hId2^{WT})$ as determined by a carboxyfluorescein succinimidyl ester (CFSE) dye dilution assay (Fig. 3B). The faster growth of $NPC^{Id2-/-}(hId2^{TPA})$ compared to $NPC^{Id2-/-}(hId2^{WT})$ was also observed in a semi-solid matrix culture and detected as an increase in the number and size of colonies observed in soft agar after 7 days (Supporting Information Fig. 1).

To ascertain whether this increased growth was a result of increased proliferation or decreased cell death, we examined cell death in these cultures using flow cytometry to simultaneously assess cellular PI and annexin V staining. There was no difference in the percentage of apoptotic cells between $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ (Supporting Information Fig. 2A, 2B) confirming an increase in proliferation. Consistent with the very low percentage of apoptotic cells in these cultures, we did not detect any Caspase-3 cleavage in $NPC^{Id2-/-}(hId2^{WT})$ or $NPC^{Id2-/-}(hId2^{TPA})$ through Western blotting (data not shown).

To examine further the effect of Id2 N-terminal phosphorylation on proliferation, we analyzed Id2 levels in $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ cultures following release from a mitotic block. After incubation of $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ cultures in nocodazole for 16 hours to block cell cycle progression, we incubated cells in media without nocodazole. We then monitored progression through the cell cycle using PI staining and flow cytometry (Fig. 3C) and compared Id2 levels by Western blotting (Fig. 3D). In proliferating cultures (Fig. 3C, untreated), prior to synchronization, $NPC^{Id2-/-}(hId2^{TPA})$ had more cells in S phase than did the culture expressing $hId2^{WT}$, consistent with the increased proliferation described in Figure 3B. In $NPC^{Id2-/-}(hId2^{WT})$ cultures (Fig. 3C, top panels), we observed a successful synchronization with nearly complete accumulation of cells in G2/M after nocodazole treatment. Following the release of this mitotic block, cells re-entered the cell cycle with an increasing proportion of cells in G1 at successive time points. The cell cycle profile after synchronization of $NPC^{Id2-/-}(hId2^{TPA})$ cultures identically treated in parallel was markedly different. These cells failed to synchronize as $NPC^{Id2-/-}(hId2^{WT})$ did. Despite nocodazole treatment for 16 hours there was a higher proportion of cells in G0/G1 in $NPC^{Id2-/-}(hId2^{TPA})$ cultures than in $NPC^{Id2-/-}(hId2^{WT})$ cultures. These differences in cell cycle distribution may be the result of a proportion of $NPC^{Id2-/-}(hId2^{TPA})$ cells overcoming the mitotic arrest and continuing to cycle. In support of this interpretation, at every time point examined there was a higher percentage of cells in S phase in $NPC^{Id2-/-}(hId2^{TPA})$ compared to $NPC^{Id2-/-}(hId2^{WT})$ (Fig. 3C). In addition, there was a lower level of Cdkn1a, the G1 cyclin-dependent kinase

inhibitor (data not shown), expressed in $NPC^{Id2-/-}(hId2^{TPA})$ cells than in $NPC^{Id2-/-}(hId2^{WT})$ cells. Interestingly, $hId2^{WT}$ protein levels fluctuate throughout the cell cycle; however, $hId2^{TPA}$ levels were maintained at a constant and high level (Fig. 3D).

Loss of Id2 N-terminal Phosphorylation Reduces Proteasome-Dependent Degradation of Id2 During NPC Cell Cycle Exit

Id2 is abundant in stem and progenitor cells of the CNS which are capable of self-renewal. However, expression of Id2 is not detected in most fully differentiated cell types, which typically have little or no proliferative capacity [47, 48]. We sought to determine how Id2 levels are regulated during cell cycle arrest of NPCs. We arrested NPCs in vitro by removing key mitogens, EGF and FGF, from the media and arresting cell growth. Cell cycle exit of NPCs, confirmed by the loss of Ki67 staining post EGF/FGF withdrawal (Fig. 4A), was associated with a decreased expression of Id2 protein (Fig. 4B). To determine whether there were also altered levels of *Id2* mRNA, we compared *Id2* at 0, 24, and 48 hours after EGF/FGF withdrawal. We found no significant difference in *Id2* mRNA expression at these times (Fig. 4C). We examined whether the decrease in Id2 following cell cycle exit was a result of proteasome-mediated degradation by comparing Id2 in WT NPCs treated with the proteasome inhibitor, epoxomicin, at a concentration of 20 uM for 1 hour following 24 hours of EGF/FGF withdrawal to vehicle dimethyl sulfoxide (DMSO) treated cells. While Id2 levels dropped precipitously in WT NPCs following growth factor withdrawal for 24 hours, Id2 levels were unchanged in epoxomicin-treated WT NPCs following EGF/FGF withdrawal (Fig. 4D). Comparable results were obtained following treatment with MG132, another proteasome inhibitor (data not shown). These findings indicated that Id2 protein levels in NPCs are regulated by proteasomal degradation following growth factor withdrawal.

To determine the effect of Id2 phosphorylation on steady-state levels of Id2, we removed EGF/FGF for 24 hours from $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ cultures and examined cell lysates for Id2 by Western blotting (Fig. 4E). We found that $hId2^{WT}$ was drastically reduced following EGF/FGF withdrawal, in spite of being under the control of the exogenous SV40 promoter encoded by our retroviral constructs. As also described above in Figure 4B, $hId2^{TPA}$ levels did not decrease to the same extent following EGF/FGF withdrawal (Fig. 4E). These data indicate a potential role for phosphorylation in the regulation of Id2 steady-state levels and support the possibility that proteasome-dependent degradation of Id2 is regulated by N-terminal phosphorylation.

