Phosphatidylinositol-3 kinase-dependent translational regulation of Id1 involves the PPM1G phosphatase

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Abstract

Id1 is a helix-loop-helix transcriptional modulator that increases the aggressiveness of malignant glial neoplasms. Since most glioblastomas (GBMs) show increased phosphatidylinositol-3 kinase (PI-3K) signaling, we sought to determine whether this pathway regulates Id1 expression. Higher basal Id1 expression correlates with dysregulated PI-3K signaling in multiple established GBM cell lines. Further characterization of PI-3K-dependent Id1 regulation reveals that chemical or genetic inhibition of PI-3K signaling reduces Id1 protein but not mRNA expression. Overall, PI-3K signaling appears to enhance Id1 translation with no significant effect on its stability. PI-3K signaling is known to regulate protein translation through mTORC1-dependent phosphorylation of 4E-BP1, which reduces its association with and inhibition of the translation initiation factor eIF4E. Interestingly, while inhibition of PI-3K and AKT lowers 4E-BP1 phosphorylation and expression of Id1 in all cases, inhibition of TORC1 with rapamycin does not consistently have a similar effect suggesting an alternative mechanism for PI-3K-dependent regulation of Id1 translation. We now identify a potential role for the serine-threonine phosphatase PPM1G in translational regulation of Id1 protein expression. PPM1G knockdown by siRNA increase both 4E-BP1 phosphorylation and Id1 expression and PPM1G and 4E-BP1 co-associates in GBM cells. Furthermore, PPM1G is a phosphoprotein and this phosphorylation appears to be regulated by PI-3K activity. Finally, PI-3K inhibition increases PPM1G activity when assessed by an *in vitro* phosphatase assay. Our findings provide the first evidence that the PI-3K/AKT signaling pathway modulates PPM1G activity resulting in a shift in the balance between hyper- and hypo-phosphorylated 4E-BP1 and translational regulation of Id1 expression.

Keywords

glioblastoma; Id1; PI-3K; PPM1G; translational regulation

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INTRODUCTION

Glioblastomas (GBMs) are the most common primary brain tumors in adults and patients with this diagnosis have an extreme poor prognosis despite aggressive multimodality therapy. Many molecular abnormalities have been associated with GBMs including amplification/mutation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) and loss of tumor suppressors such as PTEN, CDKN2A/2B, and TP53, among others, that likely drive the malignancy of these tumors.[1] In fact, comprehensive genomic characterization of GBMs as part of the Cancer Genome Atlas (TCGA) project found that abnormalities in RTK/Ras/phosphatidylinositol-3 kinase (PI-3K) signaling (including tumorigenic mutations of PTEN that results in its inactivation leading to loss of an antagonist of PI-3K) are seen in 88% of assessed samples.[2] While therapeutic approaches to target some of these abnormalities have been explored, further identification of important molecular abnormalities in GBMs may yield additional features that can be therapeutically targeted.

Id proteins are members of the helix-loop-helix (HLH) family of transcriptional regulators, acting as dominant negative repressors of basic-HLH (bHLH) transcription factors.[3, 4] Id proteins are best known for inhibiting cellular differentiation although it can affect various cellular processes including inhibition of apoptosis and promotion of cellular proliferation. Four members (Id1-4) of this family have been identified and have both common and unique activities. Id1 is overexpressed in a variety of solid tumors and appears to affect a number of cellular processes associated with malignancies.[4–6] In fact, early work in different malignancies correlated high Id1 levels with poor prognosis, chemoresistance and tumor metastases.[7, 8] In human gliomas, Id1 is expressed and its level positively correlates with tumor grade.[9] Recently, glioma-initiating cells (GICs) were found to express high levels of Id1 and, moreover, TGF-β inhibitors were found to prevent glioma initiation and recurrence by reducing Id1 expression and depleting this GIC population.[10] This work suggests that high Id1 levels may correlate with aggressive behavior and poor prognosis in glioblastoma patients. Barrett et al. found in a mouse model of gliomagenesis that a subpopulation of tumor cells displaying high levels of Id1 has high self-renewal capacity consistent with a role of Id1 in propagation of glioma stem cells.[11] More recently, Niola et al. discovered that the Id proteins are important factors in the growth of mesechymal GBMs by maintaining GICs in their perivascular.[12] Finally, our own group has identified Id1 induction downstream of cyclooxygenase-2 as the major contributory factor for producing a more aggressive glioma phenotype.[13] These studies all point to the Id proteins as critical factors in the production and maintenance of GBMs.

Given the importance of Id proteins in the pathogenesis of malignant glial neoplasms, gaining a greater understanding of the regulation of these factors may have implications for prognosis and therapeutic management of these aggressive tumors. With the importance of the PI-3K pathway in these tumors, we hypothesized a potential regulatory role for this signaling pathway on Id1 expression. In fact, we find that PI-3K signaling increases Id1 protein expression in a process that requires AKT but is not necessarily blocked by the mTOR inhibitor rapamycin. This regulation is not at the level of RNA but instead appears to involve increased translational efficiency of Id1 mRNA. Furthermore, PI-3K signaling

appears to induce Id1 expression through modulation of PPM1G, a serine/threonine phosphatase with known activity in splicing, cell cycle control and DNA damage response.[14–18] Our report establishes, for the first time, a novel mechanism whereby PI-3K signaling decreases PPM1G activity and increases 4E-BP1 phosphorylation leading to enhanced Id1 translation and accumulation of this transcriptional modulator (please see Figure 7 for our working model).

RESULTS

PI-3K signaling regulates Id1 protein expression

Since most GBM cells display both overactivity of PI-3K signaling and Id1 expression, we sought to test whether PI-3K signaling regulates Id1 expression. To this end, Id1 protein levels are assessed in several GBM cell lines with dysregulated PI-3K signaling due to mutant PTEN (mtPTEN) status as well as in an additional GBM cell line (LN229) with low levels of PI-3K activation due to wild-type PTEN (wtPTEN) status. Consistent with a regulatory role for the PI-3K pathway, basal Id1 protein levels are higher in GBM cells with mtPTEN compared to LN229 cells with wtPTEN (Figure 1a). To further confirm involvement of PI-3K signaling on regulation of Id1 expression, GBM cells with mtPTEN are treated with the PI-3K inhibitor LY294002 and assessed for Id1 expression. In each case, increasing levels of LY294002 is effective at reducing phosphorylation of AKT and decreasing protein expression of Id1 (Figure 1b). However, no corresponding change in Id1 mRNA expression is found after LY294002 treatment (Figure 1b). To rule out off-target, or non-specific effects of chemical inhibition in our system, PI-3K signaling was also suppressed genetically in four of our mtPTEN GBM lines (SF767, U251, SF763, U118) by expressing wild-type PTEN under the control of a tet-inducible promoter. Like with LY294002 treatment, induction of PTEN with doxycycline results in suppressed AKT phosphorylation and decreased protein but not mRNA expression of Id1 (Figure 1c). These results suggest that PI-3K signaling regulates Id1 expression at a post-transcriptional level.

