



Published in final edited form as:

Cell Death Differ. 2010 April ; 17(4): 677–688. doi:10.1038/cdd.2009.150.

PI3K p110 α and p110 β have differential effects on Akt activation and protection against oxidative stress-induced apoptosis in myoblasts

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Abstract

Catalytic subunits of PI3K play a critical role in growth factor signaling and survival by phosphorylating inositol lipids. We found that PI3K Class IA p110 α and p110 β have distinct functions in myoblasts. Inhibition of p110 α reduced IGF-I-stimulated Akt activity and prevented IGF-I-mediated survival in H₂O₂-treated cells; in contrast, siRNA knockdown of p110 β increased IGF-I-stimulated Akt activity. However, inhibition of p110 β catalytic activity did not increase IGF-I-stimulated Akt activity, suggesting a role for p110 β protein interactions rather than decreased generation of phosphoinositides in this effect. Increased Akt activity in p110 β -deficient myoblasts was associated with diminished ERK activation as well as ERK-dependent IRS-1 636/639 phosphorylation, findings we show to be independent of p110 β catalytic function, but associated with IGF-IR endocytosis. We also report that IGF-I protects myoblasts from H₂O₂-induced apoptosis through a mechanism that requires p110 α , but may be independent of Akt or ERK under conditions of Akt and ERK inhibition. These observations suggest that both p110 α and p110 β are essential for growth and metabolism in myoblasts. Overall, our results provide new evidence for the roles of p110 isoforms in promoting cellular proliferation and homeostasis, IGF-IR internalization, and in opposing apoptosis.

Introduction

Members of the phosphoinositide-3-OH kinase (PI3K) class of enzymes generate phosphoinositol products that act as second-messengers in a number of intracellular signaling cascades (1). PI3K catalytic enzymes are categorized into three classes by their structure, substrate specificity, and lipid products (2). Members of the Class IA PI3Ks (α , β , and δ) are heterodimers consisting of a 110 kDa catalytic subunit and an 85, 55, or 50 kDa regulatory subunit (2, 3). Activation of PI3K leads to proliferation and pro-survival effects;

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however, dysregulation of PI3K signaling can promote aberrant proliferative signals leading to cellular transformation (4-7).

Examination of the ubiquitously expressed PI3K Class IA p110 α and p110 β catalytic subunits has revealed distinct and redundant roles for each depending on cellular context (8-10). Heterozygous knock-in of catalytically-inactive p110 α or pharmacological inhibition of p110 α with a small-molecule inhibitor has been shown to negatively regulate growth, metabolism, and growth factor signaling in mice (10, 11). p110 β has been shown to mediate cell growth and development, DNA replication, insulin sensitivity, tumorigenesis, and G-protein-coupled receptor-activated signaling (12-15). Recently, a kinase-independent, scaffolding role of p110 β has been suggested; indeed, this kinase-independent function may play a role in cell proliferation and clathrin-mediated endocytosis (13, 16). Mice doubly heterozygous for p110 α and p110 β showed mild glucose intolerance and reduced sensitivity to insulin challenge associated with p85 protein instability, but showed no difference in insulin-stimulated Akt phosphorylation or activity in liver or muscle as compared to control mice (17).

Insulin-like growth factor I (IGF-I) is a hormone that promotes proliferation, differentiation, and survival in a number of cells types mediated principally through PI3K/Akt, and Ras/Raf/MEK/ERK pathways (18-20). IGF-I/PI3K signaling has been shown to promote survival in several apoptosis-inducing models such as serum withdrawal and oxidative stress (21-23); however, the specific PI3K and Akt isoforms involved in survival have yet to be established. Likewise, PI3K/Akt-independent compensatory survival mechanisms have yet to be fully resolved. We report here that C2C12 myoblasts transfected with siRNA against p110 α , p110 β , or a combination of both, displayed differential phenotypes with respect to cell growth and PI3K-dependent signaling. Furthermore, we found that IGF-I stimulation differentially regulated Akt phosphorylation and activation in a PI3K- and Akt- isoform-specific fashion, and that p110 α was the principal Class IA PI3K mediating IGF-I anti-apoptotic actions. Finally, we report that knockdown p110 β negatively regulates IGF-IR internalization and ERK activation, which was associated with relief of feedback inhibition of PI3K-dependent signal transduction.

Results

Reduced cell proliferation in cells treated simultaneously with siRNA against p110 α and p110 β is associated with increased apoptotic markers, whereas reduced cell proliferation in cells treated with siRNA against p110 β alone is not

We first determined that both p110 α and p110 β are expressed at the mRNA and protein level in C2C12 myoblasts (Figure 1a), but that p110 γ and p110 δ were not expressed appreciably. In order to establish efficacy of siRNA-mediated knockdown of p110 α and p110 β , cells were transfected with siRNA against p110 α (si-p110 α), p110 β (si-p110 β), or non-targeting control siRNA (si-Con). Addition of si-p110 α resulted in ~60-65% reduction in p110 α mRNA, while addition of si-p110 β resulted in ~65% reduction in p110 β mRNA (Figure 1b). mRNA levels of p110 β were unaffected by p110 α knockdown, and mRNA levels of p110 α were unaffected by p110 β knockdown. To determine whether cell growth was affected by knockdown of p110 α , p110 β , or the combination (si-p110 α + β), cells were

counted at 24 and 48 hours after transfection. Addition of si-p110 β and si-p110 α + β caused a reduction in cell number at 48-hours for si-p110 β ($P < 0.05$) and at 24- and 48-hours for si-p110 α + β (Figure 1c). This reduction in cell number was associated with increased cleavage of Caspase-3 and Poly (ADP)-ribosyl-polymerase (PARP), a molecule downstream of Caspase-3 and an indicator of apoptosis (Figure 1d)(24). Because knockdown of p110 α can reduce PIP(3,4,5)3 levels (11), and p110 β may act to maintain basal levels of PIP(3,4,5)3 (10), we analyzed Akt (also known as protein kinase B), an AGC kinase family members whose activation is known to be regulated by phosphoinositides (25). Forty-eight hours after transfection, levels of phospho-Akt in the hydrophobic domain (Ser473/474/472 in Akt1, Akt2, and Akt3, respectively; hereafter referred to as S473) showed decreased phosphorylation after treatment with p110 α ; conversely, phospho-Akt Ser 473 levels were increased after treatment with si-p110 β alone (Figure 1e). The levels of phosphorylated Akt did not correlate with total p85 levels, as reductions in total p85 were observed in p110 α - and p110 β - deficient cells, but were even further reduced when cells were treated simultaneously with si-p110 α and si-p110 β (Figure 1e).

RNAi knockdown of p110 α reduces, and knockdown of p110 β increases, IGF-I-stimulated Akt phosphorylation and activity

Because IGF-I can mediate pro-survival effects through Akt, we sought to determine the responsiveness of p110 α - and p110 β -deficient cells to IGF-I. Addition of IGF-I to cells transfected with either si-p110 α , si-p110 β , or si-p110 α + β resulted in increased Akt phosphorylation at the activation loop (Thr308/309/305 in Akt1, Akt2, and Akt3, respectively; hereafter referred to as T308). This increase was reduced in si-p110 α -treated cells, but further increased in si-p110 β -transfected cells (Figure 2a). IGF-I-stimulated phospho-Akt in cells treated with both si-p110 α and p110 β together was not statistically lower than si-Con cells treated with IGF-I (Figure 2a). Because the sequences surrounding the Akt active site (T308) and hydrophobic motif (S473) residues are similar between isoforms, and because the antibody cannot discriminate between isozymes, we performed *in vitro* kinase assays to determine relative contributions of each isoform. IGF-I-stimulated Akt1 activity was reduced in cells transfected with si-p110 α and si-p110 α + β ; conversely, Akt1 activity was increased in response to IGF-I in cells treated with si-p110 β (Figure 2b). Only cells transfected with si-p110 α alone showed reduced Akt3 activity, and there was no increase or decrease in Akt3 activity in cells transfected with either p110 β or p110 α + β , respectively. Akt2 activity was unchanged by any treatment, and long exposures (shown in Figure 2b) likely represent background. Taken together, these data suggest that knockdown of p110 α reduces IGF-I-stimulated Akt phosphorylation and activation of both Akt1 and Akt3. IGF-I-induced Akt1 activity was greater in si-p110 β -transfected cells than IGF-I-stimulated Akt1 activity in control cells, which correlated with increased Akt phosphorylation. Yet, IGF-I-stimulated Akt3 activity did not show this elevation, suggesting that the increase in total Akt phosphorylation may be the result of increased Akt1 activation in p110 β -deficient cells. On the other hand, in cells transfected with si-p110 α + β , Akt1 activity was diminished, but Akt3 activity was not, suggesting that the increase in IGF-I-stimulated Akt phosphorylation in si-p110 α + β -treated cells as compared to si-p110 α alone, reflects increased Akt3 activation.