One common function of post-translational phosphorylation is to modulate protein-protein interactions. To investigate the possibility that phosphorylation of Id2 alters the binding partners of Id2, we conducted a mass spectrometry experiment using SILAC to identify Id2 interacting proteins. Consistent with previous reports [25], we found that $NPC^{Id2-/-}(hId2^{WT})$ immunoprecipitated fractions were enriched for components of the APC/C. Extending these findings, we observed that in $hId2^{TPA}$ immunoprecipitates, there were significantly fewer peptides corresponding to the APC/C subunits APC1, APC2, APC5, and APC8 associated with $hId2^{TPA}$ as compared with levels of these proteins in immunoprecipitates of $hId2^{WT}$ (Fig. 4F). The lower affinity of $hId2^{TPA}$ for the APC/C

complex is consistent with the observation that hId2^{TPA} is stabilized in NPCs resulting in a higher steady-state Id2 protein level.

Id2 Phosphorylation Status Correlates with Id2 Levels in Human GSCs

GBMs are primary brain tumors that can arise from NPCs [49–51]. We sought to identify evidence for a role for phosphorylation in regulating Id2 in GBM-derived cells. We screened a panel of human GBM-derived cell lines and GSCs and found a high level of Id2 expression in nearly all of the cells examined (Fig. 5A), most of which were above that of non-transformed human NPCs, CB660. This is noteworthy because although Id2 expression can be associated with changes in mRNA encoding Id2 [19, 20, 22], we found no correlation between the level of transcript and protein in either GBM-derived cell lines or GSCs (Fig. 5A, 5B). The lack of correlation between mRNA and protein levels suggests that in GBM cells post-translational regulation may be important for modulating Id2 abundance. After analyzing GSCs for Id2 expression, we chose a GSC culture with high Id2 expression and one with low Id2 expression for further analysis. Using mass spectrometry we quantified the phosphorylation site occupancy and found that there were approximately 3, 2, and 2 times more phosphorylated peptides detected at the serine 5, serine 14, and threonine 27 positions respectively in Id2 of GSCs with naturally occurring low levels of Id2 protein (GliNS2) as compared to the levels of Id2 phosphorylation in a human GSC culture with naturally occurring high Id2 protein (GNS179) (Fig. 5C). This suggests that Id2 phosphorylation may contribute to the discrepancy between transcript and protein levels.

Inhibition of PP2A Results in a Decreased Steady-State Level of Id2 in NPCs and GSCs

Because unphosphorylated Id2 supported the proliferation of NPCs, we sought to identify a phosphatase that dephosphorylates Id2 in this cell type. Inhibition of such a phosphatase would be expected to increase Id2 phosphorylation, decrease the steady-state level of Id2 by increasing degradation, and enhance NPC proliferation. We used calyculin A to inhibit the most abundant family of serine/threonine phosphatases, the phosphoprotein phosphatase (PPP) family, which includes protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). Treatment of WT NPCs with calyculin A resulted in an accumulation of higher molecular weight Id2 and no detectable Id2 migrating at the level seen in DMSO-treated cells. We interpret these findings to indicate that the faster migrating Id2 corresponds to unphosphorylated Id2, which is supported by observing Id2 from AP-treated NPCs (Fig. 2E). When PP2A is inhibited, Id2 becomes phosphorylated and a slower migrating band becomes detectable (Fig. 6A, left panel) suggesting that among the PPP family of phosphatases were candidate enzymes that could de-phosphorylate Id2 and increase Id2 protein stability. The shift in Id2 motility seen following calyculin treatment is likely to be remaining phosphorylated Id2 that has not yet been degraded.

To examine whether the Id2 protein degradation that we observed after treatment of NPCs with calyculin A was associated with a change in Id2 phosphorylation, we analyzed calyculin A-treated NPCs by mass spectrometry. As predicted, we found that following phosphatase inhibition with calyculin A, total Id2 protein was decreased; importantly, the phosphorylation occupancy at each of the identified sites, serine 5, serine 14, and threonine 27, was increased by calyculin A treatment (Fig. 6A). To determine whether the effect of

PPP inhibition on Id2 levels was dependent on the N-terminal phosphorylation sites we identified, we treated both WT NPCs and NPC$^{Id2-/-}$(hId2^{TPA}) cultures with calyculin A. hId2^{TPA} did not decrease after calyculin A treatment of NPC$^{Id2-/-}$(hId2^{TPA}) (Fig. 6B) suggesting that N-terminal phosphorylation of Id2 can contribute to the degradation of Id2 following PPP inhibition. Although we observed a shift in NPC$^{Id2-/-}$(hId2^{TPA}) following calyculin A treatment (Fig. 6A), we interpret this result to represent phosphorylation of Id2 at sites that are not ablated in our phospho-mutant; however, these potential unknown phosphorylation sites do not appear to be important in regulating Id2 stability (Fig. 6B). We extended our analysis of the PPP family to examine the effect of PP2A, a multimeric enzyme complex, on Id2 protein levels. We inhibited the expression of the catalytic subunit of *PP2A* in WT NPCs using siRNAs targeting the catalytic isoform *PPP2CA*, and observed a decrease in Id2 levels (Fig. 6C). siRNA-mediated gene silencing of PP2A and its effect on Id2 indicates that this PPP can regulate the cellular level of Id2.

Since PP2A is able to regulate the levels of Id2 in NPCs and NPCs have been implicated in gliomagenesis [49–51], we used primary human GSCs for further analysis. GSCs were generously provided by Dr. Steven Pollard and cultured in a defined media that supports stem cells as previously described [52]. Using these GSC cultures, which are highly reminiscent of the tumors they were derived from [41], we found that in two different GSC cultures, addition of calyculin A resulted in a decrease in Id2 protein (data not shown), consistent with our findings in NPCs. To understand further the effect of PP2A activity on Id2, we examined several individual components of this enzyme complex and sought to determine which subunits can affect Id2 expression levels. PP2A is a heterodimeric complex consisting of a catalytic subunit and a structural subunit which may or may not be associated with a regulatory subunit in one of three different families, B′, B″, and B‴. There are two genes in the catalytic family, two genes in the structural family, and many different genes in the regulatory family. Therefore, a multitude of possible combinations can make up different PP2A holoenzymes, contributing to the selectivity of protein targets [53–56]. In an analysis of the 16 PP2A genes, *PPP2R1A*, *PPP2R1B*, *PPP2R3B*, *PPP2R3C*, *PPP25C*, *PPP2CA*, and *PPP2CB* were highly expressed in GNS144 human GSCs (Fig. 6D).