Id1 translational efficiency is increased by PI-3K signaling

To verify post-transcriptional regulation of Id1 by PI-3K signaling, pulse-chase analysis of Id1 is performed on the U251 and SF767 cells with or without inhibition of the PI-3K pathway by either treatment with LY294002 for 16 hours or forced expression of PTEN for 48 hours. First, Id1 levels drop over time with similar kinetics irrespective of PI-3K inhibition indicating no change in the stability of Id1 (Figure 2a–b). Of note, Id1 half-life is quite short, consistent with previous reports of ubiquitination.[19, 20] However, lower levels of Id1 were noted at the initial chase time point after PI-3K inhibition, indicating that PI-3K inhibition results in a decrease in newly synthesized Id1 protein (Figure 2a & c). These results suggest that the PI-3K pathway can alter Id1 translation but not its degradation.

To directly assess PI-3K-dependent translational regulation of Id1, polyribosome profiling of U251 and SF767 cells are analyzed for changes following PI-3K inhibition either with LY294002 treatment or PTEN induction after doxycycline treatment. In each case, the majority of Id1 mRNA associate with the translationally-active polyribosome fractions (fractions 5–10) in untreated, control cells (Figure 3a–b, upper panels). However, after

PI-3K inhibition with PTEN induction or LY treatment, Id1 mRNA clearly shifts to the translationally-inactive monosome fractions (fractions 1–4) (Figure 3a–b, middle and lower panels). Quantification of the percentage of Id1 mRNA in the polyribosome fractions 5–10 to the total Id1 mRNA present under each condition shows a definitive shift away from the translationally-active fractions with PI-3K inhibition for each cell line (supplemental Figure 1). In contrast to Id1 mRNA, 18S rRNA displays similar patterns of polyribosome association in control and PI-3K-inhibited cells (Figure 3a–b). These data further support our pulse-chase analysis showing Id1 translation is regulated by PI-3K signaling.

PI-3K-dependent regulation of Id1 is not affected by rapamycin

To further dissect the mechanism for PI-3K-dependent translational control of Id1, inhibitors of additional signaling components downstream of PI-3K are utilized. First, since AKT is a key mediator of PI-3K signaling, we assessed the effect of the allosteric AKT inhibitor MK-2206 on Id1 expression. Treatment with increasing levels of MK-2206 reduces phosphorylation of AKT, as expected for this agent, indicating effective targeting of AKT and, correspondingly, decreased Id1 expression (Figure 4a & c). Next, to assess signaling further downstream of AKT, mTOR inhibition is accomplished with the mTORC1 inhibitor rapamycin. This signaling component (mTORC1) is of particular interest because of its known role in the regulation of translation.[21, 22] Surprisingly, rapamycin has little effect on Id1 protein levels in SF767 and U251 cells (rapamycin-insensitive) despite near complete blockade of S6 phosphorylation (Figure 4b), a downstream marker of mTORC1 activity, while it reduces Id1 expression inSF763, U87, and U118cells (rapamycin-sensitive) similar to that seen with PI-3K and AKT inhibition (Figure 4c). The inconsistent effects of rapamycin on Id1 expression suggest that other factor(s) downstream of PI-3K/AKT, possibly independent of mTORC1, contributes to translational regulation of Id1.

PI-3K-dependent phosphorylation of 4E-BP1 correlates with Id1 expression

The translation repressor protein 4E-BP1 inhibits cap-dependent translation by binding to the translation initiation factor eIF4E. 4E-BP1 is a substrate for mTORC1 and its phosphorylation disrupts interactions with eIF4E resulting in activation of cap-dependent translation. Therefore, we next assessed the effects of LY294002, MK-2206 and rapamycin on phosphorylation of 4E-BP1. In the cell lines (SF763, U87 and U118) where all three inhibitors reduce Id1 expression (rapamycin-sensitive), a corresponding decrease in phosphorylation at Ser65 and Thr70 on 4E-BP1 is noted (Figure 5a). However, in the cell lines (SF767 and U251) where rapamycin (unlike LY294002 and MK-2206) is ineffective at inhibiting Id1 expression, phosphorylation of Ser65 and Thr70 on 4E-BP1 is similarly insensitive to this drug (Figure 5b). The relationship between 4E-BP1 phosphorylation and Id1 expression is further assessed with PI-3K pathway inhibition with PTEN expression or activation with myristoylated AKT (myrAKT) expression. For these experiments, SF767 and U251 cells containing PTEN under the control of the tet-inducible promoter (Figure 1c) are used. In addition, myrAKT under the control of the tet-inducible promoter is also introduced into SF767 cells. Like with chemical inhibition of PI-3K, induction of PTEN with doxycycline in SF767 and U251 cells results in decreased phosphorylation of 4E-BP1 and less Id1 expression (Figure 5c). Finally, consistent with our model, induction of myrAKT, a mutant AKT containing a myristoylation signal which produces a functionally active kinase

and increases signaling flux through the PI-3K/AKT pathway, in SF767 leads to increased phosphorylation of 4E-BP1 and more Id1 expression (Figure 5d). Based on these results, we speculate that PI-3K-dependent regulation of Id1 translation involves modulating phosphorylation of 4E-BP1.

PI-3K regulates 4E-BP1 phosphorylation and Id1 expression by the PPM1G phosphatase

The phosphorylation status of 4E-BP1 is a dynamic balance between adding (through increased kinase activity) and removing (through increased phosphatase activity) phosphates. Recently, PPM1G was identified as a phosphatase that can target 4E-BP1 in certain settings.[23] Therefore, we sought to determine if the PI-3K/AKT-dependent and rapamycin-insensitive regulation of Id1 involves PPM1G and its ability to dephosphorylate 4E-BP1. First, siRNA-dependent knockdown of PPM1G expression in SF767 and U251 cells (rapamycin-insensitive lines) results in increased 4E-BP1 phosphorylation and Id1 protein expression (Figure 6a). PPM1G knockdown in U87, SF763, and U118 cells (rapamycin-sensitive lines) similarly increased Id1 expression to a lesser extent (supplemental Figure 2). Next, PPM1G interacts with 4E-BP1 as shown by coimmunoprecipitation with either anti-PPM1G (Figure 6b) or anti-FLAG (when FLAG-PPM1G was exogenously expressed, supplemental Figure 3) antibodies and this association is enhanced by inhibition of PI-3K activity with LY294002. Since PPM1G association with 4E-BP1 appears to be regulated by PI-3K activity, we then sought to determine whether PPM1G is a phosphoprotein. After *in vivo* ^{32}P-orthophosphate labeling of SF767 cells, immunoprecipitation of both endogenous PPM1G or exogenous FLAG-PPM1G shows a radioactive signal consistent with baseline phosphorylation of this phosphatase that decreases with PI-3K pathway inhibition with either LY294002 or MK-2206 (Figure 6c). These results suggest that PI-3K activation leads to phosphorylation of PPM1G, which will reduce its association with 4E-BP1. Finally, we have also directly measured the activity of PPM1G using an *in vitro* phosphatase assay and found that LY294002 treatment results in enhanced activity as measured using a MBP substrate that has been ^{32}P-labeled with protein kinase A (Figure 6d). Therefore, our data appear to show that inhibition of PI-3K/AKT increases PPM1G activity, possibly through promotion of its binding to 4E-BP1.

