Down-Regulation of Class II Phosphoinositide 3-Kinase α Expression below a Critical Threshold Induces Apoptotic Cell Death

Winfried Elis,[1] Ellen Triantafellow,[1] Natalie M. Wolters,[3] Katie R. Sian,[3] Giordano Caponigro,[2] Jason Borawski,[1] L. Alex Gaither,[1] Leon O. Murphy,[1] Peter M. Finan,[1] and Jeffrey P. MacKeigan[3]

[1]Novartis Institutes for BioMedical Research, Developmental and Molecular Pathways; [2]Novartis Institutes for BioMedical Research, Oncology, Cambridge, Massachusetts and [3]Laboratory of Systems Biology, Van Andel Research Institute, Grand Rapids, Michigan

Abstract

Members of the phosphoinositide 3-kinase (PI3K) family collectively control multiple cellular responses, including proliferation, growth, chemotaxis, and survival. These diverse effects can partly be attributed to the broad range of downstream effectors being regulated by the products of these lipid kinases, the 3′-phosphoinositides. However, an additional layer of complexity is introduced by the existence of multiple PI3K enzyme isoforms. Much has been learned over the last years on the roles of the classes I and III PI3K members in cellular signaling, but little is known about the isoform-specific tasks done by the class II PI3Ks (C2α, β, and γ). In this study, we used quantitative reverse transcription–PCR and RNA interference in mammalian cells to gain further insight into the function of these lesser studied PI3K enzymes. We find that PI3K-C2α, but not PI3K-C2β, has an important role in controlling cell survival and by using a panel of RNA interference reagents, we were able to determine a critical threshold of PI3K-C2α mRNA levels, below which the apoptotic program is switched on, via the intrinsic cell death pathway. In addition, knockdown of PI3K-C2α to levels that by themselves do not induce apoptosis sensitize cells to the anticancer agent Taxol (paclitaxel). Lastly, we report that lowering the levels of PI3K-C2α in a number of cancer cell lines reduces their proliferation and cell viability, arguing that PI3K inhibitors targeting not only the class Iα isoform but also class IIα may contribute to an effective anticancer strategy. (Mol Cancer Res 2008;6(4):614–23)

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Note: W. Elis and E. Triantafellow contributed equally to this work.
Requests for reprints: Jeffrey P. MacKeigan, Laboratory of Systems Biology, Van Andel Research Institute, 333 Bostwick Aveneu NE, Grand Rapids, MI 49503. Phone: 616-234-5682; Fax: 616-234-5733. E-mail: jeff.mackeigan@vai.org
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Introduction

The activation of phosphoinositide 3-kinases (PI3K) in response to extracellular stimuli and the subsequent generation of 3′-phosphorylated phosphoinositides as intracellular second messengers is a hallmark of many signaling pathways (1). This evolutionarily conserved family of enzymes can be classified into three groups according to their primary structure, regulation, and *in vitro* substrate specificity (2). The vast majority of studies conducted thus far have focused on class I and class III PI3Ks, which generate PtdIns-3,4,5-trisphosphate and PtdIns-3-monophosphate, respectively. In particular, they play important roles in growth factor signaling and amino acid sensing and are dysregulated in disease states, such as cancer and diabetes (3, 4).

Fewer reports exist on the role of class II PI3Ks in cellular signaling and disease. The three enzymes (C2α, C2β, and C2γ) in this class are large molecules ranging from 166 to 190 kDa. PI3K-C2α and PI3K-C2β are widely expressed on the mRNA level, whereas expression of PI3K-C2γ is mainly restricted to the liver, breast, and prostate (5, 6). In contrast to classes I and III PI3Ks, which are both dimeric enzymes whose enzymatic activities and subcellular localizations are regulated by various types of adaptor subunits, class II PI3Ks are monomeric enzymes with extended NH_2 and COOH termini. These NH_2 and COOH termini contain multiple protein and lipid interaction domains that target them to various intracellular membranes and the nucleus (7-9).

An increasing number of stimuli have been found to activate the class II α-isoforms and β-isoforms, including growth factors (e.g., insulin, epidermal growth factor, platelet-derived growth factor; refs. 10, 11), chemokines (e.g., MCP-1; ref. 12), tumor necrosis factor-α, leptin (13), and lysophosphatidic acid (14), but the precise mechanism by which most of these upstream signals are actually transmitted to the enzymes and stimulate their lipid kinase activity *in vivo* still remains to be elucidated. Furthermore, whereas class II PI3Ks have been reported to use PtdIns and PtdIns-4-P *in vitro*, it is still controversial which of these lipids are used as substrates *in vivo* (15). Accordingly, little is known on potential downstream effectors of class II PI3Ks. Only recently have cellular roles been investigated for the β-isoform in cell migration (14, 16, 17) and the α-isoform in the regulation of vascular smooth muscle contraction (18).

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The presence of class II PI3Ks in multiple cellular compartments (6), as well as the tight regulation of PI3K-C2α expression and stability (19), argues for additional functions of these enzymes in cellular signal transduction. Furthermore, PI3K-C2α and PI3K-Cβ have been shown to be highly expressed in small cell lung carcinomas, with the β-isoform contributing to epidermal growth factor–mediated phosphorylation of Akt/PKB in these cells (20), raising the question of whether these lipid kinases might be involved in the regulation of cellular proliferation and survival. Indeed, one study recently suggested that abrogation of PI3K-C2α expression in Chinese hamster ovary–insulin receptor expressing cells leads to apoptotic cell death (21); however, concerns over the experimental approach, which included the use of a small number of sense and antisense oligonucleotides with limited sequence specificity in only one cell type, as well as contradictory results reported by other groups, questioned the relevance of these findings (6, 14).

We therefore initiated a study to comprehensively analyze the potential roles of PI3K-C2α and PI3K-C2β in cell survival. To this end, we used a panel of isoform-specific short interfering RNA (siRNA) sequences at low nanomolar concentrations and assessed their efficiency in knocking down expression of PI3K-C2α and PI3K-C2β, as well as any effect this might have on cell viability. We find that PI3K-C2α, but not PI3K-C2β, has an important role in controlling cell survival via the intrinsic cell death pathway. We were also able to quantitatively determine the critical threshold of PI3K-C2α inhibition, below which the apoptotic program is switched on, and further found that at levels above this threshold, cells are sensitized to undergo apoptosis upon exposure to the conventional chemotherapeutic agent Taxol. We expanded our findings to twenty-three additional cancer cell lines and determined that more than half of them show a significant reduction in cell proliferation and viability upon knockdown of PI3K-C2α. Our results therefore indicate that in addition to the inhibition of the class Iα isoform, interference with PI3K-C2α activity might contribute to an effective anticancer strategy in a subset of cancers.