We inhibited the expression of four candidate genes using siRNAs to evaluate whether inhibition of any of these genes may affect Id2 levels. The candidate genes included *PPP2R1A*, *PPP2R3C*, *PPP2R5C*, and PPP2CA. *PPP2R1A* was the scaffolding subunit with the highest expression in GNS144 and *PPP2R3A* had the highest level of expression of the B″ regulatory subunit family. *PPP2R5C* had the highest level of expression of the B′ regulatory subunit family. We chose to examine further the catalytic alpha subunit of PP2A, *PPP2CA*, because although it was expressed at a similar level as *PPP2CB*, in a publicly available database, The Cancer Genome Atlas, it significantly predicts a lower survival in GBM patients [57] and mediates Id2 protein levels in NPCs (Fig. 6C). We found that inhibiting the expression of the structural subunit, R1A, the regulatory subunit R3C, and the catalytic a subunit, led to a reduction in Id2 protein (Fig. 6E) suggesting that they may be part of the PP2A complex which de-phosphorylates Id2. Our findings in both NPCs and GSCs provide evidence that protein phosphatases may be key regulators of proliferation in both normal and malignant CNS cells.

Discussion

Complex molecular pathways regulate NPC proliferation during normal neural development, and when dysregulated, these pro-growth pathways can contribute to neoplastic disease [49–51]. We and others have found that Id2 functions to promote cell cycle progression in NPCs [14, 15, 40]. More recently, we found that enhanced expression of Id2 is oncogenic in a PDGF-enriched microenvironment of the CNS [38] and ongoing work in our laboratory has identified a subset of murine GBM tumors in which inhibition of Id2 diminishes tumorigenicity (data not shown). Levels of Id2 protein are regulated at the post-translational level [25], but the molecular mechanisms mediating this process have not been characterized in the CNS. Because of the importance of Id2 in both normal developmental processes and oncogenesis [2, 14, 15, 38], we sought to understand better the cellular mechanisms regulating its level in proliferating cells of the CNS. We used mass spectrometry to characterize post-translational modifications of Id2 in NPCs and GSCs. Among the many post-translational modifications that our approach could have readily identified, we only detected phosphorylation. These phosphorylation sites occur in the poorly characterized N-terminal region of the Id2 protein and include two novel sites, serine 14 and threonine 27, which were observable in both normal and malignant cells of CNS origin (Figs. 1 and 6).

We analyzed polyclonal cultures expressing Id2 protein harboring mutations in individual residues where phosphorylation was identified and were able to identify statistically significant changes in Id2 protein levels in $NPC^{Id2-/-}(hId2^{S5A})$ and $NPC^{Id2-/-}(hId2^{S14A})$ (Fig. 2D). Although our studies cannot rule out the possibility that different combinations, including double mutants, of phosphorylation occupancy at the three N-terminal residues will alter the steady-state level of Id2 in unexpected ways, we anticipate that double mutants would provide an intermediate effect on Id2 levels between that seen with the mutation of a single phosphorylation site (Fig. 2D) and that seen with loss of all three sites (Fig. 2C). Our current study suggests an effect mediated by multiple sites based on the observation that cultures expressing $hId2^{TPA}$, which cannot be phosphorylated at any of the three N-terminal sites we studied, consistently expressed significantly increased steady-state levels of Id2 compared to any of the individual mutants. This phenomenon of multisite phosphorylation being important for protein degradation is similar to that observed in studies of transcription factors and cell cycle regulators [58–60].

Id2 phosphorylation at the serine 5 position has been identified and analyzed previously in mesenchymal cells [31–33], a residue conserved between each of the 4 Id family members. Hara et al. observed a decrease in the number of colonies formed by U2OS human osteosarcoma cells that were transfected with a S5A-Id2 mutant construct [32]. They interpreted this finding to indicate that S5A-Id2 inhibited growth of these cells. Matsumura et al. and Butler et al. also found that substitution of serine 5 with alanine in Id2 was growth-suppressive, as indicated by reduced BrdU incorporation, in rat aortic smooth muscle cells and mouse myoblasts, respectively [31, 33]. In our analysis of CNS cells, we found that Id2 is phosphorylated at two additional sites, serine 14 and threonine 27, which are not conserved in any of the other Id proteins and thus may regulate Id2 uniquely. $hId2^{TPA}$ was expressed at very high levels and consistent with the pro-proliferative function of Id2, these post-translational modifications of Id2 were found to enhance proliferation in NPCs (Figs. 2

and 3). These cell-type and context-dependent activities for Id proteins are expected based on their ability to interact with both ubiquitously expressed E-proteins and tissue-specific bHLH transcription factors, each of which can have diverse functions [61–63] and different effects on cellular proliferation [3, 64, 65]. Here we provide the first cellular and molecular data indicating that in CNS cells, N-terminal phosphorylation acts to target Id2 for degradation and that loss of Id2 phosphorylation results in accumulation of Id2 and enhanced cellular proliferation.

We found phosphorylation near the N-terminus to function at least in part to regulate the steady-state level of Id2 protein by altering the rate of proteasome-dependent degradation. A previous study identified a functional C-terminal DEAD-box motif in Id2, a well-characterized targeting motif used by the APC/C complex [25]. We found that even with the DEAD-box targeting motif intact, loss of N-terminal phosphorylation of Id2 was sufficient to alter the ability of APC/C to bind Id2 (Fig. 4). Although we were able to detect an interaction between Id2 and APC/C using sensitive mass spectrometry techniques, this interaction is likely to be very weak, as we were unable to detect an interaction by co-immunoprecipitation using antibodies against anaphase-promoting complex subunits. Similarly, Lasorella et al. were only able to detect an Id2-APC/C interaction after overexpressing APC/C subunits at high levels. This mechanism of post-transcriptional control of Id2 levels in NPCs contributes to the observation that *Id2* mRNA and protein levels do not correlate to one another in a panel of human GBM cells (Fig. 5). In this regard, the phosphorylation status of Id2 may be more informative in predicting protein expression than transcript levels in human GSCs.