DISCUSSION

Id1 has been implicated in the development and maintenance of a variety of malignancies likely through its effects at promoting cancer stem cell initiation and propagation. In particular, the Id proteins, especially Id1, can enhance the aggressiveness, or malignancy of glioblastoma cells. Since overactivity of PI-3K signaling is one of the most prominent molecular features in malignant glial neoplasms,[2, 24] it is not surprising to find that this pathway also regulates Id1 expression. Basal Id1 protein level is increased in glioma cell lines that have increased flux through the PI-3K pathway from PTEN loss. Blocking PI-3K/AKT signaling by forced expression of wtPTEN or treatment with inhibitors for PI-3K or AKT results in decreased Id1 expression at the protein but not mRNA level, suggesting possible translational regulation of Id1, which was confirmed by pulse-chase assay and polyribosome profile analysis.

We have now uncovered more mechanistic details regarding PI-3K/AKT-dependent regulation of Id1 translation. The PI-3K signaling is known to regulate protein translation through activation of mTORC1 which phosphorylates 4E-BP1, leading to its dissociation with eIF4E and facilitation of translation initiation.[21] Interestingly, while PI-3K and AKT inhibition reduces 4E-BP1 phosphorylation and Id1 expression in all cases, inhibition of mTORC1 with rapamycin does not have a consistent similar effect. SF767 and U251 cells display little change in the phosphorylation of 4E-BP1 at Ser65 and Thr70 and expression of Id1 after treatment with rapamycin despite almost complete abolishment of S6 phosphorylation, another marker of mTORC1 activity. These results are not completely unexpected since studies have shown that mTORC1 may have some rapamycin-resistant functionality.[25] However, due to these results, we postulated that a PI-3K/AKT-dependent, mTORC1-independent mechanism for Id1 translational control exists. In fact, we now identify a mechanism for translational regulation of Id1 expression involving the serine-threonine phosphatase PPM1G. First, PPM1G knockdown increases 4E-BP1 phosphorylation and Id1 expression. Furthermore, PPM1G is not only phosphorylated but PI-3K signaling regulates this phosphorylation. More interestingly, PI-3K blockade enhances the physical interaction of PPM1G with 4E-BP1 and increases PPM1G activity. Thus, PI-3K signaling can alter the phosphorylation of 4E-BP1 not only through activation of mTORC1, which increases 4E-BP1 phosphorylation but also through inactivation of PPM1G, which reduces 4E-BP1 dephosphorylation. A schematic of our model for PI-3K pathway regulation of 4E-BP1 phosphorylation and Id1 translation is shown (Figure 7).

PPM1G, formerly known as PP2Cγ, is a member of the PP2C family of serine/threonine phosphatases.[26] Diverse functions have been ascribed to PPM1G including effects on mRNA splicing[14, 16], snRNP assembly[27], histone exchange[15], DNA damage response[17, 18], and cellular survival.[28] More recently, PPM1G was shown to be capable of dephosphorylating 4E-BP1 and regulating protein translation.[23] Our results that PPM1G is capable of regulating Id1 expression through changes in translational efficiency are consistent with these findings by Liu et al. Beyond these range of functions, PPM1G is capable of being phosphorylated. Matsuoka et al. found in large-scale proteomic evaluation of ATM and ATR substrates that PPM1G is phosphorylated on Ser183.[29] Beli et al. confirmed phosphorylation at Ser183 as well as identified additional sites at Ser201 and Ser527 after treatment with ionizing radiation.[17] Ser195 has also been identified as a possible phosphorylation site.[30] Finally, Khoronenkova et al. found that ATM-dependent PPM1G phosphorylation results in activation of the PPM1G phosphatase which dephosphorylates and downregulates USP7S leading to Mdm2 downregulation and p53 accumulation.[18] By contrast, our data suggest that PI-3K/AKT-dependent phosphorylation of PPM1G decreases its activity against 4E-BP1 since PI-3K inhibition results in enhanced phosphatase activity *in vitro* and reduced 4E-BP1 phosphorylation state *in vivo*. In addition, incorporating a point mutation at Ser183 (S183A), Ser195 (S195A), or Ser527 (S527A) results in no appreciable change in PPM1G phosphorylation in SF767 cells after *in vivo* ^{32}P-labeling (unpublished data). Given that PI-3K/AKT-dependent phosphorylation of PPM1G inhibits rather than activates this phosphatase (as is the case with ATM-dependent phosphorylation of PPM1G)[18], we predict that PI-3K/AKT-dependent phosphorylation site(s) will differ from those defined after ATM activation. While work to map these

phosphorylation site(s) is (are) ongoing, their exact identification is beyond the scope of the present study.

The TCGA has been a resounding success at characterizing the genomic landscape of a variety of malignancies including GBMs.[2, 31–44] In the analysis of GBMs, mRNA expression profiling has been particularly important in defining a classification scheme (classical, mesenchymal, neural, proneural) based on unsupervised hierarchical clustering.[45] In addition, while expression levels of numerous transcripts are prognostically significant, Id1 mRNA expression levels have not proven to be such a factor based on analysis of the TCGA GBM database (data not shown). However, lack of significance in mRNA expression should not be considered in isolation since changes in protein expression is what ultimately produces functional consequences. As our results here illustrate, PI-3K activation increases Id1 protein expression due to enhanced translational efficiency without changes in Id1 mRNA expression. Thus, the prognostic significance of Id1 expression would be missed if only transcript levels are considered.

Because PI-3K pathway activation is such a prominent feature of GBMs, translational regulation should be taken into consideration when evaluating gene expression in these tumors. Rajasekhar et al. first demonstrated a major role of translational control on gene expression in GBMs when they found that signaling from oncogenic Ras and Akt had potentially greater effects on recruitment of specific mRNA to polysomes than on mRNA transcription itself.[46] More recently, Helmy et al. profiled global changes in translational efficiency of specific mRNAs in murine glioma models.[47] In their supplemental data, translation efficiency of Id1 mRNA was actually increased in their PDGF-expressing glioma tumor cells in comparison to normal brain cells which supports our finding that PI-3K signaling regulates Id1 expression since expression of PDGF ligand would be expected to activate its receptor tyrosine kinase (PDGF-R) leading to increased PI-3K signaling.[47] Although other mechanisms exist, PI-3K is thought to primarily control translation by altering the phosphorylation state of 4E-BP1 through mTORC1.[21, 22] Our work now invokes a novel PI-3K-dependent means for altering the phosphorylation state of 4E-BP1 by modulating the activity of the PPM1G phosphatase and its association with 4E-BP1.

In summary, our findings provide the first evidence that PI-3K signaling translationally regulates Id1 expression. While the mechanism for this effect was expected to involve mTORC1-dependent phosphorylation of 4E-BP1, we find that, in many situations, PI-3K/ AKT-dependent modulation of PPM1G phosphatase activity and its association with 4E-BP1 is also an important factor in shifting the balance between hyper- and hypo-phosphorylated 4E-BP1 and regulating cap-dependent translation. Finally, our results suggest, for first time, that PPM1G is phosphorylated in a PI-3K/AKT-dependent fashion resulting in inhibition of its phosphatase activity and reduced ability to suppress EIF4E-dependent translation.