Results

PI3K Isoform Expression Profile

The three classes of human PI3K are composed of eight different catalytic subunits: class I (C1α, C1β, C1γ, C1δ), class II (C2α, C2β, C2γ), and class III (C3 or Vps34). Although expression of some of these enzymes can be restricted to specific cells, most cell types coexpress multiple isoforms (22). The expression profile of all eight isoforms relative to one another has not been shown, and we therefore did real-time quantitative reverse transcription–PCR (qRT-PCR) to determine their relative mRNA levels in HeLa cells (Fig. 1A) and U2OS cells (data not shown). In HeLa cells, the class Iβ, class IIα, and class III isoforms were the predominantly expressed PI3K family members. Remarkably, transcript levels for C2α were six times higher than those for C1α and 33% higher than those for C1β. In addition, the C1β (or p110β) isoform is four times more abundant than the frequently mutated and widely studied C1α (or p110α) isoform (23). qRT-PCR in U2OS cells showed that C2α was again the most abundant PI3K isoform, with mRNA levels 2-fold higher than for C1β or C1δ and 10-fold higher than for C1α. Our results in both cell lines also highlight the restricted expression profile of the PI3Kγ isoforms (C1γ and C2γ), which are mainly found in cells of hematopoietic origin in the case of C1γ (24) and liver, breast, and prostate for C2γ (22, 25-27).

Inhibition of PI3K-C2α Function Leads to Apoptosis via the Intrinsic Cell Death Pathway

To better understand the function of the highly expressed C2α isoform, we transfected HeLa cells with two siRNAs that target unique regions of C2α. Under conditions of >99% transfection efficiency (28), the degree of mRNA knockdown was measured by qRT-PCR, and the physiologic consequences were assessed. Treatment of cells with the isoform-specific C2α-1 and C2α-2 siRNAs resulted in 85% and 86% knockdown of C2α transcript levels, respectively (Fig. 1B), resulting in a dramatic decrease in cell viability and increase in apoptosis with 52% and 46% of the cells being TUNEL positive as measured by flow cytometry (Fig. 1C and Table 1).

To further characterize these effects on a molecular level, we used the siRNA duplexes to target and knockdown PI3K-C2α in HeLa cells and measured the cleavage of poly(ADP-ribose) polymerase (PARP) as a hallmark of apoptosis. In accordance with the observed decrease in cell viability, down-regulation of C2α resulted in an increase in the amount of cleaved PARP relative to that in control cells (Fig. 2A) starting as early as 24 hours after siRNA treatment (Fig. 2B). We also investigated whether any of the initiator caspases were involved in the sequence of events that lead to apoptosis upon knockdown of PI3K-C2α and found that caspase-9 is strongly activated under these conditions and with the same kinetics as PARP cleavage (Fig. 2A and B), indicating a role for the intrinsic cell death pathway that is being activated by various intracellular stresses (29). This was further corroborated by the fact that siRNA-mediated knockdown of the downstream proapoptotic members Bax and Bak resulted in a dramatic suppression of the PI3K-C2α loss of function phenotype (Fig. 2C), implying that ablation of this lipid kinase constitutes intracellular stress leading to mitochondrial dependent apoptosis. This is also supported by the observation that signaling pathways which relay extrinsic signals emanating from cell surface receptors to the interior of the cell via Akt or GSK-3 are unaffected by the knockdown of PI3K-C2α (Fig. 2D).

C2α RNA Interference–Induced Apoptosis Is Specific to C2α Down-Regulation

Although RNA interference (RNAi) is a powerful research tool for selectively silencing the target mRNA and the protein encoded, it is imperative to follow-up initial observations made using a limited number of siRNAs with additional siRNAs to confidently understand the function of a single gene product (30). With this in mind, we used a total of six unique C2α siRNA sequences in a DNA fragmentation assay as another hallmark of apoptosis to exclude the possibility of off-target effects contributing to our observations. Again, each of the six individual C2α siRNAs exhibited a significant increase in apoptosis over control (scrambled) siRNA (Fig. 3A).

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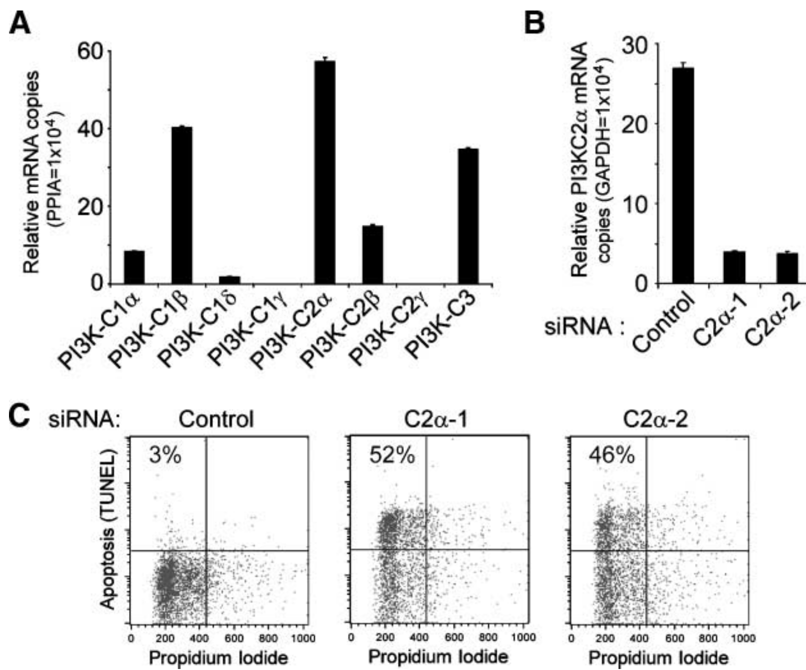

FIGURE 1. PI3K class II α inhibition leads to decreased cell viability in HeLa cells. **A.** PI3K isoform expression in HeLa cells. Total RNA was isolated, and primers are designed to specifically amplify each of the indicated PI3K classes I, II, and III isoforms. Expression levels were determined using qRT-PCR and comparative C_t method for relative mRNA expression to PPIA. **B.** Real-time qRT-PCR knockdown analysis using C2α siRNA duplexes. HeLa cells were transfected with two different siRNA duplexes (25 nmol/L) directed against C2α, and target knockdown was measured by qRT-PCR. **C.** TUNEL analysis of cell death. HeLa cells were transfected with either control siRNA or two individual C2α siRNA duplexes (25 nmol/L) as indicated. After 72 h of C2α knockdown, the cells were examined for cell viability by phase contrast and the level of apoptosis determined by TUNEL staining.