To test whether this increased Akt activity in p110 β knockdown cells correlated with increased p110 association with components of the IGF-IR signaling complex, we performed immunoprecipitation experiments. Treatment of cells with IGF-I promoted p110 α and p110 β association with IRS-1 and IGF-IR at early time points following administration (Figure 3a, left). In p110 α -deficient cells, IGF-I-induced association of both p110 α and p110 β with IGF-IR and IRS-1 was reduced, whereas in p110 β -deficient cells, association of p110 α with IGF-IR and IRS-1 was enhanced (Figure 3a, right). Simultaneous knockdown of p110 α and p110 β prevented IGF-I induced association of both p110 α and p110 β with IGF-IR and IRS-1. These findings suggest that IGF-I-induced association of p110 α with IGF-IR and IRS-1 is enhanced when p110 β levels are reduced. We next determined whether p110 α or p110 β deficiency affected IGF-I-stimulated PI(3,4,5)P3 production. IGF-I promoted PI(3,4,5)P3 generation from p110 α and p110 β immunoprecipitates, and this effect was reduced when cells were deficient in the respective isoform (Figure 3b). Levels of PI(3,4,5)P3 in IGF-I-treated cells transfected with si-p110 β were intermediate between unstimulated and IGF-I-stimulated control cells. Altogether, these data suggest that Akt activation correlates with levels of p110/IGF-IR complex, but in cells deficient in p110 β , mechanisms other than PI(3,4,5)P3 production may contribute to elevated Akt activation.

Knockdown of p110 β is associated with decreased ERK signaling and IGF-IR internalization

To further elucidate the mechanism whereby Akt phosphorylation and activity is increased in p110 β -deficient cells, as well as to further characterize intracellular responses to p110 catalytic subunit deficiency, we examined other molecules involved in canonical IGF-I signaling pathway. In cells deficient in p110 β alone, or deficient in p110 α and p110 β , we found that phosphorylation of ERK was reduced in the basal state as well as after stimulation with IGF-I (Figure 4a). Additionally, IGF-I-stimulated phosphorylation of IRS-1 as Ser 636/639 was attenuated under conditions of reduced p110 β , a finding consistent with previous work suggesting a dependence on ERK for IRS serine phosphorylation at this site (26).

To determine whether decreased ERK phosphorylation seen in p110 β -deficient cells was due to an overall decrease in p110 β protein levels or due to decreased catalytic activity, cells were treated with increasing concentrations of TGX-221, an inhibitor of p110 β catalytic function. Addition of TGX-221 reduced LPA-induced Akt phosphorylation in a dose-dependent fashion, but did not affect IGF-I-stimulated ERK phosphorylation at any concentration tested (Figure 4b). These data suggest that the decreased levels of phosphorylated ERK observed in p110 β -deficient cells may result from a loss of total p110 β rather than loss of catalytic function, a finding consistent with a scaffolding action of p110 β .

To address whether this catalytic-independent function of p110 β mediates IGF-IR internalization, we examined the presence of IGF-IR at the cell surface after IGF-I treatment in the absence or presence of TGX-221 or si-p110 β . Treatment of myoblasts with IGF-I resulted in a reduction in cell-surface IGF-IR that was maximal after 30-minutes (Figure 4c, left). In IGF-I-stimulated cells pre-treated with 100 nM TGX-221, IGF-IR internalization

was similar to IGF-I treatment alone; however, in cells deficient in p110 β , IGF-IR did not internalize in response to IGF-I (Figure 4c, right). These findings suggest that at least one kinase-independent role of p110 β in myoblasts is to mediate IGF-IR internalization.

IGF-I acts through PI3K p110 α to prevent H₂O₂-induced apoptosis

To establish whether the differential signaling effects of p110 α and p110 β knockdown extend to physiological actions in a pro-apoptotic milieu, cells were examined to determine whether knockdown of p110 isoforms was sufficient to inhibit IGF-I from preventing oxidative stress-induced cell death. To first test this, we employed an inhibitor specific to PI3K p110 α (“p110 α i”) (27), since broad-spectrum PI3K pharmacological inhibitors such as LY294002 and wortmannin do not discriminate between isoforms. Caspase-3 and PARP cleavage were increased in cells treated with 400 μ M H₂O₂ for 4-hours, an effect that was completely prevented by a 30-minute pre-treatment with IGF-I (Figure 5a and 5b, lanes 1-4). Caspase-3 and PARP cleavage was increased in p110 α i and p110 α i + H₂O₂ -treated cells, and p110 α i completely prevented IGF-I from inhibiting apoptosis (Figure 5a and 5b lanes 7-8; compare to lanes 1-2). In contrast, treatment of myoblasts with IGF-I in the presence of the p110 β inhibitor TGX-221 did not prevent IGF-I from reducing H₂O₂-stimulated Caspase-3 and PARP cleavage (Figure 5c, right).

To confirm these results, we employed siRNA directed against p110 α and p110 β . Cells were pre-treated with IGF-I, treated with H₂O₂, or both in the presence or absence of si-p110 α , si-p110 β , or si-p110 α + β . Similar to the inhibitor results, IGF-I was unable to prevent H₂O₂-induced cleavage of caspase-3 and PARP in cells deficient in p110 α whether transfected with si-p110 α alone (Figure 5d left) or in combination with si-p110 β (Figure 5d, right). si-p110 β -treated cells appeared partially resistant to H₂O₂-induced apoptosis (Figure 5d, middle), possibly a result of increased Akt activation (Figure 2). Taken together, these data suggest that IGF-I acts through PI3-K p110 α isoform to prevent H₂O₂-induced apoptosis in myoblasts exposed to oxidative stress.

Since reduction of p110 β effectively inhibited phosphorylated ERK levels while simultaneously increasing Akt activation, we next tested whether Akt was a primary survival intermediate in p110 β knockdown cells. Maximal inhibition of IGF-I-stimulated phosphorylation of Akt S473 was obtained at a concentration of 10 μ M using a compound specific for Akt (“Akti;” ref. (28)) (Figure 5e, left). IGF-I was unable to prevent H₂O₂-induced apoptosis in p110 β -transfected cells (Figure 5e, right) also treated with Akti, thus confirming that a functional Akt pathway is necessary for survival in p110 β -deficient myoblasts.