Our findings (Fig. 3) and the findings of others [7, 17, 32] indicate dynamic modulation of Id2 levels during cell cycle transit. Based on our observation that Id2 is phosphorylated in proliferating CNS cells, we sought to identify a phosphatase that can regulate Id2 de-phosphorylation, and thereby contribute to the rapid alteration in the intracellular concentration of Id2 (Fig. 6). Protein phosphatases function as multiprotein complexes comprised of combinations of structural, catalytic, and regulatory proteins. Each of these families of subunits contains multiple different proteins, allowing for numerous possible combinations, and the mechanisms that determine the ability of these subunits to interact with specific targets are complex and poorly understood [53–55]. GBM patients with PP2A activity levels above 160 pMP have a significantly worse survival rate than patients with levels below this threshold [39]. In addition, a small molecule inhibitor of PP2A, LB100, delayed the growth of GBM cells in vitro and in vivo in combination with radiation therapy [66]. In our study, we found that inhibition of select PP2A subunits in GSCs provides a novel mechanism to decrease Id2 protein levels by causing rapid degradation of Id2. Furthermore, it was recently reported that downregulation of Id2 increases the chemosensitivity of glioma [67], providing additional evidence that Id2 may be an effective drug target in this disease. Our work can inform future therapies that might target PP2A to enhance proteasomal degradation of Id2 and decrease the proliferation of cancer cells.

Conclusion

In summary, we elucidated a role for novel Id2 phosphorylation sites in modulating Id2 protein degradation and NPC proliferation. PP2A, a phosphatase, contributes to the maintenance of high levels of Id2 in GBM cells. These findings suggest that inhibitors of phosphatase activity provide a novel mechanism by which to regulate Id2 levels.

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Significance Statement

Glioblastoma (GBM), the most common and aggressive brain tumor of adults, arises from neural precursor cells (NPCs). Id2 is a pro-proliferative transcription factor that is highly expressed in NPCs and when overexpressed is tumorigenic in a mouse model of GBM. We have identified novel Id2 phosphorylation sites which regulates its steady-state level by modulating proteasome-dependent degradation and thereby affecting NPC proliferation. Importantly, phosphorylation of pro-proliferative phosphoproteins has emerged as an important therapeutic strategy in many different tumors, and an understanding of Id2 phosphorylation may identify new targets for therapy.

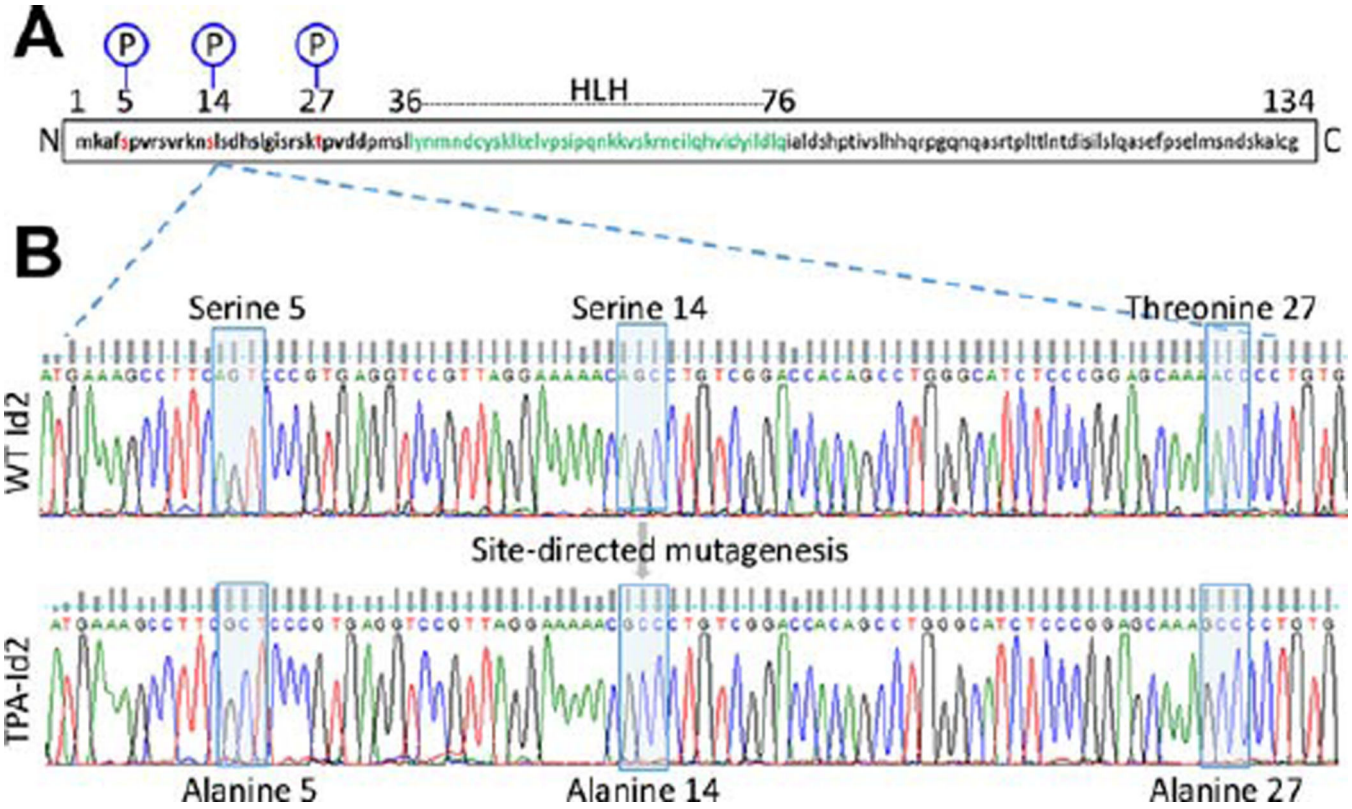

Figure 1.

Identification of three phosphorylation sites near the N-terminus of Id2: serine 5, serine 14, and threonine 27, and characterization of a phospho-mutant Id2 vector. **(A)**: Amino acid sequence map of the location of three Id2 phosphorylation sites. **(B)**: DNA sequence of wild type Id2 (above) and a mutant triple phospho-ablated-Id2 construct (below) in which each of the three phosphorylation sites has been converted to an alanine. Abbreviation: HLH, helix-loop-helix.