Besides using multiple siRNA duplexes to corroborate our observations, we also wanted to establish the lowest effective siRNA concentration to rule out potential off-target effects. To this end, we selected the most potent siRNA (C2α-1) from our panel of C2α siRNAs, titrated in the amount of siRNA from 1 to 10 nmol/L, and again did the ELISA for histone-DNA fragmentation. The level of mRNA knockdown with 1, 5, and 10 nmol/L C2α-1 siRNA was 57%, 75%, and 80%, respectively, with a concomitant increase in apoptotic cell death (Fig. 3B). Importantly, the apoptotic phenotype correlates with the degree of C2α suppression. From these and earlier experiments, we have determined a 25 nmol/L concentration of C2α siRNA, corresponding to an 85% knockdown, as the lowest effective C2α siRNA concentration which gives a maximal C2α suppression.

Lastly, we wanted to determine whether the C2α siRNAs were having any off-target effects on other PI3K family members due to partial sequence conservation that might account for the apoptotic phenotype. Thus, HeLa cells were transfected with C2α-1 siRNA at 25 nmol/L for 30 hours, and transcript levels of each PI3K isoform were measured. As shown in Fig. 3C, levels of all PI3K isoforms expressed in HeLa cells remained virtually unchanged in the presence of C2α-1 siRNA, whereas the level of C2α mRNA is dramatically reduced. Note that there is a slight decrease in the PI3K-C1β mRNA level with the C2α-1 siRNA. However, using four specific C1β siRNAs that each knockdown mRNA levels by 90%, 87%, 91%, and 88%, we did not observe any effect on cell viability, indicating that the apoptosis phenotype observed is a result of selective PI3K-C2α reduction and not due to some off-target effect on PI3K-C1β. Furthermore, knockdown of mRNA levels of additional PI3K isoforms (C1α, C1δ, and C3) with siRNAs neither displayed off-target effects with regard to PI3K-C2α transcript levels nor did these siRNAs induce cell death.

Table 1. Time Course of PI3K-C2α siRNA-Mediated Apoptosis

siRNA	Time (h)	% TUNEL+
Control	—	3-7
C2α-1	24	50
C2α-2	24	51
C2α-1	48	82
C2α-2	48	93
C2α-1	72	52
C2α-2	72	46

Critical Threshold of C2α Reduction Required for Apoptosis

It is well-established that the levels of signaling proteins within a cell play an important role in setting the threshold for their action in many cellular functions, including apoptosis. However, whereas class I PI3K-specific inhibitors have been described, no class II PI3K-specific compounds exist which could be used to investigate whether cells can be sensitized to apoptosis by inhibiting PI3K-C2α function (6, 31). The use of two common PI3K inhibitors, LY294002 and wortmannin, has identified class I PI3Ks as playing an important role in cell survival signaling. Many studies seem to attribute the effects of these pan-PI3K inhibitors to the inhibition of class I PI3K alone, although the sensitivity of PI3K-C2β and PI3K-C2γ to these inhibitors is similar to that of human class I and class III PI3Ks (32). Furthermore, many studies use compound concentrations far higher than normal for class I PI3K inhibition, which begin to inhibit even the more resistant PI3K-C2α enzyme (33).

We therefore investigated whether we could establish a threshold of PI3K-C2α inhibition and determine whether HeLa

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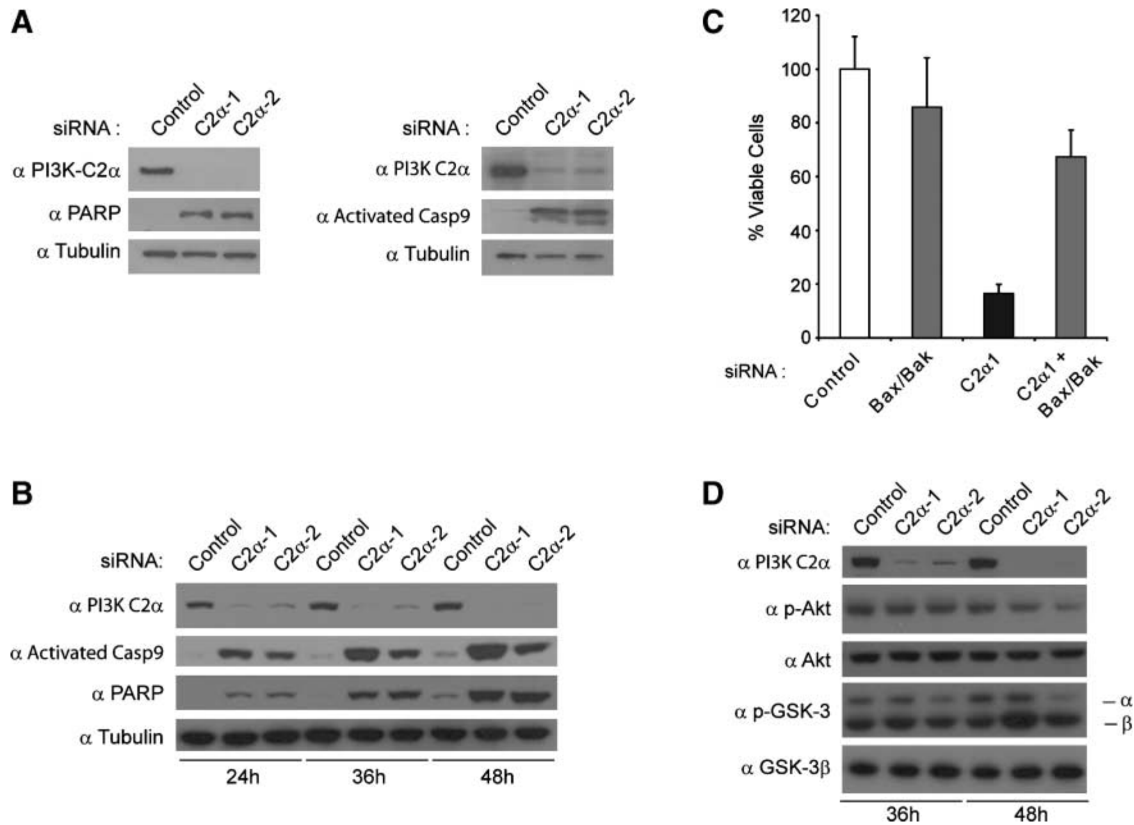