MATERIAL AND METHODS

Plasmids

Human wild-type PTEN (wtPTEN) (OriGene Technologies Inc., Rockville, MD) and myristoylatedAKT (myrAKT, kindly provided by Dr. Tschilis) cDNAs were inserted into

pRetroX-Tight-Pur expression plasmid (Clontech, Mountain View, CA, USA) to achieve inducible expression. A lentiviral expression vector containing epitope-tagged human PPM1G (pLenti-PPM1G, lentiviral vector encoding Myc-DDK-PPM1G) was also obtained from Origene.

Cell lines, culture conditions and transfections

Glioma cell lines (LN229, SF539, SF763, SF767, U87, U118, U251, and their derivatives) were cultured in high glucose DME media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma), Na pyruvate (1mM) and penicillin (100U/ml)/ streptomycin (100μg/ml) unless otherwise indicated. Glioma cell lines were originally obtained from the Brain Tumor Research Center at UCSF with authentication by STR analysis (tested in 3/2013 and 6/2015). In addition, all parental glioma cells used for experiments in this study have been assessed for mycoplasma contamination by PCR testing at some point since the beginning of 2015. Packaging cell lines (Phoenix or 293T cells) were cultured as above without Na pyruvate. Each retroviral vector was transiently transfected into Phoenix cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's recommendation. To achieve inducible gene expression, pRetroX-Tet-On (Clontech) and pRetroX-tight-Pur containing genes of interest were sequentially introduced by infection into glioma cell lines and selected with 1mg/ml G418 and 2μg/ml puromycin (Invitrogen), respectively, to obtain stable cell lines. To generate stable expression of PPM1G, pLenti-PPM1G along with lentiviral packaging plasmids for *env* (VSV-G), *gag* and *rev* were transiently transfected into 293T cells and conditioned supernatants were used to infect the glioma cell of interest (SF767). For transient knockdown of PPM1G by siRNA suppression, SF767 and U251 cells were transfected with commercially available siRNAs against PPM1G (Integrated DNA Technologies, Coralville, IA) using Lipofectamine 2000 (Invitrogen). Stealth RNAi medium GC duplex-negative control (Invitrogen) was used to control for sequence-independent effects of introducing short RNA duplexes into cells.

Immunoblotting and antibodies used

All Western blots were done according to standard procedures. Blots or immunoprecipitations were probed with antibodies against PTEN (sc-6817) and Id1 (sc-488) (Santa Cruz Biotechnology, Santa Cruz, CA), P-AKT (T308, #9275 & S473, #5012), AKT (#4691), P-4E-BP1 (S65, #9451 & T70, #13396), 4E-BP1 (#9644), and P-S6 (#4857) (Cell Signaling Technology, Danvers, MA), PPM1G (A300-880A) (Bethyl Laboratories, Inc., Montgomery, TX), and FLAG (antibody only, F1804 & M2 affinity gel, A2220) (Sigma). As loading controls, blots were also probed with antibodies against EIF5α (sc-282) (Santa Cruz). Blots were detected using appropriate secondary antibodies conjugated with horseradish peroxidase and chemiluminescent substrate according to standard procedures.

RNase protection assay (RPA)

RPA was performed by standard techniques as previously described.[48] Riboprobe templates for detection of Id1 and 18S were generated from vectors termed pKS/ribo/Id1 and 18S contain 348 base pairs of the human Id1 cDNA and 125 base pairs of human 18S rDNA, respectively. Riboprobe template for detection of cyclophilin A (normalization control) was obtained commercially (Ambion, Austin, TX). Protected riboprobes were resolved on 5%

urea/polyacrylamide gel and detected by phosphorimaging on a Typhoon 9210 system using ImageQuant software from Molecular Dynamics (GE Healthcare, Piscataway, NJ) for quantitation of signal.

Pulse-chase assay

Cells were incubated in Met/Cys-free (starvation) DME media for 30 minutes, pulsed with 200μCi of ^{35}S-labeled Met/Cys in 2ml starvation media/60mm dish for 10 minutes, and chased with DME media supplemented with 5mM cold Met/Cys for the indicated times. Lysates was generated with 1ml of IP buffer containing 50mM Tris-Cl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100, and 10μl protease inhibitor cocktail (Sigma). Immunoprecipitation using an anti-Id1 antibody and protein A beads (Roche Diagnostics, Indianapolis, IN) was performed by standard procedures, resolved by SDS-PAGE and electrotransferred to PVDF membrane. Membranes were detected by phosphorimaging as described above.

Polyribosome profiling

Assay was performed essentially as previously described.[49] Briefly, SF767/tetPTEN and U251/tetPTEN cells were incubated with cycloheximide (100μg/ml) for 15 minutes to arrest polyribosome migration after pretreatment, when indicated, with 10μM LY294002 for 16 hours or 1μg/ml doxycycline for 48 hours. Cells were lysed in 800μl of lysis buffer containing 20mM Tris (pH 7.0), 100mM KCl, 5mM MgCl$_2$, 0.5% Triton X-100 and 8μl protease inhibitor cocktail (Sigma), incubated on ice for 20 minutes and spun at 13K rpm at 4°C for 30 minutes to isolate cytoplasmic extracts. Cytoplasmic extracts loaded on a 15–45% (wt/vol) sucrose gradient were centrifuged at 39K rpm in a SW41 rotor for 60 minutes at 4°C and fractionated. Total RNA was extracted from each fraction and Id1/18S RNA levels per fraction were determined by RPA.

Coimmunoprecipitation

SF767 cells were pretreated with 10μM LY294002 for 2 hours prior to lysis in 1ml of IP buffer as described above. Coimmunoprecipitation was accomplished by incubating lysates with anti-PPM1G antibody/protein A beads or anti-FLAG M2 affinity gel (as appropriate), analyzing bound proteins by SDS-PAGE and immunoblotting with anti-PPM1G or anti-4E-BP1 antibody.

In vivo ^{32}P-labeling

Glioma cells were pretreated, as indicated, with vehicle, LY294002, or MK-2206 for 1 hour followed by incubation in phosphate-free media containing vehicle/inhibitor(s) for 30 minutes. 200μCi ^{32}P-orthophosphate/ml was then added for 2 hours. After labeling, cells were harvested/lysed in buffer containing 50mM Tris-Cl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, protease inhibitor cocktail 111 (Sigma), and phosphatase inhibitor cocktail 1 and 11 (Sigma). Immunoprecipitation with anti-PPM1G or anti-FLAG antibody was then performed, resolved by SDS-PAGE and electrotransferred to PVDF membrane. ^{32}P-labeling of PPM1G was detected by phosphorimaging as described above.

Immunoblotting was then performed using antibodies against PPM1G by standard procedures.