IGF-I acts through alternative signaling pathways to prevent apoptosis induced by deficiency of p110 α alone, p110 α + p110 β , or by H₂O₂

We noted that knockdown of p110 α or p110 α + β increased Caspase-3 and PARP cleavage (Lanes 1-2 compared to lanes 5-6 Figure 5a, 5b, and 5d), an effect that was reversed by IGF-I administration. To determine the identity of pathway(s) or molecule(s) involved in this effect, cells were treated with an inhibitor of Akt (Akti) or of MEK (U0126). Cells were pre-incubated with inhibitors of increasing concentrations for one hour, and then exposed to

IGF-I for 4.5 hours. Ten micromolar U0126 completely prevented IGF-I-stimulated phosphorylation of p42/p44 ERK at 30-minutes, and 4.5-hours after IGF-I administration, levels of phospho-ERK remained slightly below that of IGF-I-naive cells (Figure 6a, left). Cells treated with si-p110 α or si-p110 α + β were pre-incubated with either Akti or U0126 and then stimulated with IGF-I for 4.5 hours. Pharmacological blockade of MEK in IGF-I-treated p110 α -deficient cells prevented IGF-I-induced survival, and blockade of Akt increased p110 α -deficiency induced apoptosis beyond that of the MEK inhibitor (Figure 6a, right). However, in p110 α + p110 β -deficient cells, inhibition of Akt, but not MEK, fully prevented IGF-I pro-survival signaling. These data suggest that when p110 α , and by extension Akt activation, is reduced, IGF-I survival signaling compensates by increased flux through MAPK pathway. On the other hand, when cells are deficient in both p110 α and p110 β , IGF-I signals primarily through Akt to promote survival.

Because IGF-I differentially acts through Akt and MEK pathways to promote survival from p110 α and p110 α + p110 β - deficiency-induced apoptosis, we sought to determine whether these pathways were also involved in preventing H₂O₂-induced apoptosis. Blockade of either Akt, ERK, or the concurrent inhibition of both, did not prevent IGF-I from inhibiting Caspase-3 or PARP cleavage (Figure 6b). These data suggest that, although Akt and ERK pathways can compensate for each other in response to IGF-I, at least one more mechanism exist through which IGF-I can exert anti-apoptotic effects in response to oxidative stress in myoblasts.

Discussion

In this work, we report that myoblasts deficient in p110 α and/or p110 β show disparate growth and intracellular signaling phenotypes, corresponding with differential regulation of Akt and ERK signaling pathways. One key observation was that knockdown of p110 α inhibited, but knockdown of p110 β promoted, the activation of Akt in an isoform-specific manner. Although p110 β may play a secondary role in growth factor signaling by maintaining a basal pool of PI (3, 4, 5)P3 (10, 13), it is not generally thought to play a primary role in RTK-instigated signaling except under certain conditions such as PTEN loss, or in certain cell types (2, 29). Our results suggest that p110 β deficiency leads to increased Akt activation through two separate mechanisms: First, reduced p110 β levels enhance the IGF-I-induced association of p110 α with IGF-IR and IRS-1. Second, reduced p110 β levels inhibit phosphorylation of ERK and IRS-1 at S636/639, thereby relieving IRS-associated negative feedback. One intriguing finding was that increased IGF-I-stimulated Akt activation in p110 β -deficient cells did not correlate with increased PI(3,4,5)P3 production, a finding consistent with previous results in skeletal muscle of insulin-stimulated *p110 α ^{+/-} / p110 β ^{+/-}* mice (17). Those authors suggested that decreased p85 levels may contribute to insulin-sensitivity, and indeed, in our study we observed lowered p85 in p110 β -deficient cells (Figure 1e).

Furthermore, our finding that serine phosphorylation of IRS-1 at S636/639, a site dependent on ERK (26) is reduced, is consistent with a relief of negative PI3K-signaling feedback (30). Since IGF-I-stimulated Akt activity did not statistically differ between control and p110 α + p110 β -deficient cells (Figure 2b), yet there was virtually no p110/IGF-IR/IRS-1 association

(Figure 2c, right), the loss of feedback inhibition in p110 α + p110 β -deficient myoblasts may explain the mechanism underlying the similarity in Akt activation. Indeed, in skeletal muscle of insulin-stimulated p110 α ^{+/-}/p110 β ^{+/-} mice, Akt phosphorylation was similar to that observed in muscle of insulin-treated wild type mice. It is possible that other mechanism(s) contribute to increased Akt activation in p110 β -deficient cells, including decreased actions of Akt-specific phosphatases (31, 32), but whether activation of these phosphatases is reduced under these conditions remains to be investigated. Altogether, our observations suggest that increased IGF-I-stimulated Akt activity in p110 β -deficient myoblasts may result from both an increase in p110 α /IRS-1/IGF-IR association as well as decreased negative feedback to IRS-1 secondary to reduced ERK activation. That distinct Akt isoforms are differentially regulated suggests a high degree of fine-tuning in these processes. Figure 7 presents this signaling model diagrammatically.

The lower levels of ERK phosphorylation in p110 β deficient cells may be reflective of decreased association of p110 β with Ras, an upstream molecule in the MAPK signaling pathway; indeed, ERK activation has been shown to be attenuated under conditions where p110 β cannot bind to Ras (4, 5). However, use of TGX-221, a compound that inhibits p110 β catalytic activity, did not prevent ERK phosphorylation in response to IGF-I in this study. These results suggest that non-catalytic functions of p110 β may be essential for ERK activation in myoblasts; in support of this, cells deficient in p110 β also showed decreased IGF-IR internalization in addition to attenuated ERK signaling. This is in agreement with previous work suggesting a requirement for IGF-IR internalization for activation of upstream MAPK pathway components in CHO cells (33); additionally, p110 β itself has been shown to modulate EGFR and transferrin uptake (13, 16). Taken together, these data and ours support the hypothesis of a kinase-independent function of p110 β in IGF-IR internalization and signaling. Consequently, p110 β may play a role in IGF-I-mediated processes in skeletal muscle such as progenitor cell proliferation, survival, and differentiation, which occur during muscle development and repair/regeneration.

In light of the findings above, it was not surprising that inhibition of p110 α by means of pharmacological blockade or siRNA completely prevented IGF-I from reducing Caspase-3 and PARP cleavage in response to H₂O₂, whereas TGX-221- or RNAi-induced silencing of p110 β did not (Figure 5a-d). Akt is a critical mediator of anti-apoptotic signaling, acting to suppress apoptosis by phosphorylation and inactivation of pro-apoptotic molecules such as apoptosis-signal regulating kinase-1 (Ask1) (34), Bad (35), and FoxO3a (36). Because Akt signaling was reduced after p110 α knockdown or treatment with p110 α i, it would logically follow that suppression of Akt-targeted pro-apoptotic molecules would also be attenuated. Likewise, because Akt activation was elevated with p110 β knockdown, and this was found to be necessary for survival, IGF-I-stimulated Akt actions would also be elevated.

Myoblasts deficient in p110 α or p110 α and p110 β together showed increased Caspase-3 and PARP cleavage, an effect fully reversed by IGF-I. We found that inhibition of MEK prevented IGF-I from attenuating p110 α -deficiency-induced Caspase-3 and PARP cleavage, and that inhibition of Akt caused apoptosis in excess of p110 α deficiency alone. Inhibition of Akt, but not MEK, prevented IGF-I from attenuating p110 α + p110 β -deficiency-induced Caspase-3 and PARP cleavage (Figure 6a). These data suggest that IGF-I can act through a

MEK-dependent pathway to rescue cells from apoptosis associated with p110 α deficiency, but that myoblasts under these conditions are more sensitive to loss of Akt, possibly resulting from the reduced Akt activation associated with p110 α deficiency. Likewise, in cells simultaneously deficient in p110 α and p110 β , blockade of Akt prevented IGF-I from opposing apoptosis, whereas blockade of MEK had minimal effects. This is consistent with our findings that ERK activation is inherently reduced under conditions of p110 β deficiency, whether alone or in combination with p110 α reduction.