FIGURE 2. Knockdown of PI3K class II α leads to apoptosis via the intrinsic pathway. **A.** Activated caspase-9 and the caspase substrate PARP were detected by Western blot analysis. HeLa cells were transfected with either control or two individual C2α siRNAs (25 nmol/L) for 72 h and immunoblotted for PI3K-C2α knockdown, cleaved PARP, and caspase-9 activation. **B.** Kinetics of PI3K-C2α knockdown, caspase-9 activation, and cleaved PARP. **C.** Knockdown of proapoptotic factors Bax and Bak enhances survival of cells with depleted PI3K-C2α levels. HeLa cells were cotransfected with either siRNAs against C2α or Bax/Bak or cotransfected with all three siRNAs, and cell viability was measured after 72 h. **D.** Knockdown of PI3K-C2α does not affect the PI3K class I/Akt cell survival pathway. HeLa cells were transfected with either control or two individual C2α siRNAs (25 nmol/L) and immunoblotted for PI3K-C2α knockdown, phosphorylated Akt (S^{473}), Akt, phosphorylated Gsk-3α/β ($S^{21/9}$), and Gsk-3β at the indicated time points (36 or 48 h) after siRNA transfection.

cells could be "sensitized" to undergo apoptosis. To achieve this, we used three cell lines stably expressing different C2α short hairpin RNAs (shRNA; ref. 34). The shRNAs used reduced PI3K-C2α mRNA and protein levels to varying degrees (Fig. 4A). We then titrated varying amounts of C2α-1 siRNA (1-25 nmol/L) and did the DNA fragmentation ELISA. At the lowest dose (1 nmol/L), the mRNA level for PI3K-C2α in control cells was reduced by >50%, although we did not observe any increase in apoptosis (>2-fold increase). However, at 5 nmol/L or higher of C2α-1 siRNA, transcript levels were reduced by 75% and cell death could be observed (Fig. 4B and C). In the shRNA-1 sensitized cells, the basal level of apoptosis was already higher than in control cells and similar to the extent of apoptosis observable after treatment of these control cells with 5 nmol/L C2α-1 siRNA. In addition, exposure of the sensitized HeLa cells to as little as 5 or 10 nmol/L of C2α-1 siRNA further decreased cellular viability (Fig. 4C). We also tested whether cells with reduced levels of PI3K-C2α are more sensitive to conventional chemotherapeutics and found that a combination of Taxol and PI3K-C2α knockdown is much more potent than Taxol alone (Fig. 4D). Taken together, these results show that a decrease in PI3K-C2α expression sensitizes cells to undergo apoptosis, with a target knockdown of ≥75% required to switch-on apoptosis in all C2α RNAi-sensitized cell lines tested.

Depletion of C2β Does Not Induce Apoptosis

The class II PI3Ks are large monomeric enzymes with similar domain structures and *in vitro* substrate specificity. Unlike other PI3Ks, which were identified based on their cellular function, all three class II PI3K members were identified through lipid kinase domain homology searches and cloned by PCR due to the presence of C2 and PX domains at their carboxyl terminus (25-27, 33). To investigate whether the other ubiquitously expressed C2 enzyme, C2β, may have a role similar to that of C2α in regulating cell survival, four different siRNA duplexes against C2β were used to knockdown mRNA levels in HeLa cells. Each siRNA effectively suppressed both mRNA transcript (Fig. 5A) and protein levels (Fig. 5B), but had no effect on cell viability as measured by DNA fragmentation ELISA (Fig. 5C). Thus, our findings, as well as those of other research groups, suggest that the PI3K isoforms C2α and C2β have distinct functional roles in cells. Indeed, although both isoforms are highly conserved, they also contain divergent NH_2 terminal regions that may account for isoform-specific protein-protein interactions, resulting in distinct roles in cellular

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signaling. These data show that the α-isoform of class II PI3K, but not the β-isoform, is an important survival kinase.

PI3K-C2α Is Required for Cell Survival in Many Cancer Cell Lines

Having established that PI3K-C2α plays an isoform-specific role in cellular survival in at least HeLa cells, we wanted to determine whether this is a general hallmark of cancer lines. To further investigate this question, 23 cancer cell lines from bladder, breast, brain, colon, kidney, liver, lung, ovarian, pancreas, prostate, skin, and thyroid carcinomas were infected with a PI3K-C2α shRNA lentivirus (C2α-shRNA). The C2α-shRNA potently reduces PI3K-C2α mRNA levels, and the effects on cell viability were measured (Table 2). As expected, not all of the cancer cell lines tested were equally susceptible to the down-regulation of PI3K-C2α. Importantly, more than half (13 of 23) of them showed a reduction of 50% or more in cell survival when compared with the parental cell line that was infected with a scrambled shRNA control lentivirus. In total, 21 of 23 cell lines from various tumor types had decreased cell viability to some extent after PI3K-C2α RNAi-mediated down-regulation. However, sensitivity to reduced levels of PI3K-C2α was also apparent in one of the normal cell lines tested (human umbilical vascular endothelial cell), but not in two additional cell lines, normal human bladder smooth muscle cells, and human lung epithelial fibroblast (WI-38) cells (Fig. 6B). This raises the possibility that C2α may have a tissue specific role in the survival of endothelial cells and carcinoma cells. Indeed, these data confirm and extend our previous observations to show that PI3K-C2α is required for cell survival in a large number of carcinoma cell lines.