PPM1G phosphatase assay

Phosphatase activity assay were performed as described with some modifications.[50] Briefly, myelin basic protein (MBP) was used as substrate of PPM1G. 1mg MBP (Sigma) was radiolabeled by incubating with 200U protein kinase A (PKA) (Sigma), 1mM ATP, 50μCi of γ^{32}P-ATP, 10mM $MgCl_2$, 2.5mM DTT, 10mM β-mercaptoethanol, and 50mM Tris-Cl (pH 7.4) in a final volume of 500μl for 2 hours at 37°C. Labeled MBP was then precipitated with 170μl ice-cold trichloroacetic acid (TCA) on ice for 10 minutes and centrifuged at 15,000g for 10 minutes at 4°C. Pellet was resuspended in 500μl of 40mM Tris-Cl (pH7.4). TCA precipitation was repeated x3 to effectively remove unincorporated ATP. Lysates containing phosphatase activity were obtained from SF767/FLAG-PPM1G cells treated with vehicle or 10μM LY294002 for 2 hours. Lysis was performed in IP buffer containing both protease and phosphatase inhibitor cocktails as described above. FLAG-tagged PPM1G proteins were immunoprecipitated using anti-FLAG antibody, extensively washed, and then eluted in 50μl phosphatase assay buffer containing an overexcess of 3x FLAG peptide. Phosphatase reaction involved mixing 2μl of ^{32}P-labeled MBP and 40μl of the 3x FLAG-eluted, PPM1G supernatants with 360μl of additional phosphatase assay buffer (50mM TrisCl, pH 7.4, 20% glycerol, 1mM $MnCl_2$, 100mM NaCl, 1mM DTT) and incubating for 1 hour at 37°C. Reaction without PPM1G supernatants was used as a blank control. Reactions were terminated with 130μl of ice-cold 100% TCA and 100μl BSA (0.9mg/ml), incubated on ice for 10 minutes, and centrifuged at 15,000g for 10 minutes at 4°C. 250μl of the supernatant was transferred to scintillation vials for measuring radioactivity as a readout of hydrolyzed radioactive phosphate. The remainder of each immunoprecipitate was assessed by immunoblotting with an anti-PPM1G antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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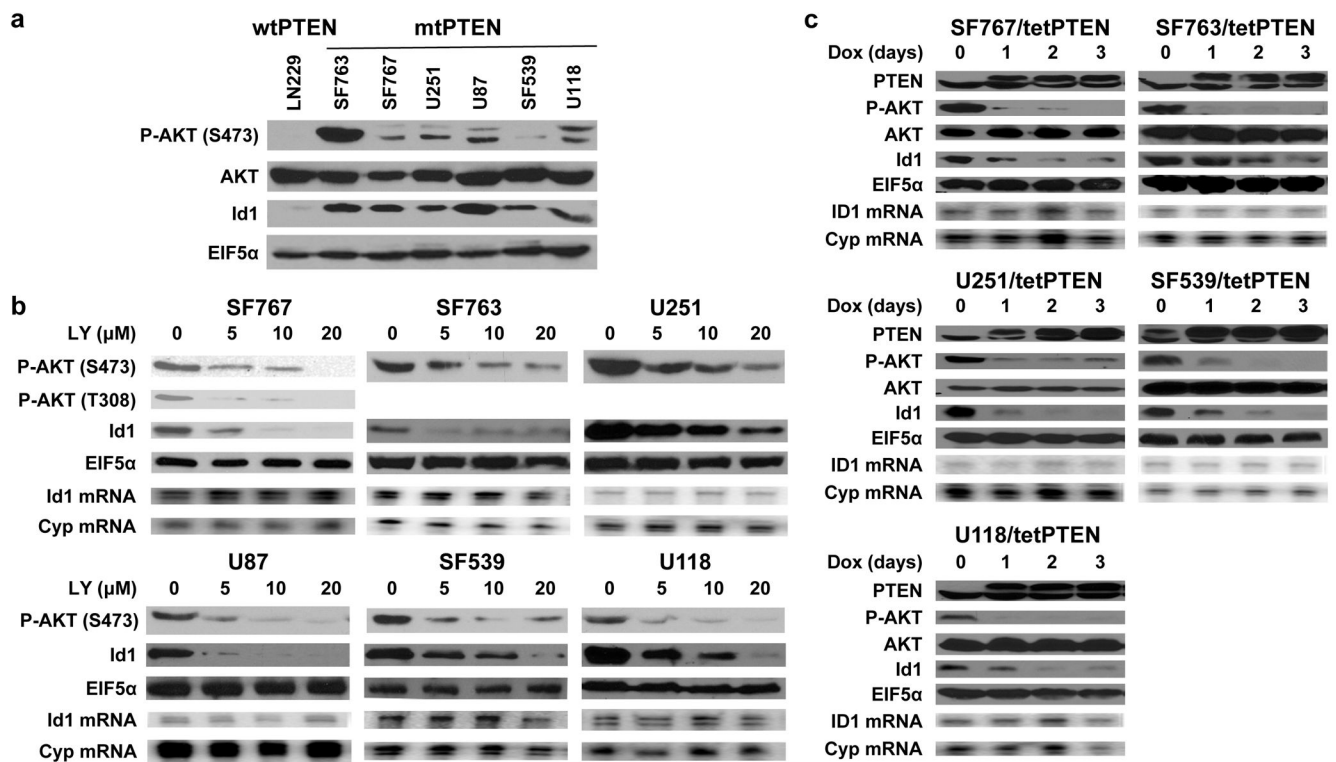**Figure 1.**
PI-3K signaling increases Id1 protein expression. (**a**) multiple human GBM cell lines with wild type (wtPTEN) or mutant PTEN (mtPTEN) status were assessed by immunoblot (IB) for expression of phosphorylated AKT (P-AKT), AKT and Id1. (**b**) mtPTENGBM cells were treated with increasing levels of LY294002 for 16 hours and assessed for Id1 protein and mRNA expression by IB and RNase protection assay (RPA), respectively. P-AKT levels were used to assess PI-3K inhibition by LY294002 in SF767 cells and similar drug levels were used in the other cell lines. (**c**) mtPTEN GBM cell derivatives