We found that IGF-I was still able to prevent H₂O₂ -induced apoptosis even under conditions of Akt and MEK blockade. However, our results also suggest a dependence on p110 α . Therefore, our data collectively suggest that IGF-I is capable of preventing H₂O₂-induced apoptosis through an Akt- and MEK-independent, but p110 α -dependent mechanism. Since other AGC kinases are also regulated through p110 α in response to IGF-I, it is possible that one or several may contribute to opposing apoptosis. For example, serum- and glucocorticoid-inducible kinase 1 (SGK1) can phosphorylate and inhibit pro-apoptotic FoxO3a at the same sites as Akt, although there exists a preference for some sites over others (37).

In conclusion, we found that knockdown of p110 α and p110 β have differential effects on cell growth, survival signaling, and Akt activation. We have defined a kinase-independent role for p110 β in IGF-IR internalization and ERK phosphorylation, which is associated with negative feedback to the IGF-IR/IRS-1 signaling complex. Additionally, we identified p110 α as the primary Class IA PI3K involved in IGF-I-mediated rescue from oxidative stress-induced apoptosis and that, in the absence of Akt and MEK-dependent signaling, IGF-I can still prevent H₂O₂-induced apoptosis, but through a mechanism that requires p110 α .

Materials and Methods

Materials

C2C12 murine myoblasts were purchased from ATCC (Manassas, VA). H₂O₂ and bovine serum albumin (fraction V) was purchased from Sigma (St. Louis, MO). rhIGF-I was purchased from Austral Biologicals (San Ramon, CA). Primary antibodies directed against p110 β and GAPDH and HRP-linked secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA); all other antibodies were obtained from Cell Signaling Technologies (Danvers, MA). PI3K α inhibitor IV ("p110 α i" in this manuscript, corresponds to figure 15e in ref (27)), TGX-221, Akt 1/2 inhibitor VIII ("Akti"), and U0126 were purchased from Calbiochem (San Diego, CA). LPA (18:1 oleoyl) was purchased from Avanti Polar Lipids (Alabaster, AL).

Cell culture conditions and siRNA transfections

C2C12 myoblasts were maintained in high-glucose DMEM containing 10% FBS and antibiotics (growth medium), with medium being replenished after 24-hours. Forty-eight hours after initial seeding cells were ~95% confluent and unless otherwise indicated, experiments were conducted under these conditions (in growth medium). Pre-designed Silencer Select siRNAs for mouse p110 α (ID# s71604), p110 β (ID# s93108), and negative

controls (non-targeting siRNA ID# 4390843) were purchased from Ambion Inc. (Austin, TX). Cells (1.8×10^5 /well in 6-well culture dish) were reverse-transfected with double-stranded siRNA in antibiotic-free DMEM plus 10% FBS using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Twenty-four hours after reverse transfection, medium was changed to DMEM with 10% FBS containing antibiotics. RNA was isolated 48-hours after transfections using RNA-STAT (Tel-test, Friendswood, TX), quantified, and the level of silencing quantitated by real-time PCR as described later in this section. In some cases, protein was isolated 48h after transfection and the level of silencing determined by Western Blot.

Cell Counts

Medium was removed from plates and monolayers detached with 0.05% Trypsin-EDTA (Gibco). Cells were suspended in growth medium and counted in a hemacytometer chamber.

RNA Isolation, cDNA synthesis, and real-time PCR

Conversion of total RNA to single-strand cDNA was accomplished using the High-Capacity cDNA Archive Kit (P/N 4322171; Applied Biosystems, Foster City, CA). Briefly, 2 μ g total RNA was reverse transcribed using random primers for the following incubation times: 25° C for 10 minutes, then 37° C for 2 hours. cDNA samples were stored at -80° C until use. TaqMan-MGB p110 α (Mm00435673_m1), p110 β (Mm00659576_m1), p110 γ (Mm00445038_m1), p110 δ (Mm00435674_m1) and B2M (Mm00437762_m1) probe and primers were purchased from Applied Biosystems as "Gene Expression Assays." The real-time PCR reaction was performed within an ABI 7500 thermal cycler. The fluorescence of 3 to 15 cycles was set up as background. Data was collected at the annealing step of each cycle, and the threshold cycle (Ct) for each sample calculated by determining the point at which the fluorescence exceeded the threshold limit. The standard curve was calculated automatically via software by plotting the Ct values against each standard of known concentration and calculation of the linear regression line of this curve. Serially diluted amounts of RNA were used to establish standard curves. All samples were run in duplicate.

Protein extraction, immunoprecipitation, and Western immunoblot

C2C12 cells were harvested in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml Leupeptin, 1 mM PMSF, and 1: 100 dilution of phosphatase inhibitor cocktails 1 and 2 (Sigma)). Homogenates were triturated through a small-bore needle, incubated on ice for 30 min, and then centrifuged at 14,000 \times g for 10 min at 4°C. Protein concentrations were determined by the method of Bradford (38). For immunoprecipitations, 200 μ g of protein lysate were co-incubated with 4 μ L of respective antibody in 200 μ L cell lysis buffer with gentle rotation overnight at 4°C. 20 μ L of Protein A/G agarose slurry (Santa Cruz) were then added and rotated an additional 2 - 4 hours at 4°C. Complexes were centrifuged at 14, 000 \times g and washed 5 times in wash buffer before addition of 20 μ L 2 \times Laemmli buffer and then boiled for 5 minutes. For Western blotting, equal amounts of cell lysate proteins (typically 25 μ g) were electrophoresed through denaturing SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore Corp.,

Bedford MA). Membranes were incubated for 1 h in 5% dry milk solution in Tris-buffered saline plus 0.5% Tween-20 (TBST) and then incubated with the appropriate primary antibody at an appropriate dilution (as described in Table 1) overnight in 5% BSA in TBST. Membranes were washed three times in TBST followed by incubation with the appropriate secondary antibody and again washed three times. Membranes were incubated with enhanced chemiluminescence reagents (Thermo Fisher, Rockford, IL) and exposed to film.

Akt kinase assay

Immune complex-kinase assays were performed by a protocol from an assay kit purchased from Cell Signaling technologies. Cell lysates (200 μ g) were incubated with anti-Akt1, anti-Akt2, or anti-Akt3 antibody overnight at 4°C in cell lysis buffer. Immune complexes were then rotated for 2 – 4 h at 4°C with protein A-conjugated agarose beads (20 μ l of 50% slurry/reaction) before being washed twice in cell lysis buffer and twice in kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 , 10 mM MgCl_2). After resuspension in kinase assay buffer containing ATP and a GST-GSK-3 α/β fusion protein (residues surrounding GSK-3 α/β Ser21/9 (CGPKGPGRRGRRRTSSFAEG)), the reaction was allowed to proceed at 30°C for 30 min. After the reaction was stopped by addition of concentrated SDS-PAGE loading buffer, samples were separated by SDS-PAGE and transferred to PVDF membranes as described above. Immunoblotting was performed using primary antibodies to phospho-GSK-3 α/β provided in the kinase assay kit, followed by addition of HRP-conjugated secondary antibodies, detection by ECL, and exposure to X-ray film. Results were quantitated by densitometry.

Cell Surface Receptor

Analysis of IGF-IR at the cell surface was accomplished through use of a commercially available cell surface protein isolation kit (Pierce). Briefly, for each treatment, four 100mm culture dishes were seeded with C2C12 cells and transfected as described above. Cells were labeled with Sulfo-NHS-SS-biotin for 30-minutes and the reaction quenched with the provided quenching solution. Cells were transferred to 50 mL conical tubes, centrifuged, and washed with TBS. Lysis buffer provided in the kit was added to cell pellets and transferred to microfuged tubes and briefly sonicated every 10-minutes for 30-minutes at a power of 1.5 followed by clarification of supernatant which was quantified and 1.4 g added to provided spin columns. At this point, some protein lysate was stored at -80° C for analysis of total IGF-IR or GAPDH. Biotin-labeled proteins were washed and isolated with NeutrAvidin Agarose (provided in kit) with end-over-end mixing for one hour. Agarose/biotinylated complexes were washed and incubated in sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 20% glycerol) for one hour, centrifuged, and the eluate collected. A small amount of bromophenol blue was added to eluate and proteins were separated by SDS-PAGE followed by Western blotting for IGF-IR or GAPDH as described above.