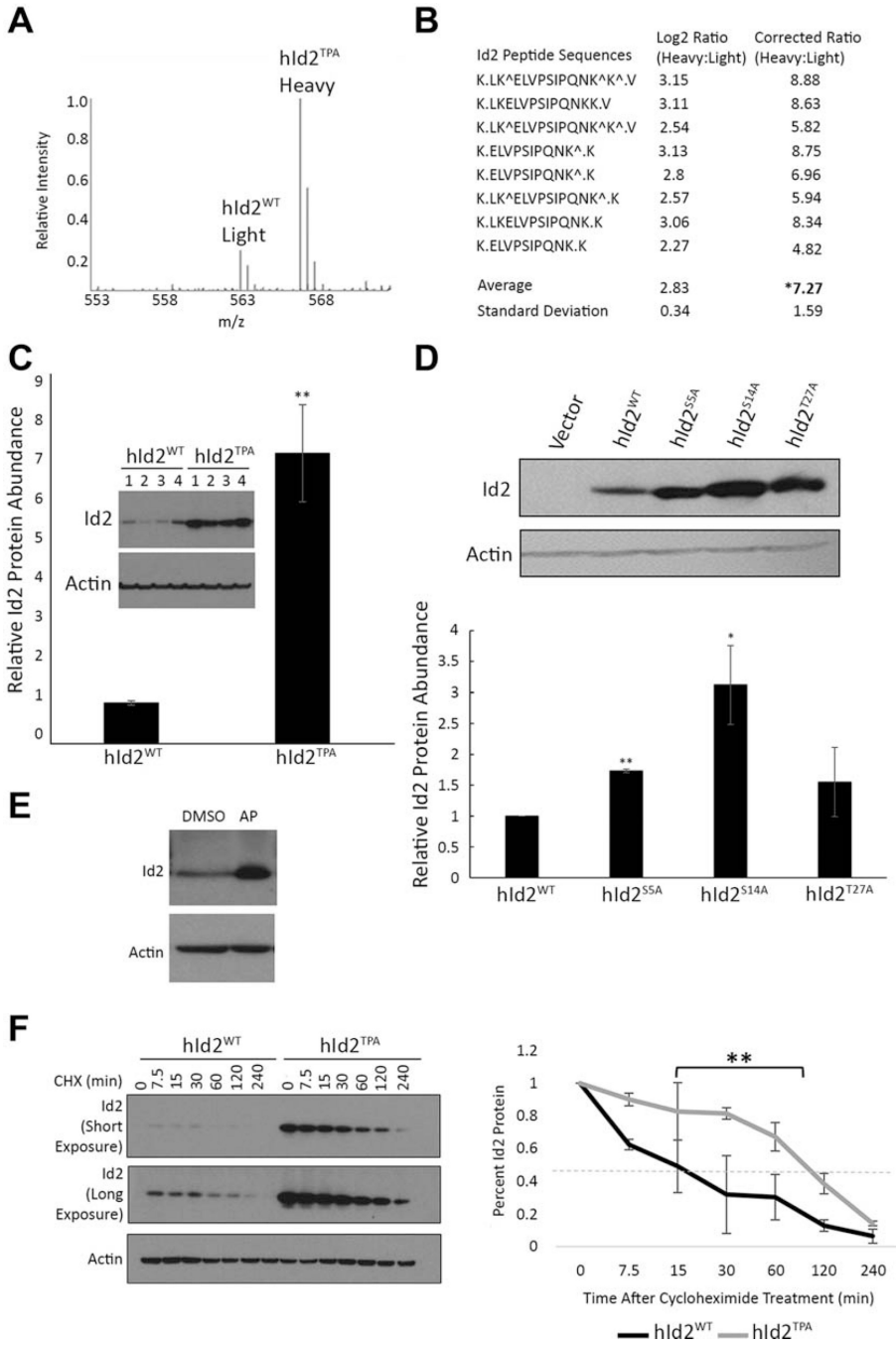

Figure 2.

Phosphorylation regulates the steady-state level of Id2 in NPCs. **(A)**: Representative m/z scan view of heavy labeled hId2^{TPA} and light labeled hId2^{WT} peptides from a SILAC analysis. **(B)**: Table of the Id2 peptide sequences, average Log2 ratios, and corrected ratios of Id2 peptides in NPC$^{Id2-/-}$(hId2^{WT}) and NPC$^{Id2-/-}$(hId2^{TPA}) from a SILAC analysis. **(C)**: Relative protein abundance of Id2 compared to actin in NPC$^{Id2-/-}$(hId2^{WT}) and NPC$^{Id2-/-}$(hId2^{TPA}) from multiple independent Western blotting experiments. ** $p < .01$. Inset: Representative Western blot of Id2 in four independent polyclonal cultures of

$NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$. **(D)**: Top: Representative Western blot of Id2 in $NPC^{Id2-/-}$(vector), $NPC^{Id2-/-}(hId2^{WT})$, $NPC^{Id2-/-}(hId2^{S5A})$, $NPC^{Id2-/-}(hId2^{S14A})$, and $NPC^{Id2-/-}(hId2^{T27A})$. Bottom: Relative protein abundance of Id2 compared to actin in $NPC^{Id2-/-}(hId2^{WT})$, $NPC^{Id2-/-}(hId2^{S5A})$, $NPC^{Id2-/-}(hId2^{S14A})$, and $NPC^{Id2-/-}(hId2^{T27A})$. $hId2^{S5A}$, ** $p < .01$; $hId2^{S14A}$, * $p < .05$. **(E)**: Western blot analysis of Id2 and actin in WT NPCs after a 1 hour incubation with DMSO or alkaline phosphatase. **(F)**: Left: Representative Western blot of Id2 in $Id2^{-/-}$ NPCs expressing $hId2^{WT}$ or $hId2^{TPA}$ after treatment with cyclohexamide for 0, 7.5, 15, 30, 60, 120, or 240 minutes. Right: Percent Id2 protein from three independent Western blotting experiments, ** $p < .01$.