containing tetracycline-inducible wtPTEN were treated with doxycycline (Dox, 1μg/ml) for 1, 2 or 3 days. IB was used to assess PTEN, P-AKT (S473), AKT and Id1 protein expression and RPA was used to assess Id1 mRNA expression. EIF5α and cyclophilinA (Cyp) mRNA levels were used as normalization controls for IB and RPA, respectively. In all cases, representative results from three independent experiments are shown.
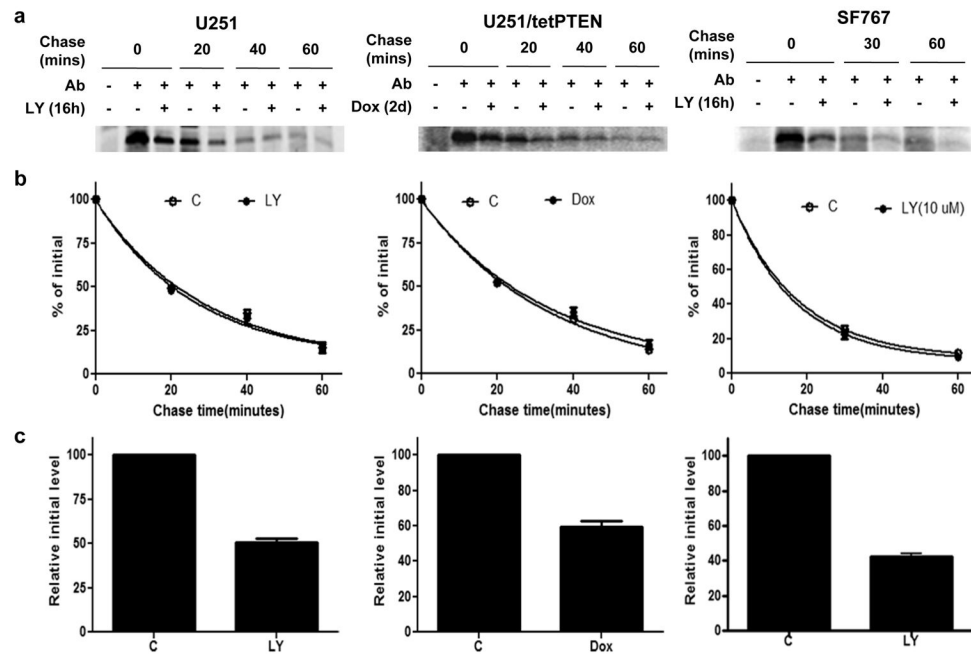
Figure 2.
PI-3K signaling does not alter Id1 protein stability. (**a**) indicated cells were treated with LY (10μM) for 16 hours or Dox (1μg/ml) for 2 days prior to pulsing with ^{35}S-Met/Cys and chasing with unlabeled media as described in Methods. Immunoprecipitated Id1 was resolved by SDS-PAGE and detected by phosphor imaging. A no Id1 antibody (Ab) control (contains rabbit IgG only) was included for each cell line. Representative results of three independent experiments are shown. (**b**) average values of Id1 band intensities from three independent experiments were quantitated by phosphor imaging and normalized to 100% value for each initial time point. Error bars are ± standard error of the mean (SEM). (**c**) average Id1 band intensities from three independent experiments for initial, or 0 minute time point were quantitated by phosphor imaging and normalized to 100% value for the initial time point of each untreated cell line. Error bars are ± SEM.
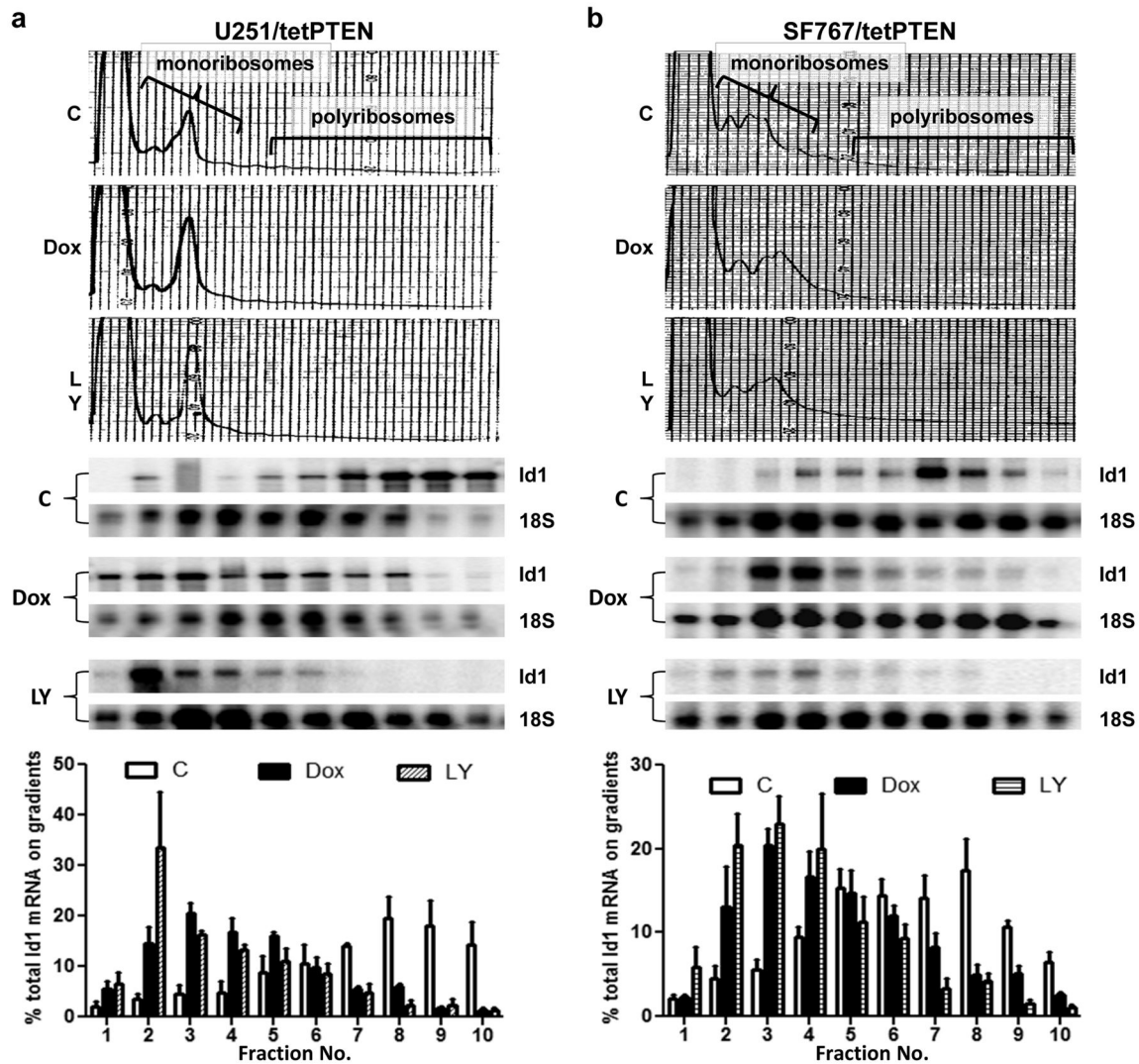
Figure 3.
PI-3K signaling enhances Id1 translational efficiency. Polyribosome profile analyses were
performed in (**a**) U251/tetPTEN and (**b**) SF767/tetPTEN cells as described in Methods.
Cytoplasmic extracts from untreated (C, upper panels), Dox-treated (1μg/ml x 2 days,
middle panels) or LY-treated (10μM x 16 hours, lower panels) cells were prepared in the
presence of $MgCl_2$ for linear sucrose gradient fractionation (15–45%) to separate translating
(polyribosomes, fractions 5–10) from nontranslating (monoribosomes, fractions 1–4)
components, monitored by absorption at OD_{254} (top panels). Amount of Id1 mRNA and
housekeeping 18S rRNA in each fraction was determined by RPA to assess the distribution
of RNAs in translating or non-translating fractions (middle panels). The displayed phosphor
images were representative of three experiments. Graphs show average relative Id1
expression in each fraction from three experiments with error bars ± SEM (bottom graphs).
Quantification of percentage of translating Id1 (fractions 5–10) to total Id1 (fractions 1–10)
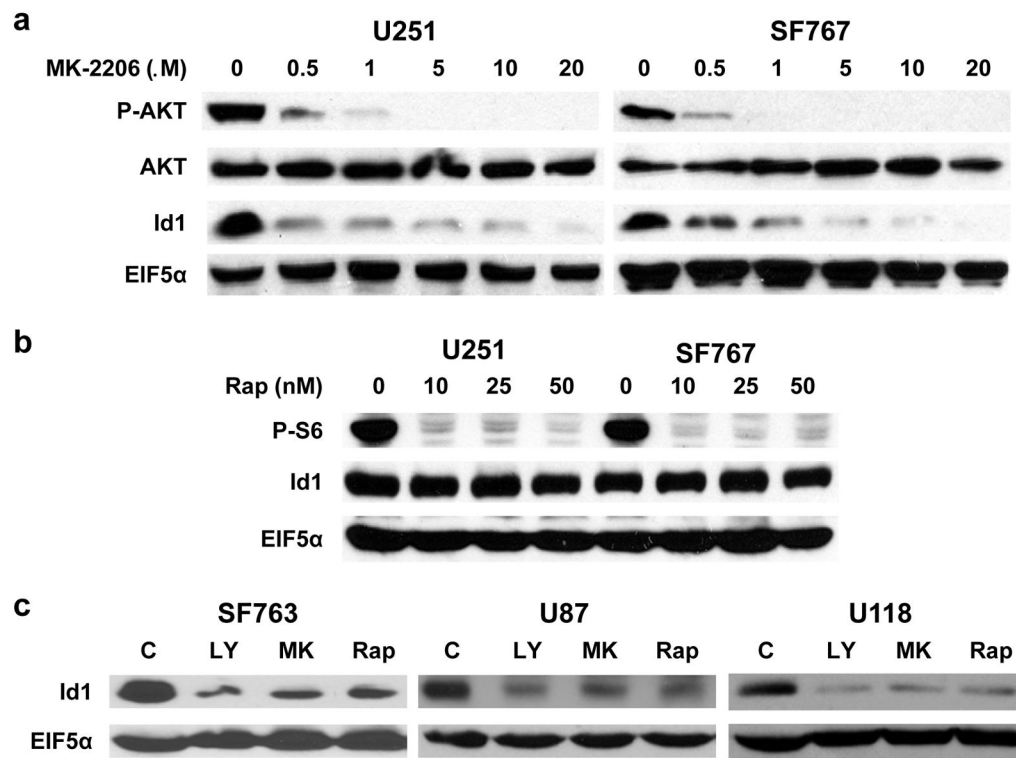for each condition is shown in supplemental Figure 1.