Detection of PI(3,4,5)P3

Levels of PI(3,4,5)P3 were determined by using a commercially-available ELISA assay (Echelon Biosciences, Salt lake City, UT; P/N K-1000) following the manufacturers instructions. Briefly, after treatment, cells were washed three times in Buffer A (20 mM

Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1 mM sodium orthovanadate) and incubated at 4-degrees C for 30-minutes in Buffer A plus 1% NP-40 and 1 mM PMSF. Insoluble material was removed by centrifugation and clarified lysates quantified. 125 µg was immunoprecipitated with either p110 α or p110 β antibody for 2-hours at 4-degrees C, followed by mixing with protein A/G agarose beads (Santa Cruz) for 1-hour. Immunocomplexes were washed three times with Buffer A plus 1% NP-40, three times with 0.1 M Tris-HCl, pH 7.4; 5 mM LiCl, and 0.1 mM sodium orthovanadate, and twice in TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate). Kinase reactions were performed in triplicate by incubation in PI3K reaction buffer (20 mM Tris, pH 7.4, 10 mM NaCl, 4 mM MgCl₂, and 25 µM ATP) and 100 µM PI(4,5)P₂ substrate for 2-hours, followed by centrifugation to stop the reactions and addition of PI(3,4,5)P₃ detector for 1-hour. At this time, PIP₃ standards (provided) were set up using concentrations of 200, 100, 50, 25, 12.5, and no lipid control. Reacted mixtures and standards were then transferred to the detection plate for 60-minutes, washed once in TBS-T, and secondary detector added for 30-minutes. Wells were washed three times with TBS-T and then 100 µl of TMB solution added to induce color change. Color development was stopped by addition of 1 N H₂SO₄ stop solution when color development was within the linear range of standards, followed by reading at 450 nM.

Statistics

Data are presented as means \pm S.E.M. Statistics were performed using either one-way or two-way ANOVA and multiple range tests *a posteriori*, as described in figure legends. Densitometric analysis was performed using Image J 1.60 (NIH). A P-value <0.05 was considered significant.

Acknowledgments

The authors wish to thank Dr. John C. Lee of the Department of Biochemistry, University of Texas Health Science Center at San Antonio, for many helpful discussions during the preparation of this work.

This work was supported by NIA grant R01AG026012 to MLA. RWM was supported by pre-doctoral award from NIA training grant T32 AG021890-08.

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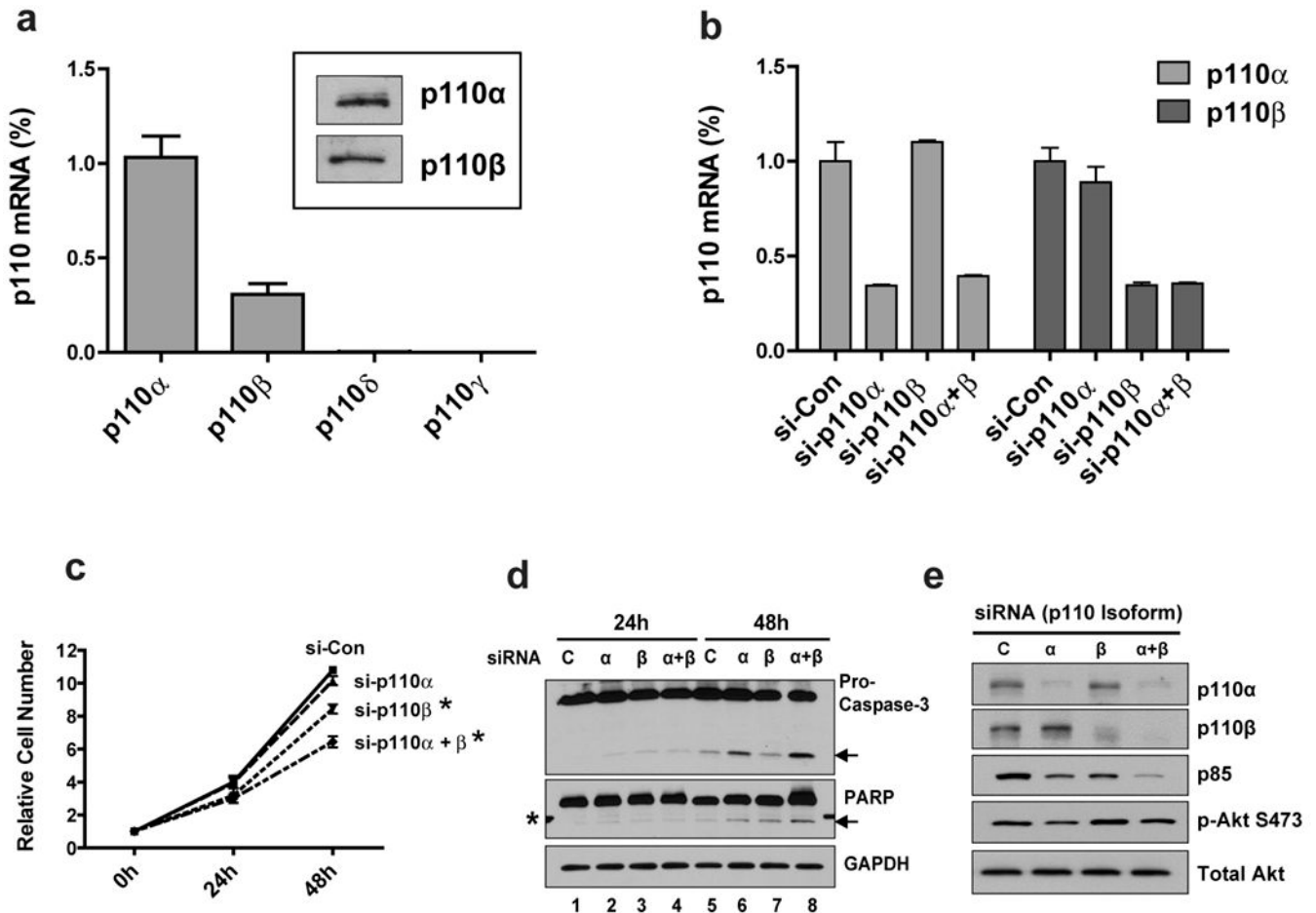
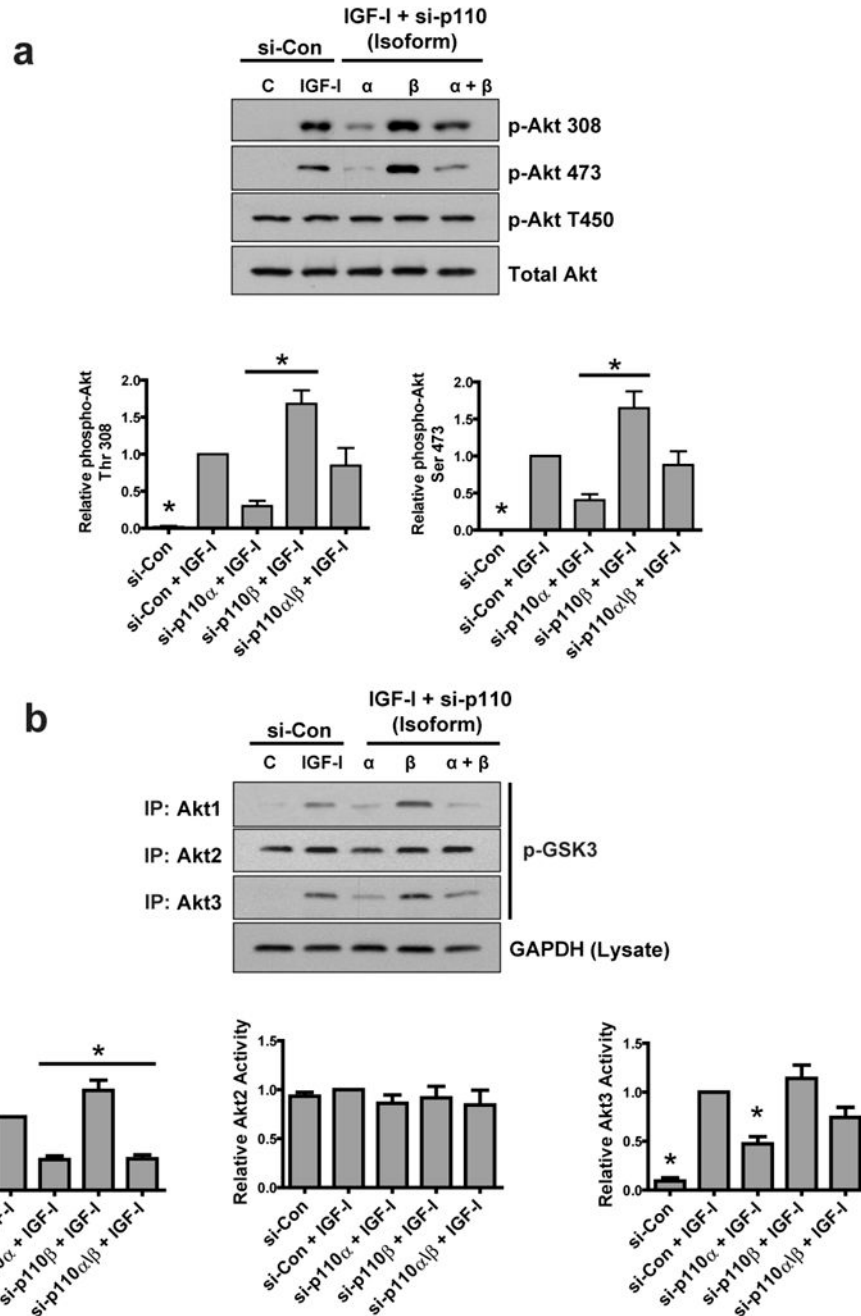