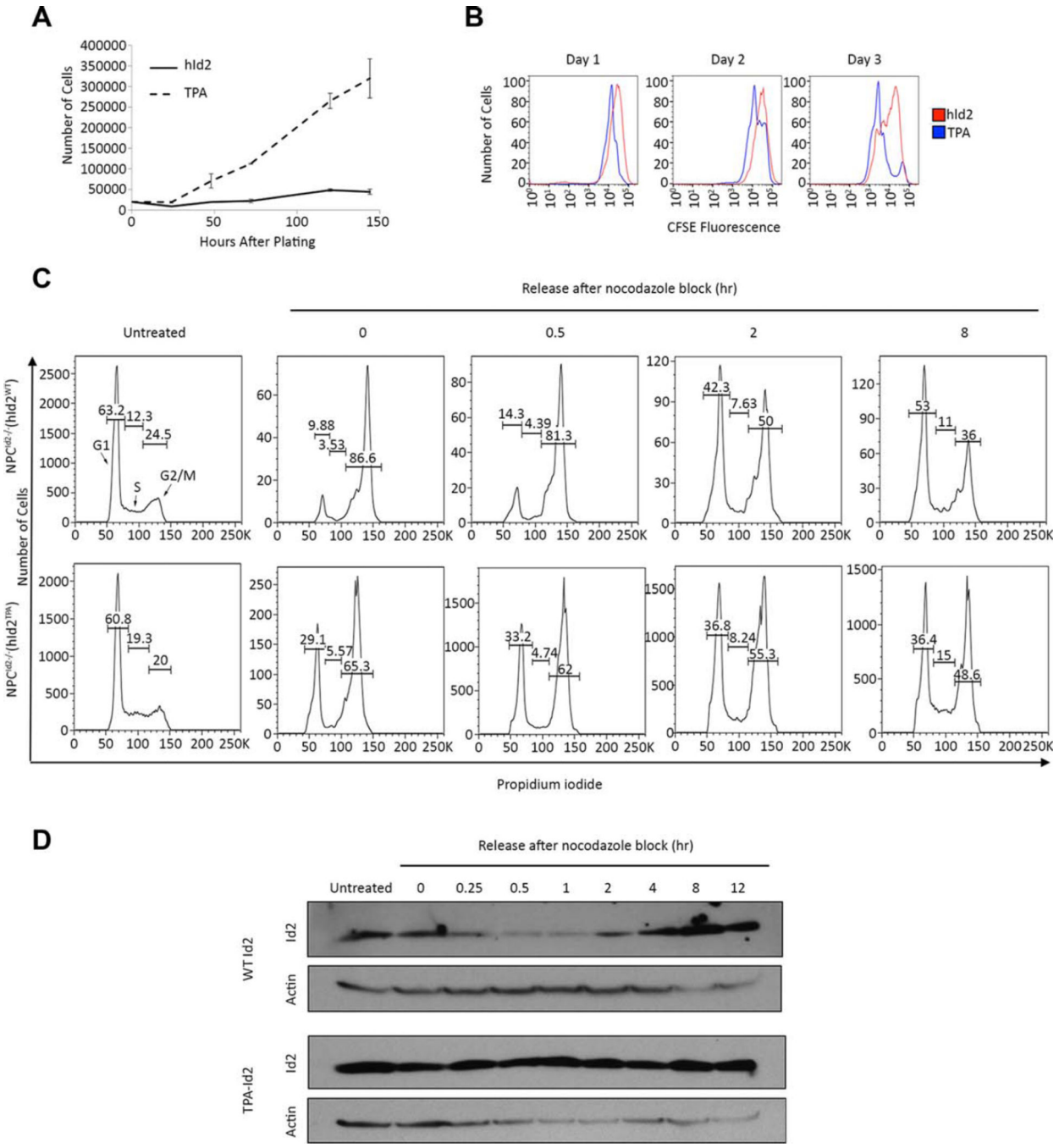

Figure 3.

Lack of Id2 phosphorylation enhances NPC proliferation. **(A)**: Growth curve of $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$. **(B)**: Carboxyfluorescein succinimidyl ester dye dilution assay of cultured $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$. **(C)**: Flow cytometry analysis of the cell cycle distribution of $NPC^{Id2-/-}(hId2^{WT})$ and $NPC^{Id2-/-}(hId2^{TPA})$ proliferating cultures ($t = 0$) that were treated for 16 hours with nocodazole and released from the block for 0.5, 2, or 8 hours. **(D)**: Western blot of Id2 and actin levels in wild type (WT) NPCs and $NPC^{Id2-/-}(hId2^{TPA})$ proliferating cultures ($t = 0$)

that were treated for 16 hours with nocodazole and released from the block for the indicated time points. Abbreviations: hId2, human Id2; TPA, triple phospho-ablated.