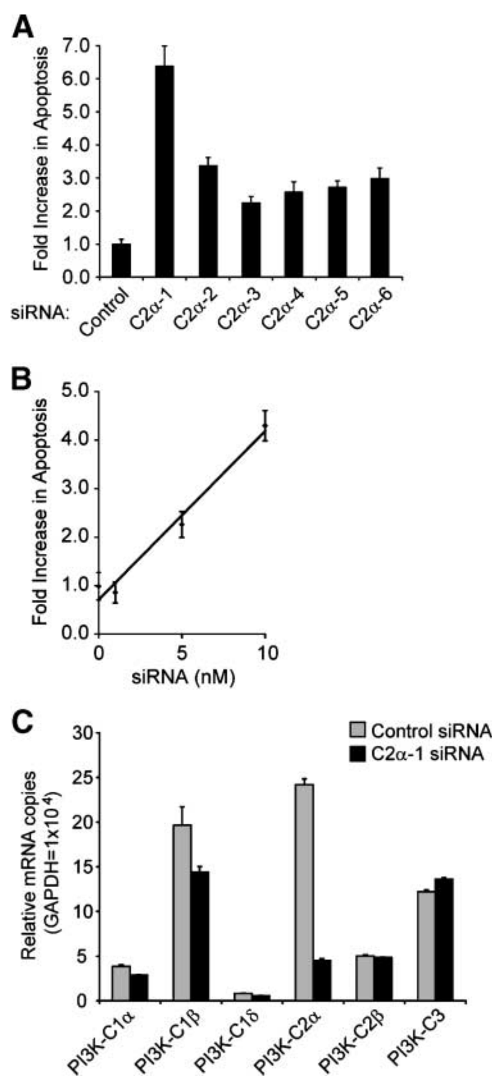

FIGURE 3. Induction of apoptosis is due to specific knockdown of PI3K class II α expression. **A.** Apoptosis confirmed with multiple siRNA duplexes directed against C2α. HeLa cells seeded in 96-well plates were transfected with control (scrambled) siRNA or six unique C2α siRNA duplexes. Cells were incubated for 72 h to allow for target knockdown, and apoptosis was measured by a DNA-fragmentation ELISA. Basal apoptosis is set at one and relative apoptosis for the six C2α siRNA duplexes was determined. **B.** C2α siRNA dose-response curve. HeLa cells were transfected with 0, 1, 5, or 10 nmol/L C2α-1 siRNA duplex and apoptosis measured as indicated above. **C.** Measurement of C2α siRNA off-target effects on PI3K family members using qRT-PCR. HeLa cells were transfected with either C2α-1 siRNA or control siRNA duplexes, total RNA was isolated, and primers were used to specifically amplify each of the indicated PI3K classes I, II, and III isoforms. Expression was determined using qRT-PCR and comparative C_t method for relative mRNA expression to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Discussion

Previous studies have shown that class II PI3Ks act downstream of several growth factors (10, 11) and chemokines (12), but the cellular responses that are controlled by these lipid kinases have only slowly begun to emerge. Thus far, both PI3K-C2α and PI3K-C2β have been implicated in the regulation of membrane trafficking (9, 35), whereas the β-isoform has been described to play an additional isoform-specific role in cellular migration (14). PI3K-C2α has also been found to be rapidly degraded in proliferating cells upon exposure to genotoxic stress and at the G_2-M transition during the cell cycle (19), leading to speculation that class II PI3Ks could be involved in the regulation of cellular proliferation or survival. Indeed, earlier reports had shown that the β-isoform is activated by insulin (10), an important growth factor, and in turn plays a role in the regulation of Akt/PKB (20), a known survival kinase.

In this study, we investigated the effect of ablation of expression of the class II PI3Ks on cell viability, and our results identify PI3K-C2α as a crucial cellular survival factor. Whereas HeLa cells can tolerate a strong decrease in the expression of the β-isoform, a reduction in the α-isoform by 75% is sufficient to induce apoptosis. Furthermore, our study indicates that PI3K-C2α fulfills this crucial role not only in HeLa cells, but also in a significant number of cancer cell lines from various organs and tumor stages. At the same time, the human umbilical vein endothelial cells are susceptible to low levels of PI3K-C2α whereas the human bladder smooth muscle cells and human lung epithelial fibroblast cell lines (WI-38) cells are not, so clearly other factors exist that can be exploited in certain cancer contexts. The cellular mechanism by which PI3K-C2α contributes to cell survival is thus far unknown, but seems to involve suppressing or countering caspase-9 activation, thus

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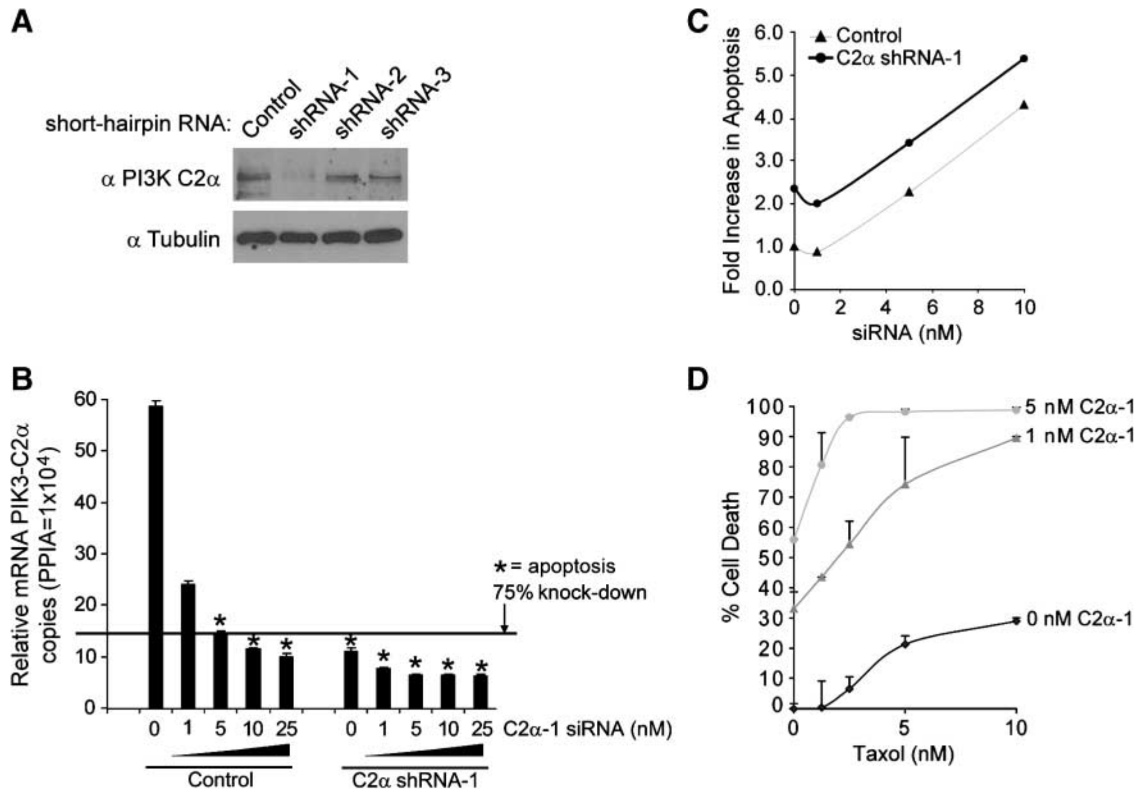