Figure 1.

PI3K p110 α and p110 β are expressed in C2C12 myoblasts and reduction of p110 β or p110 α and β simultaneously reduced cell growth. **(a)** PI3K p110 catalytic subunit expression in myoblasts. Real-time PCR was used to determine mRNA transcript level as described in materials and methods. Ct values were first normalized to invariant endogenous beta-2 microglobulin (B2M), and then values were expressed relative to p110 α which was arbitrarily set at 1.0. Inset: detection of expressed p110 isoforms by Western blot. **(b)** Efficacy of RNAi-mediated knockdown of p110 α and p110 β was determined by real-time PCR using B2M as a reference gene, as in **(a)**. Cells were reverse-transfected with 10nM negative control siRNA (si-Con) or 5nM siRNA against p110 α (si-p110 α) plus 5nM si-Con, 5nM p110 β (si-p110 β) plus 5nM si-Con, or simultaneous transfection (co-transfection) with 5nM p110 α and 5nM p110 β (si-p110 α + β). Medium was changed to antibiotic-containing media 24-hours after plating, and cells were harvested for RNA 24-hours following media change (48-hours after initial reverse-transfection). Values are expressed relative to p110 α which was arbitrarily set at 1.0. Columns represent averages of two independent experiments and error bars indicate S.E.M. **(c)** Cells were reverse-transfected as described in **(b)** at day 0. Twenty-four and 48-hours after plating, cells were trypsinized and counted in a hemacytometer and expressed as fold-changes relative to initial plating density, which was set at 1.0. Asterisks next to treatments indicate significant difference at $P < 0.05$ after two-

way ANOVA followed by Bonferroni post-test. **(d)** Cells were transfected as described in **(b)**, and myoblasts were harvested for protein lysates 24- and 48-hours after plating. Western blotting was performed to analyze levels of Caspase-3, PARP, and GAPDH as described in materials and methods. Uncleaved Caspase-3 and PARP are labeled; cleaved Caspase-3 and PARP are indicated by arrows. Numbers 1 – 8 (bottom) indicate lanes. The asterisk (*) next to the PARP image indicates protein ladder (98 kDa) that reacted with antibody in this and some subsequent runs. Abbreviations: C (non-targeting control siRNA), α (siRNA against p110 α), β (siRNA directed against p110 β), $\alpha + \beta$ (siRNA directed against p110 α co-transfected with siRNA directed against p110 β). **(e)** Cells were treated and maintained as described in **(b)**. Western blots of various proteins involved in PI3K signal transduction are shown. Each blot is representative of two independent experiments. Abbreviations are identical to those described in **(d)**.

**Figure 2.**

RNAi-mediated knockdown of p110 α reduces Akt phosphorylation Thr 308 and Ser 473 and activation of Akt1 and Akt3, while p110 β knockdown does not. **(a)** Cells were reverse-transfected and maintained as described in Figure 1b. Forty-eight hours after transfection, cells were treated with 125ng/ml IGF-I for 30-minutes and then harvested for protein lysates upon which Western blotting (left upper) and in-vitro kinase (IVK; right upper) assays were performed as described in materials and methods. For Western blots, the antibody used is indicated to the right. Blots are representative of 3-5 independent experiments. For in vitro

kinase assays, the antibody used for immunoprecipitation is indicated to the right, and experiments were performed 3-5 times. Levels of GAPDH protein were determined by Western blotting on protein lysates. **(b)** Quantification of blots depicted in **(a)**. Quantification of phospho-Akt (upper graphs) and isoform-specific Akt activities was performed using Image J software (NIH) as described in materials and methods. Levels of phosphorylated Akt were normalized to total Akt for each specific run, and the level of IGF-I-stimulated Akt phosphorylation was set to 1.0. Isoform-specific activated Akt levels were normalized to GAPDH for each specific run, and the level of IGF-I-stimulated Akt phosphorylation was set to 1.0. Columns in graphs represent averages of 3-5 independent experiments, and error bars represent S.E.M. Asterisks indicate significant difference from IGF-I-stimulated values ($P < 0.05$; one-way ANOVA followed by Dunnett's multiple comparisons test), and horizontal bars directly below asterisks span columns statistically different from IGF-I-stimulated values.