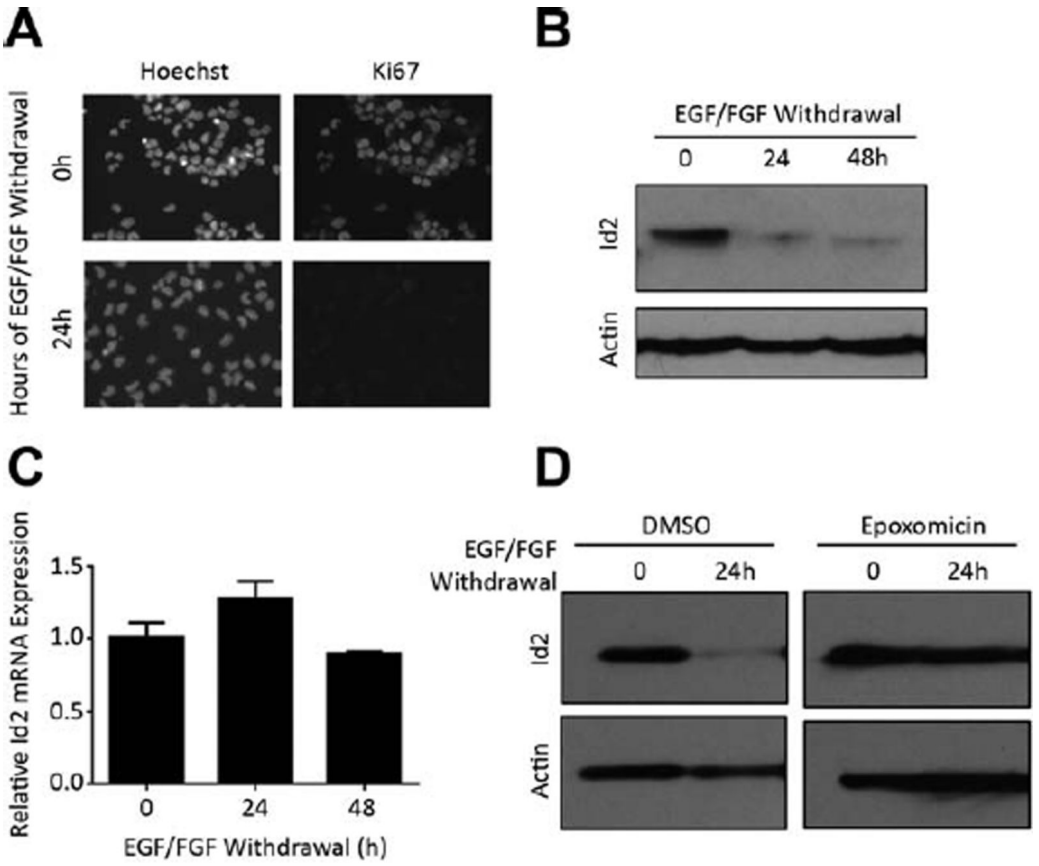

Figure 4.

Loss of Id2 N-terminal phosphorylation reduces proteasome-dependent degradation of Id2 during NPC cell cycle exit. **(A)**: Ki67 expression in NPC$^{Id2-/-}$(hId2^{WT}) 0 and 24 hours after epidermal growth factor (EGF)/fibroblast growth factor (FGF) withdrawal. **(B)**: Western blot of Id2 at 0, 24, and 48 hours after EGF/FGF withdrawal. **(C)**: mRNA expression levels of Id2 relative to actin in NPCs 0, 24, and 48 hours post cell cycle exit. Error bars represent SEM. **(D)**: Western blot of Id2 in wild type (WT) NPCs treated with 20 uM DMSO or epoxomicin for 1 hour following 0 or 24 hours of EGF/FGF withdrawal. **(E)**: Western blot

analysis of Id2 0 and 24 hours after induction of cell cycle exit of $NPC^{Id2-/-}$ ($hId2^{WT}$) and $NPC^{Id2-/-}$($hId2^{TPA}$). **(F)**: Table of APC/C subunits that were differentially bound to Id2 in a SILAC analysis of $NPC^{Id2-/-}$($hId2^{WT}$) and $NPC^{Id2-/-}$($hId2^{TPA}$).

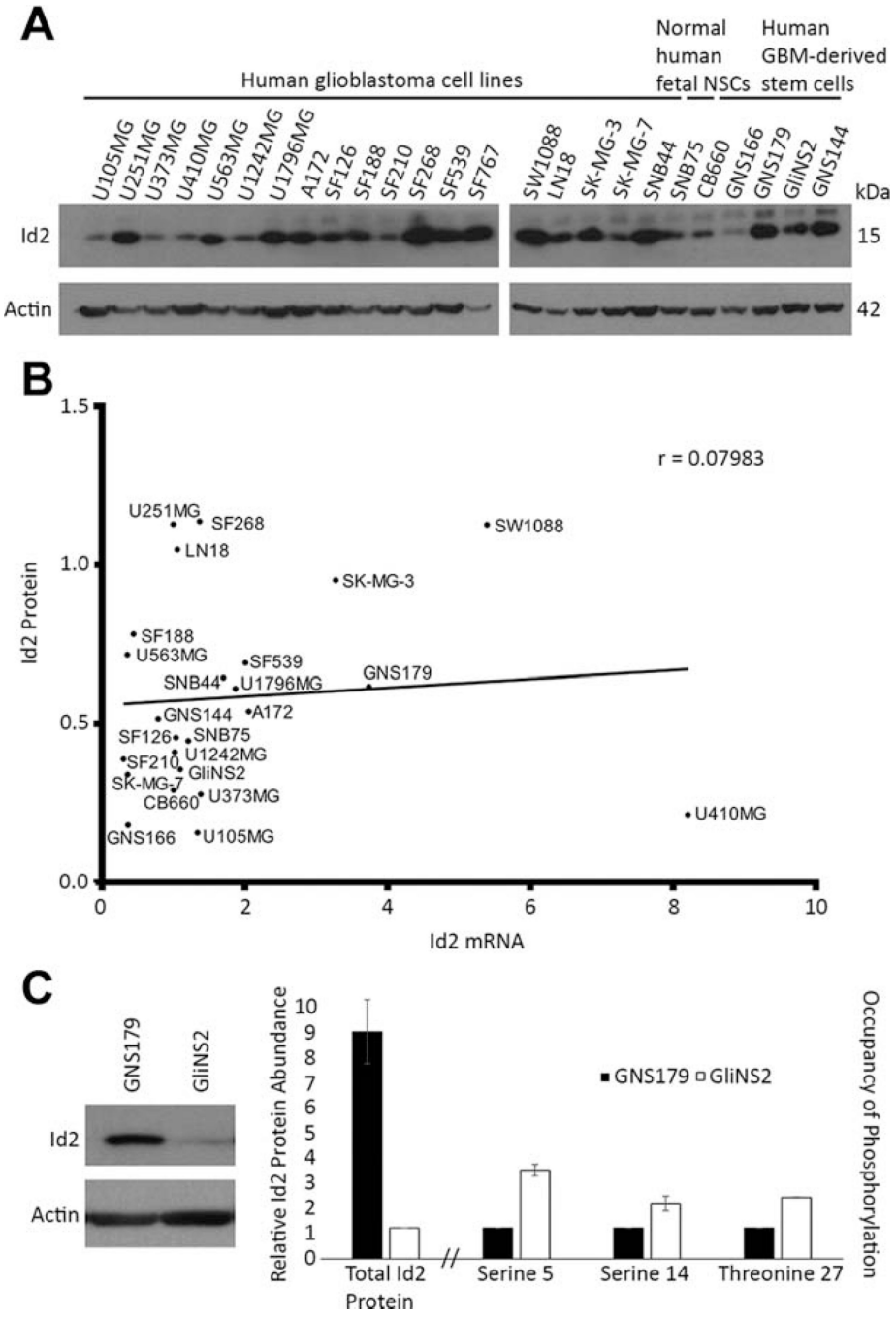

Figure 5.