FIGURE 4. Critical threshold of C2α target inhibition required to switch on apoptosis. **A.** HeLa cells were infected with either control or three different C2α shRNA-encoding lentiviruses and PI3K C2α protein levels were detected by Western blot analysis. **B.** Control or C2α shRNA-1 expressing cells were incubated with C2α-1 siRNA (0, 1, 5, 10, or 25 nmol/L) to measure target knockdown by qRT-PCR and apoptosis by a DNA fragmentation ELISA. Expression was determined using qRT-PCR and comparative C_t method for relative mRNA expression to PPIA. *, indicates >2-fold DNA fragmentation relative to control cells. **C.** C2α shRNA suppression sensitizes cells to low nanomolar C2α siRNA-induced apoptosis. HeLa cells were infected with either control or C2α shRNA-1 virus followed by increasing concentrations of C2α-1 siRNA, and apoptosis was measured by ELISA. **D.** PI3K-C2α siRNA sensitizes cells to the chemotherapeutic agent Taxol (paclitaxel). HeLa cells were transfected with suboptimal levels of C2α-1 siRNA (0, 1, or 5 nmol/L) for 48 h and then treated with increasing concentrations of Taxol (0, 1.25, 2.5, 5, or 10 nmol/L) for an additional 24 h, and percentage of cell death was measured.

potentially linking PI3K-C2α to the intrinsic cell death pathway and mitochondrial integrity. Interestingly, the presence of several other class IA and class III PI3K family members is not sufficient to protect the cells from the effects of PI3K-C2α down-regulation, arguing for an important and specific role of this lipid kinase in cell survival.

Our findings fit nicely with the observations made by other groups that expression of PI3K-C2α is not only highly regulated in time (i.e., during the cell cycle and upon exposure of cells to genotoxic stress), but also in space (as evidenced by the existence of distinct cytoplasmic and nuclear compartments; refs. 7, 19). Indeed, it is probably this tight regulation that blocked our attempts, as well as those of other groups, to stably express exogenous protein (19), which would have allowed us to undertake a rescue experiment designed to save cells from apoptosis that were treated with siRNAs directed against the 3′ untranslated region of the mRNA coding for PI3K-C2α. Nevertheless, through the identification of several different siRNAs that at low nanomolar levels were able to abolish both mRNA and protein levels as well as the use of shRNAs that either sensitized cells to siRNA-induced apoptosis or caused cell death themselves, we have increased the likelihood that the apoptotic phenotype we observe can be attributed to on-target down-regulation of PI3K-C2α and not to an off-target effect on some other factor.

Similar to the three class IA PI3Ks, it is thus far unknown why many cells coexpress at least two class II PI3Ks. PI3K-C2α and PI3K-C2β show many similarities in their primary structure. The human proteins are 44% identical and 71% similar on the amino acid sequence level, although these numbers are only 19% and 47% in the first 400 amino acids, respectively. The same can be said for their domain structure and subcellular localization, which helps to explain their overlapping function in membrane trafficking. At the same time, the sequence differences have the potential to also link the two isoforms to different signaling pathways, thus accounting for their nonredundant roles in cell survival and migration.

The identification of class II PI3Ks as key players in cellular functions ranging from cell migration and vascular smooth muscle contraction to cell survival merits follow-up studies aimed at identifying the factors acting immediately upstream and downstream of these lipid kinases, as well as characterizing the domains responsible for these interactions. It is also worth keeping in mind that these functions of class II PI3Ks might not merely rely on their lipid kinase activity, because PI3K-C2α and PI3K-C2β are large enzymes that could also serve as platforms for the assembly of protein complexes. Together, such studies will not only shed light on the mechanisms by which class II PI3Ks contribute to the above-mentioned cellular

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functions, but they might also uncover novel roles for these long-neglected lipid kinases in cell signaling.

Materials and Methods

Cell Culture

HeLa, A2058, A549, MDA-MB-468, PC-3, RKO, SW579, and T24 cells were maintained in DMEM, HEPG2, J82, SK-HEP-1, and U-87MG cells were cultured in Eagle's MEM; Mia PaCa-2 cells were grown in DMEM containing glutamax; and 786-O, A-375, BxPC-3, DU145, HCT116, HT-29, MDA-MB-231, NCI-H1299, NCI-H838, and SK-OV-3 cells were maintained in RPMI 1640 and supplemented with 10% heat-inactivated fetal bovine serum. LN-18 cells were cultured in DMEM supplemented with 5% fetal bovine serum. Human umbilical vein endothelial cells were grown in EGM-2, bladder smooth muscle cell in SmGM2, and WI-38 in DMEM with 10% fetal bovine serum and nonessential amino acids.

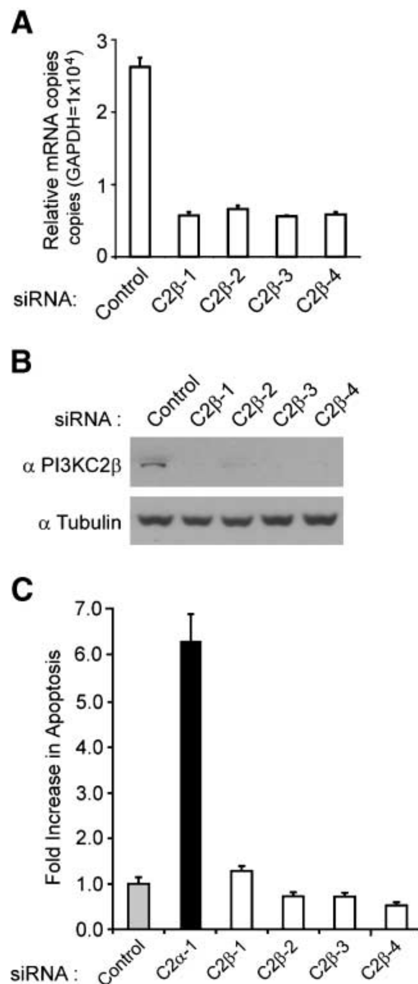

FIGURE 5. C2β does not have a functional role in apoptosis. **A** and **B.** qRT-PCR and Western blot analysis of C2β siRNA duplexes. HeLa cells were transfected with four different siRNAs (25 nmol/L) directed against C2β or control siRNA. Total RNA was isolated 30 h after transfection to measure C2β mRNA levels and cell lysates were generated after 72 h for C2β Western blot analysis. **C.** Suppression of C2β does not result in apoptosis. Apoptosis was measured 72 h after C2α or C2β siRNA transfection by ELISA that detects DNA-histone fragmentation, and the data are expressed as fold increase in absorbance relative to control-treated cells.