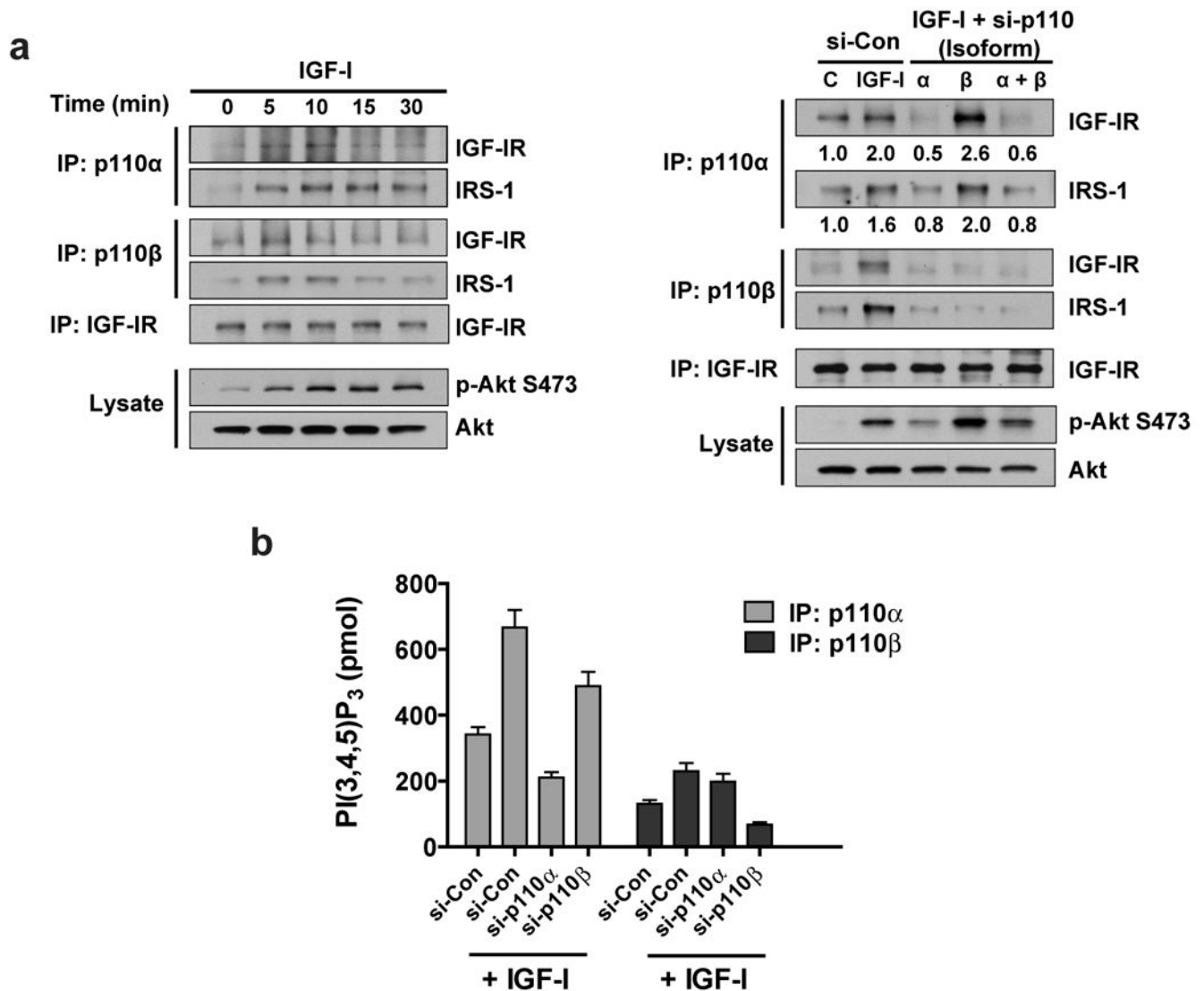


Figure 3. IGF-I effects on p110 subunit association with IGF-IR signaling complex and PI(3,4,5)P₃ generation. **(a)** Left: Cells were treated with 125 ng/mL IGF-I and then harvested at the indicated times. Immunoprecipitation (IP) with antibodies against p110α, p110β, or IGF-IR is indicated at the left of the images. Western blotting using the primary antibodies noted to the right of the images was performed as described in Materials and Methods. For phospho-Akt and total Akt, Western blotting was performed without immunoprecipitation (Lysate). Right: Cells were treated with 125 ng/mL IGF-I for 7-minutes before harvesting. Immunoprecipitation and Western blotting was performed as described directly above. Numbers below blots indicate band density as compared to unstimulated control cells, which was set at 1.0. Shown are representative results from three independent experiments. **(b)** Cells were reverse-transfected as described in Figure 1b. Forty-eight hours after transfection, cells with were treated with 125 ng/mL IGF-I and then harvested after 7-minutes. Immunoprecipitations were performed on 125 μg cell lysate using antibodies against p110α

or p110 β followed by kinase reactions and detection of PI(3,4,5)P3 levels by ELISA as described in Materials and Methods. Data represent means of triplicate experiments, and error bars indicate SD.

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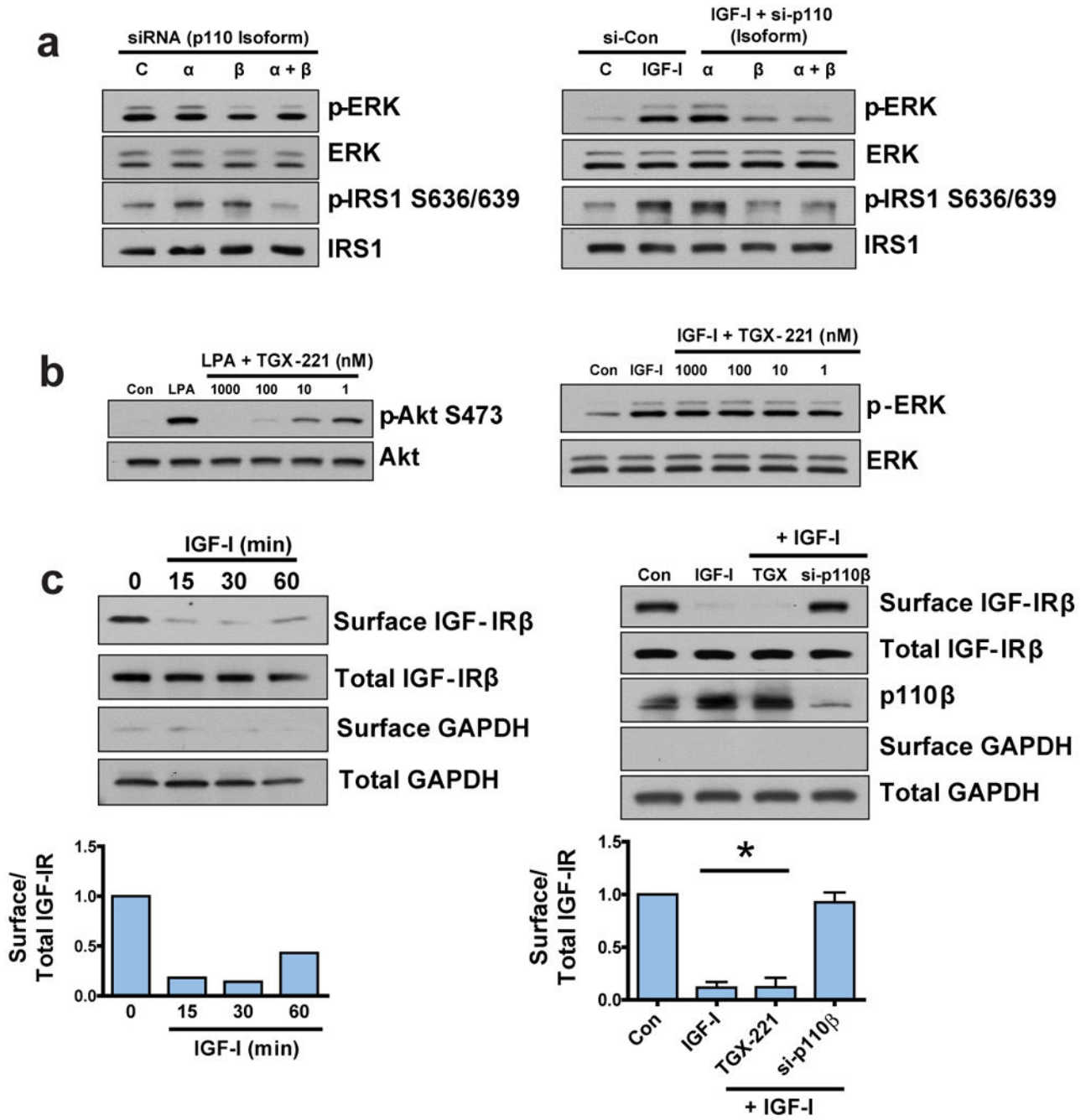
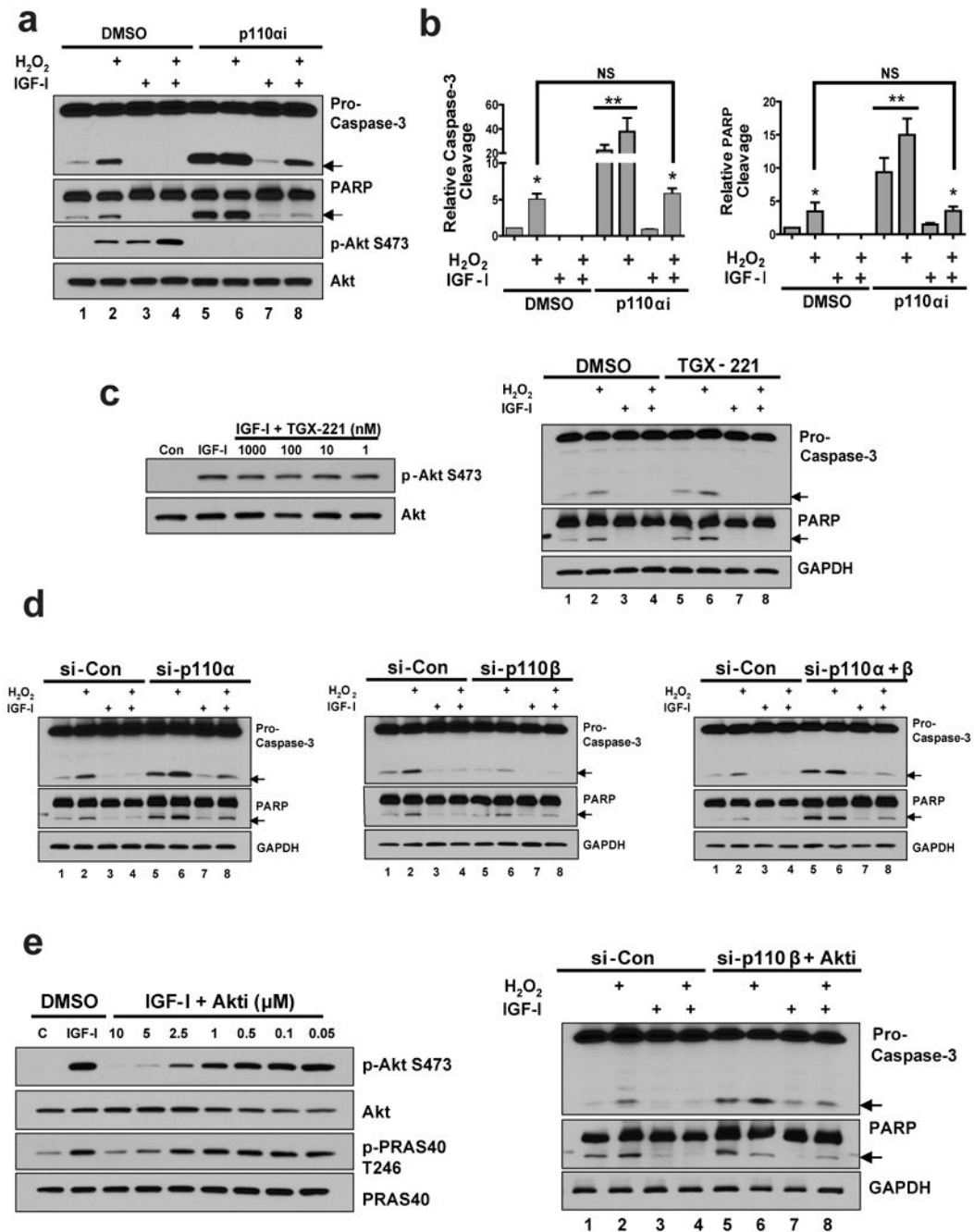