Figure 4.
PI-3K-dependent regulation of Id1 expression is not inhibited by rapamycin. U251 and SF767 were treated with increasing levels of (**a**) the allosteric AKT inhibitor MK-2206 (MK) or (**b**) the mTORC1 inhibitor rapamycin (Rap) for 16 hours and assessed by IB for expression of P-AKT (S473), AKT, phosphorylated S6 (P-S6) and Id1, as indicated. These cells had no significant change in Id1 expression with rapamycin treatment (rapamycin-insensitive). (**c**) SF763, U87 and U118 were treated with LY (10μM), MK (1μM) and Rap (10nM) for 16 hours and assessed by IB for Id1 expression. These cells had significant Id1 repression after treatment with all three inhibitors including rapamycin (rapamycin-sensitive). EIF5α was used as normalization controls. In all cases, representative results from three independent experiments are shown.
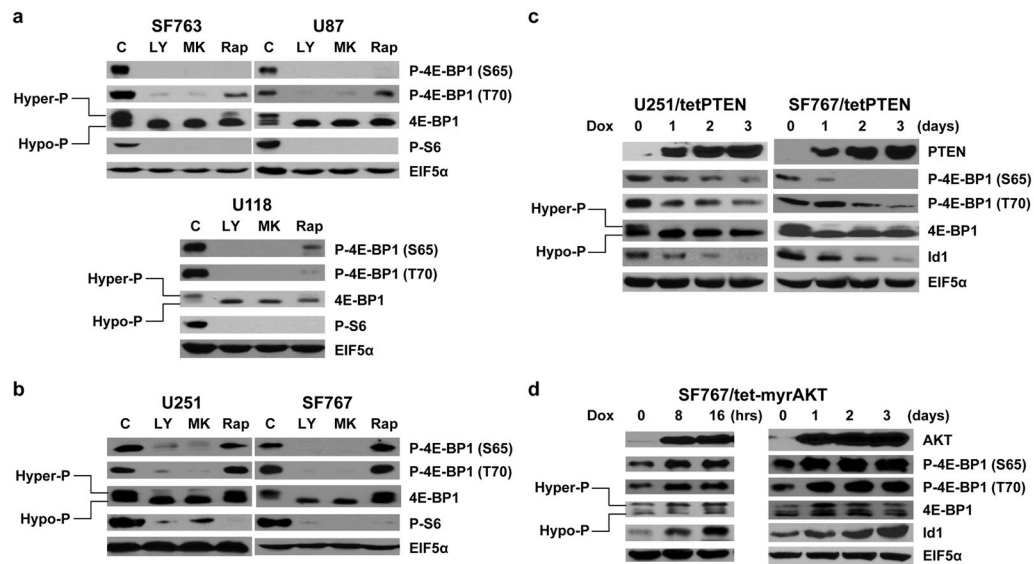
Figure 5.
PI-3K-dependent regulation of Id1 correlates with phosphorylation of 4E-BP1. (**a**) SF763, U87 and U118 (rapamycin-sensitive) or (**b**) U251 and SF767 (rapamycin-insensitive) cells were treated with LY (10μM), MK (1μM) and Rap (10nM) for 16 hours and assessed by IB for expression of phosphorylated 4E-BP1 (P-4E-BP1) (S65 & T70), 4E-BP1 and P-S6. (**c**) U251/tetPTEN and SF767/tetPTEN cells were treated with Dox (1μg/ml) for the indicated days and assessed by IB for PTEN, P-4E-BP1, 4E-BP1 and Id1. (**d**) SF767/tet-myrAKT cells were treated with Dox (1μg/ml) for the indicated times and assessed by IB for AKT, P-4E-BP1, 4E-BP1 and Id1. In all cases, 4E-BP1 doublet bands are marked as slower migrating hyperphosphorylated (Hyper-P) and/or faster migrating hypophosphorylated (Hypo-P) forms. EIF5α was used as normalization controls. Representative results from three independent experiments are shown.
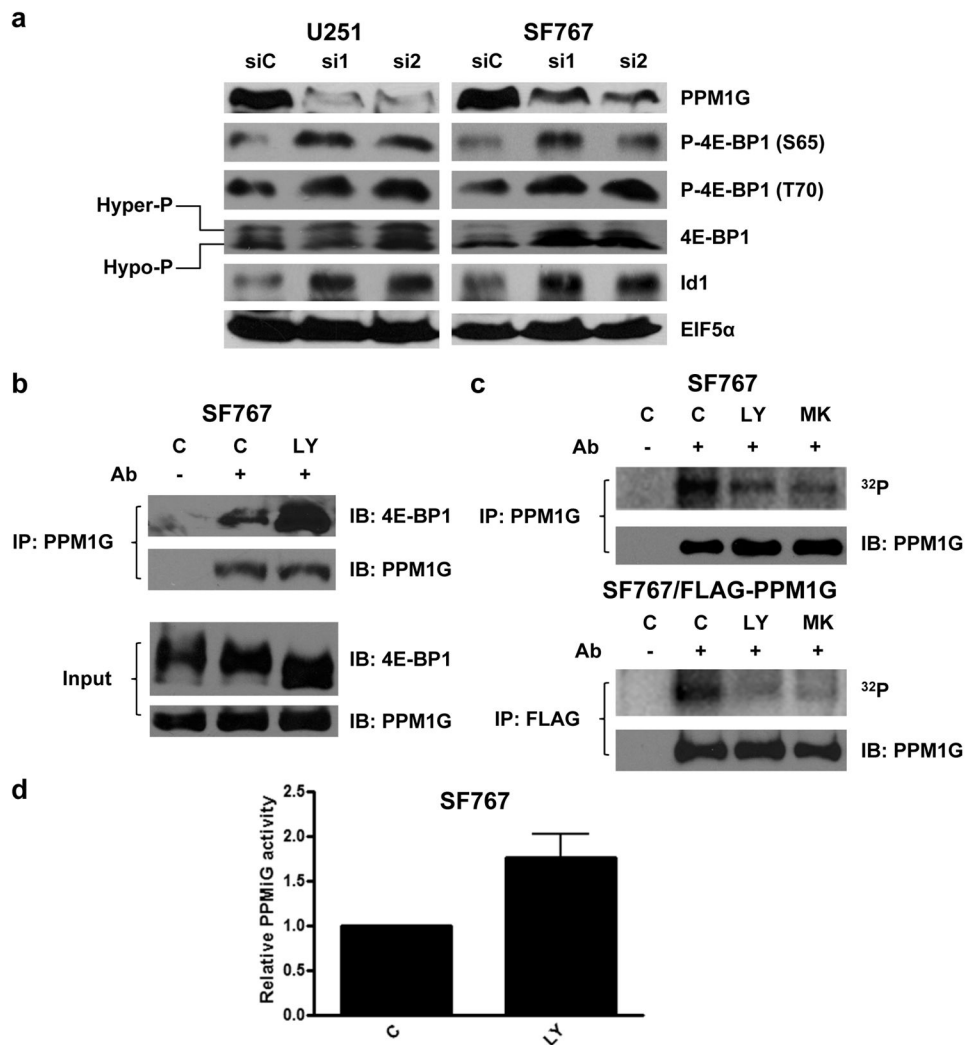
Figure 6.
PPM1G is involved in PI-3K-dependent regulation of 4E-BP1 phosphorylation and Id1
expression. (**a**) U251 and SF767 cells were transiently transfected with siRNA control (siC)
and two different siRNA targeting PPM1G (si1 & si2) and assessed after 48 hours by IB for
PPM1G, P-4E-BP1, 4E-BP1 and Id1. EIF5α was used as normalization controls. (**b**) SF767
cells were treated with or without LY (10μM) for 2 hours, lysed for PPM1G
immunoprecipitation (IP) and assessed by IB for 4E-BP1 (to detect co-association, upper
panels) and PPM1G (lower panels). A no antibody (Ab) control was included. IB assessment
for 4E-BP1 and PPM1G was also performed against the lysates only (input) without IP
(lower panels). (**c**) indicated cells were treated with LY (10μM) and MK (1nM) for 1 hour
prior to *in vivo* labeling with ^{32}P-orthophosphate for 2 hours as described in Methods.
Lysates were harvested and subjected to PPM1G or FLAG IP, resolved by SDS-PAGE and
transferred to PVDF membrane. Membranes were assessed by phosphor imaging to
detect ^{32}P-labeled PPM1G and by IB analysis to detect total PPM1G. Representative
pictures from three independent experiments are shown for A-C. (**d**) Purified PPM1G
activity was analyzed using ^{32}P-labeled MBP as substrate (described in Materials and