Table 2. Cell Viability and Knockdown of Carcinoma Cell Lines after PI3K-C2α RNAi

Cell line	Carcinoma	% Viability	% Knockdown
MDA-MB-231	Breast	51 ± 1	80
HCT 116	Colon	49 ± 1	78
RKO	Colon	51 ± 5	76
NCI-H838	Lung	44 ± 3	90
NCI-H1299	Lung	85 ± 6	82
MiaPaca-2	Pancreas	22 ± 1	79
PC-3	Prostate	55 ± 5	82
A2058	Skin	49 ± 4	91
A375	Skin	68 ± 3	75

RNAi Sequences

The siRNA sequences were synthesized by Dharmacon or Qiagen as 21-mer oligoribonucleotides with a 19-bp duplex region and two deoxynucleotide overhangs on the 3′ terminus of each strand. The Dharmacon siRNA sequences are a set of four siGENOME duplexes (MQ-006771-00-0020 for PIK3C2A and MQ-006772-00-0020 for PIK3C2B). The Qiagen siRNA sequence design uses a BIOPREDsi potency predictor algorithm to score 21-mer oligoribonucleotides (36).

The cDNA sequences against which siRNAs were prepared are C2α-1 (agaggaagtgctgcagaataa), C2α-2 (ttgaagagagatcgacagcaa), C2α-3 (aaggatttcagctaccagtta), C2α-4 (cacaaggaagcttacctatct), C2α-5 (cagaatcagtaaagaagctaa), C2α-6 (ccagaacttacaagtattcaa), C2β-1 (tggccggaagcttctgggttt), C2β-2 (gacatcaacactttctctttg), C2β-3 (ctcaagagctctggccgaatc), and C2β-4 (atgctgagaccctgcgtaaga).

shRNA clones containing the following sequences coding for shRNAs against PIK3C2A were obtained from The RNAi Consortium: shRNA-1 (ccggccacttatgctttaccttctactcgagtagaaggtaaagcataagtggttttt) TRCN0000002228, shRNA-2 (ccgggctagtgtgaaggtctccattctcgagaatggagaccttcacactagcttttt) TRCN0000002229, shRNA-3 (ccggcaaagaagtatggaacgagtactcgagtactcgttccatacttctttgttttt) TRCN0000002231, shRNA-4 (ccggcgagcagtagatcaagtaattctcgagaattacttgatctactgctcgttttt) TRCN0000002230.

siRNA Transfection and shRNA Infection

For siRNA transfection, HeLa and U2OS cells (2×10^5) were cultured in six-well plates for 24 h and transfected with siRNA duplexes using Oligofectamine (Invitrogen) at the indicated concentrations. Cells were lysed after 30 h for RNA isolation and after 72 h for protein levels. shRNA lentivirus production and infection conditions for HeLa cells were optimized in six-well plates (34, 37). Cells were seeded at a density of 2×10^5 per well in a six-well plate, incubated for 24 h, infected using 1 mL of shRNA lentiviral supernatant, and incubated for 7 d and more using 2 μg/mL puromycin selection. Viral supernatants for all other cell lines were produced in 293T cells using a modified version of the three plasmid transfection protocols described in (refs. 38, 39). Briefly, 293T cells were transfected with a mixture of two

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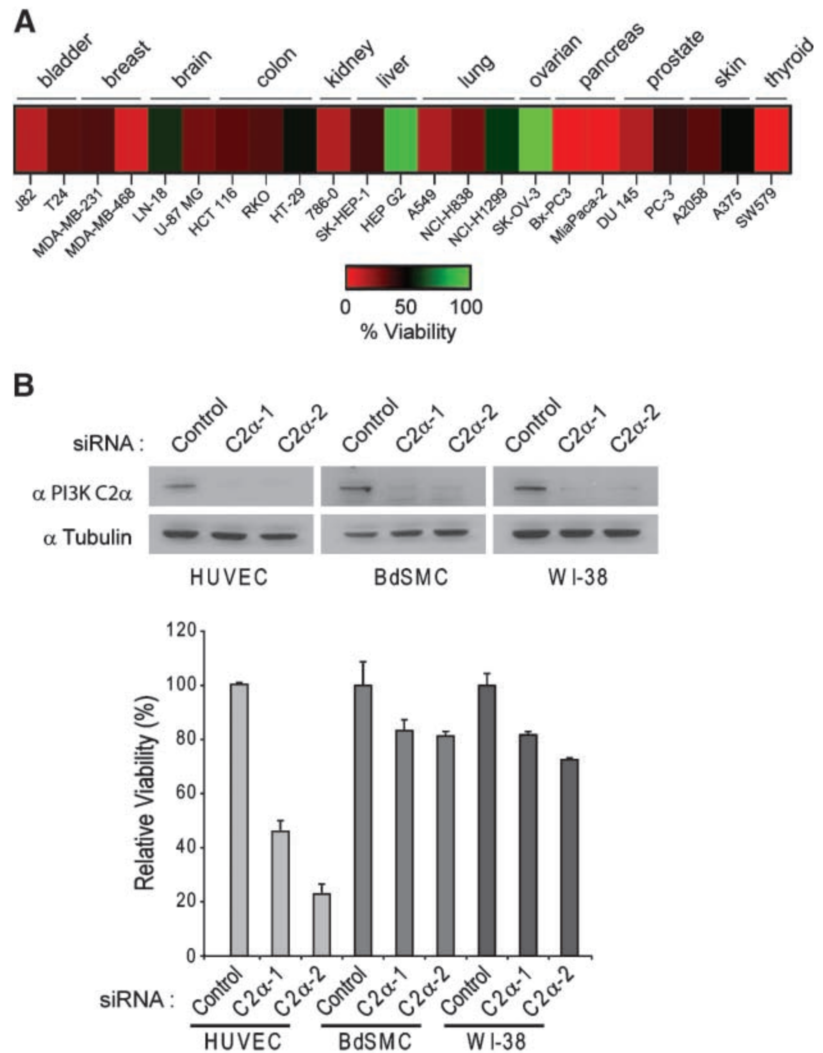