Phosphorylation status is predictive of Id2 protein abundance in human GSCs. **(A)**: Western blot of Id2 and actin protein levels in a panel of human glioblastoma (GBM) cell lines and human GSCs. **(B)**: Scatterplot of Id2 mRNA expression and protein expression relative to actin in a panel of human GBM cell lines and human GSCs. r=0.07983. p = .7108. **(C)**: Left: Western blot of Id2 and actin in GNS179 and GliNS2. Right: Mass spectrometry analysis of Id2 protein abundance and phosphorylation occupancy at serine 5, serine 14, and threonine 27 in GNS179 and GliNS2 human GSCs. Abbreviation: NSCs, neural stem cells.

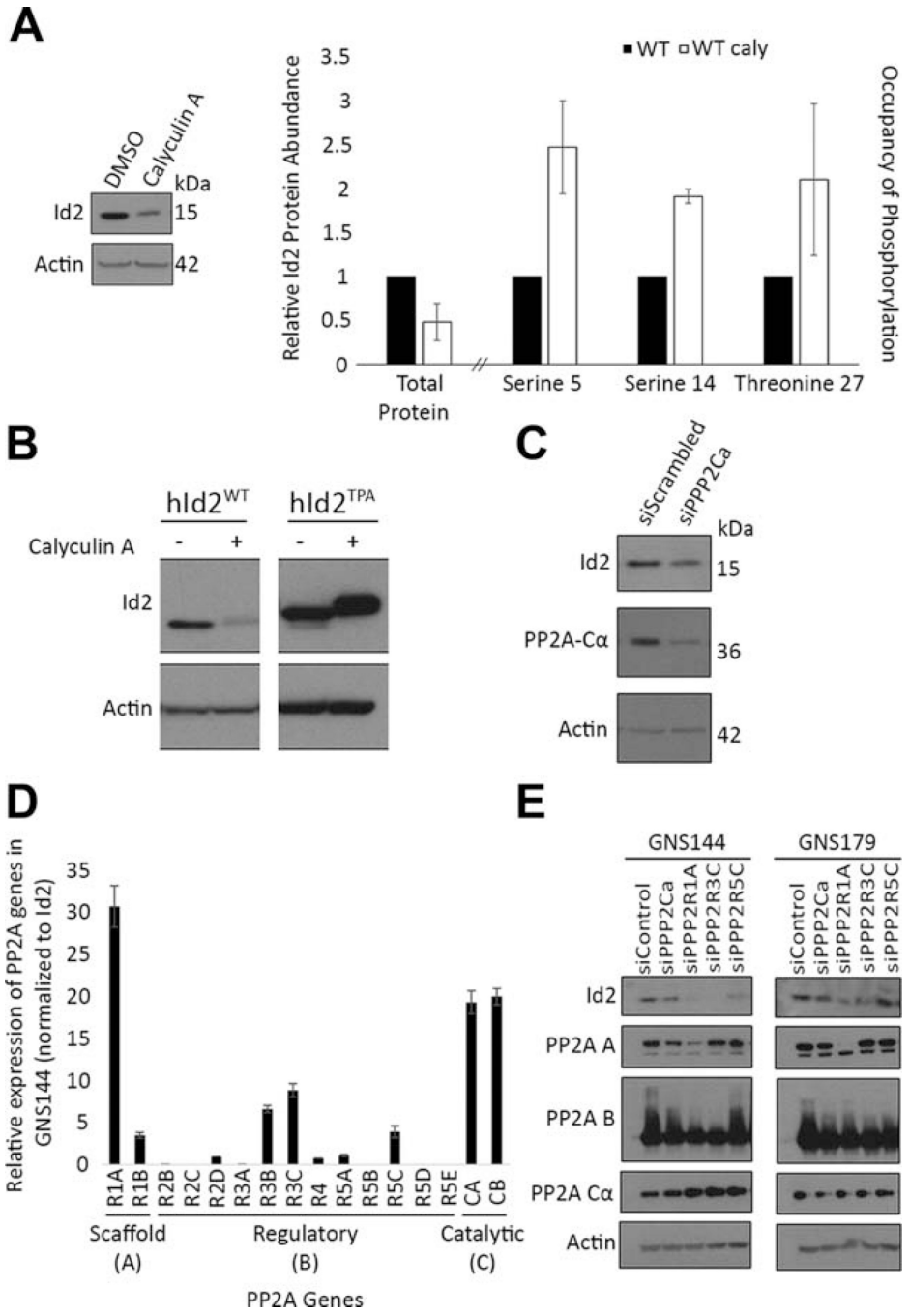

Figure 6.

Inhibition of PP2A results in decreased Id2 protein expression in NPCs and GSCs. **(A)**: Left: Western blot of Id2 and actin in DMSO or calyculin A treated NPCs. Right: Mass spectrometry analysis of total Id2 protein and occupancy of phosphorylation at serine 5, serine 14, and threonine 27 in wild type (WT) NPCs treated with DMSO or calyculin A. **(B)**: Western blot analysis of Id2 and actin in WT NPCs or $NPC^{Id2-/-}$($hId2^{TPA}$) after 1 hour incubation with DMSO or calyculin A. **(C)**: Western blot analysis Id2, PP2A, and actin in WT NPCs/siScrambled or WT NPCs/siPP2A. **(D)**: Relative mRNA expression of PP2A

genes in GNS144 cells using RT-PCR. **(E)**: Western blot analysis of Id2, PP2A A, PP2A B, PP2A C, and actin in GNS144 and GNS179 after electroporation using siRNAs as indicated in the figure.