Methods). Graph represents fold increase in PPM1G activity after treatment with LY (10μM) for 2 hours. Bars represent average of three independent values with error bars representing ± SEM. Activity for control (C) samples (not treated with LY) was arbitrarily set at one.
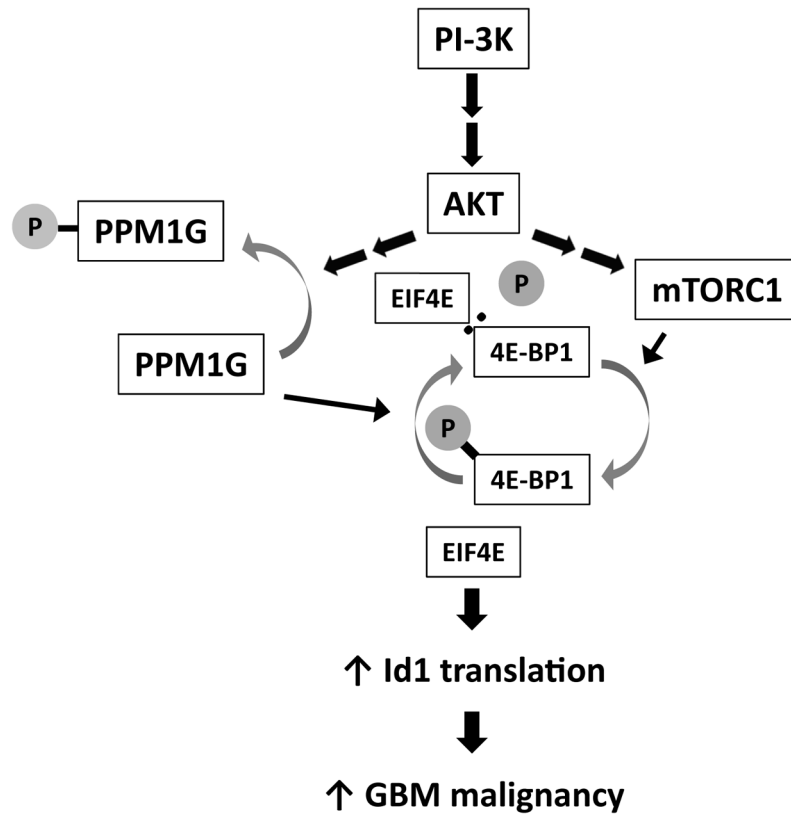
Figure 7.
Working model of PI-3K-dependent Id1 expression is shown. PPM1G is phosphorylated in a PI-3K/AKT-dependent, mTORC1-independent fashion leading to a reduction in phosphatase activity, an increase in the phosphorylation state of 4E-BP1 and activation of Id1 translation resulting in enhanced Id1 expression.