FIGURE 6. Susceptibility of carcinoma and normal cell lines to knockdown of PI3K-C2α. **A.** Heatmap depicting cell viability of twenty-three carcinoma cell lines following P13K-C2α RNAi. Decreased cell viability compared with nontargeting control shRNA is in red and those unchanged or up-regulated are in green. The cell viability/proliferation assay is a measurement of the change in cell number over either a 5-d or 9-d period. Numbers given represent percentage of viability of C2α shRNA-infected cells compared with cells that were infected with a virus expressing a nontargeting control shRNA (Table 2). Percentages of knockdown of PI3K-C2α mRNA levels are indicated where available (Table 2). **B.** Human umbilical vein endothelial cells (*HUVEC*), human bladder smooth muscle cells (*BdSMC*), and human lung epithelial fibroblast cell lines (WI-38) were transfected with 25 nmol/L of either control siRNA or two individual C2α siRNA duplexes, and cell viability was measured. In a parallel experiment, PI3K-C2α protein levels were detected by Western blot analysis after 72 h of C2α knockdown.

helper plasmids, pVSV-G (40) and pCMVΔR8.9 (39), and the specific pLKO-shRNA expressing vector (34) along with Transit-293 lipid transfection reagent (Mirus). After 18 to 24 h, the medium was replaced, and after an additional 72h, supernatants containing viral particles were harvested, filtered through 0.45 μmol/L cellulose-acetate and filtrates frozen down at −80°C until use. Cells to be infected were plated in black-sided clear-bottomed 96-well plates at densities ranging from 500 to 4,000 cells per well depending on the particular cell type 18 to 24 before infection with virus. Infections were done in 200 μL of media supplemented with 8 μg/mL polybrene at 37°C and 5% CO_2 for 4 h using between 0.1 and 2.0 μL of titer-normalized viral supernatant/well depending on the particular cell type. Medium was replaced 12 to 24 h later with 100 μL fresh medium and puromycin added to the wells after an additional 12 to 24 h (final concentration, 0.5-2.0 μg/mL).

Real-time qRT-PCR

Total RNA was extracted 30 h after siRNA transfection and purified using the RNeasy mini kit (Qiagen). Subsequent cDNA synthesis was done using the StrataScript first strand cDNA synthesis kit (Stratagene). For real-time qRT-PCR, 81 ng of total cDNA was mixed with 10 μL of 2× Taqman Universal PCR master mix (Applied Biosystems) and 1 μL of 20× Taqman gene expression assay (Applied Biosystems; see below for respective catalogue number) in a total volume of 20 μL. Also included were no template controls and no reverse transcriptase controls preceding cDNA synthesis step to check for contamination by genomic DNA. Samples were analyzed in a 384-well plate using the ABI Prism 7900HT with the following cycling variables: (*a*) 50°C for 2 min, (*b*) 95°C for 10 min, (*c*) 95°C for 15 s, (*d*) 60°C for 1 min, repeat steps 3 to 4 for a total of 40 times. Each reaction was run in triplicate, with cyclophilin A (PPIA) or glyceraldehyde-3-phosphate dehydrogenase used as endogenous controls. The samples were then analyzed using default settings (background: cycles 3-15, threshold set at 0.2), and a comparative C_t method was used to obtain relative copy numbers. The following Taqman gene expression assays were acquired from Applied Biosystems: PI3KC1α (Hs00180679_m1), PI3KC1β (Hs00178872_m1), PI3KC1δ (Hs00192399_m1), PI3KC1γ

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(Hs00176916_m1), PI3KC2α (Hs00153223_m1), PI3KC2β (Hs00153248_m1), PI3KC2γ (Hs00362135_m1), PI3KC3 (Hs00176908_m1), PPIA (Hs99999904_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1).

TUNEL Analysis

Floating and adherent cells were collected, washed in PBS, and fixed in 1% paraformaldehyde followed by 70% ethanol. Cells were washed and DNA breaks were incubated in DNA labeling solution containing TdT and bromo-dUTP at 37°C for 60 min. Cells were then incubated for 30 min with anti-BrdUrd FITC-conjugated antibody (Becton Dickinson), washed in 0.5% Tween 20 plus 1% bovine serum albumin in PBS, resuspended in propidium iodide stain buffer (0.5% Tween 20, 250 μg/mL DNase-free RNase A, 20 μg/mL propidium iodide). After staining, samples were analyzed by fluorescence-activated cell sorting with a FACScan flow cytometer (Becton Dickinson), and data were acquired and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

Apoptosis ELISA

HeLa cells seeded 24 h earlier in 96-well plates (3,000 per well) were transfected with the indicated siRNAs. In each siRNA experiment, scrambled siRNA duplexes were used as negative controls. After 72 h of target knockdown, quantification of apoptotic cell death was determined by an ELISA that measures cytoplasmic histone-DNA fragments produced during apoptosis (Roche). The 96-well plates were centrifuged (200 × *g*) for 10 min, the supernatants were discarded, and lysis buffer was added. After lysis, the samples were centrifuged and 20 μL of the supernatant were transferred to a strepavidin-coated microtiter plate. Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well, and the plate was incubated at room temperature for 2 h. After three washes with buffer, the peroxidase substrate was added to each well. After 10-min incubation, the plates were read at 405 nm in a microplate reader. The enrichment of histone-DNA fragments is expressed as fold increase in absorbance compared with control (nonsilencing) siRNA.

Cell Viability Assay

To determine growth of the various cell lines after knockdown of PIK3C2α, relative cell number was measured at two different times using Cell Titer Glo or Cell Titer Blue (Promega) according to the manufacturer's instructions. The initial measurement of cell number was taken 72 h after the addition of puromycin, once uninfected cells had been removed via selection. The second measurement was taken either 5 or 9 d after the initial measurement, once control cells had doubled three to five times. For experiments using siRNAs, relative cell number was quantified 72 h after target knockdown. All luminescence measurements were taken using an Envision plate reader (Perkin-Elmer) and were done on three replicate wells. For each infection, the fold change in cell number over the time course was determined by calculating the ratio of the first and second cell number measurements and comparing it to the ratio obtained for cells infected with a virus encoding a nontargeting shRNA.

Cell Lysis and Immunoblotting

Cell extracts were prepared by washing cells in ice-cold PBS and harvesting in lysis buffer (pH 7.2; 10 mmol/L KPO_4, 1 mmol/L EDTA, 10 mmol/L $MgCl_2$, 50 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 0.5% NP40, 0.1% Brij-35, 1 mmol/L sodium orthovanadate, 40 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 5 μg/mL pepstatin A). Extracts were centrifuged at 15,000 rpm for 10 min at 4°C, and cell lysates were immunoblotted using standard procedures using anti-PI3K p170, anti-PI3K class II β, anti-GSK-3β (BD Biosciences), anti-PARP, anti-caspase-9, anti-pGSK3α/β(S21/9), anti-Akt, and anti-pAkt(S473) (Cell Signaling Technology), and anti-tubulin (Sigma) antibodies.

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