Figure 4. p110β knockdown, but not catalytic inhibition, reduces IGF-I-mediated ERK phosphorylation and IGF-IR internalization. (a) Cells were reverse-transfected and maintained as described in Figure 1b. Forty-eight hours after transfection, cells were harvested or treated with 125ng/ml IGF-I for 30-minutes and then harvested for protein lysates. Western blotting was performed using antibodies indicated to the right of the respective images. Blots are representative of two independent experiments. (b) 95% confluent C2C12 myoblasts were pre-treated with TGX-221 at the indicated concentrations

for 1-hour before addition of 20 μ M LPA or 125 ng/mL IGF-I for 30-minutes. Western blots using the antibody against the indicated protein were then performed. (c) Quantity of IGF-IR at the cell surface after IGF-I administration was determined by biotin labeling and immunoprecipitation of surface proteins. Left Panel: cells were treated with 125 ng/ml IGF-I for the indicated times, labeled with sulfo-NHS-SS-biotin, and isolated according to the procedure described in "Materials and Methods." The amount of IGF-IR located at the cell surface determined by Western blot after immunoprecipitation with NeutrAvidin Agarose; levels of total IGF-IR and GAPDH were determined by analyzing total cell lysate prior to immunoprecipitation. Columns represent quantified levels of surface IGF-IR divided by total IGF-IR normalized to untreated control, which was set at 1.0. Right Panel: cells were treated with 125 ng/ml IGF-I for 30-minutes after pre-treatment with negative control siRNA (unlabeled) for 48-hours, si-Con for 48-hours and TGX-221 for 1-hour ("TGX-221"), or 5 nM siRNA against p110 β ("si-p110 β ") for 48-hours. Surface and total levels of indicated proteins were determined as described above. Columns represent quantified levels of surface IGF-IR divided by total IGF-IR from two independent experiments normalized to untreated control, which was set at 1.0. Asterisk indicates significant difference from unstimulated cells as determined by ANOVA with Tukey's HSD test post hoc ($P < 0.05$). Error bars represent SD.

**Figure 5.**

p110 α is necessary for IGF-I-mediated protection from H₂O₂-induced apoptosis. (a) Approximately 95% confluent myoblasts were treated with 125ng/ml IGF-I for 30-minutes in the indicated lanes. Some cells were then treated for an additional 4-hours with 400 μ M H₂O₂, as indicated. For cells treated with p110 α inhibitor (p110ai), cells were pre-treated for an additional hour prior to IGF-I, H₂O₂, or IGF-I + H₂O₂ addition. Control cells were treated with DMSO (p110ai diluent) and/or water (H₂O₂ and IGF-I diluent), as appropriate. Four hours after H₂O₂ addition, cells were harvested and Western blots performed to detect

levels of Caspase-3, PARP, phospho-Akt Ser 473, or total Akt (cleaved Caspase-3 – 17 kDa, cleaved PARP – 85 kDa). Numbers 1-8 along the bottom of the Western blots indicate lane numbers. Blots are representative of 3-5 independent experiments. **(b)** Amounts of cleaved Caspase-3 and PARP were quantified. Due to heteroscedasticity, values were transformed as $y = -1 * \log(y)$, and significant differences determined by one-way ANOVA followed by Bonferroni test post-hoc. Values are expressed relative to unstimulated controls, and the original, non-transformed data is shown. Asterisks indicate significant difference from control, and double asterisks indicate significant differences from control and H₂O₂-treated cells. Horizontal bars directly below asterisks span columns statistically different from control and H₂O₂-treated cells. “NS” – not significant. **(c)** Left panel: Approximately 95% confluent myoblasts were pre-treated with DMSO (unlabeled) or TGX-221 for 1-hour before addition of IGF-I for 30-minutes. Western blots were performed using the indicated antibodies. Right panel: Cells were treated with IGF-I, H₂O₂, or TGX-221 in place of p110 α i exactly as described in **(a)**. **(d)** Cells were reverse-transfected with 5 nM negative control siRNA (si-Con) or 5 nM siRNA against p110 α (si-p110 α ; Left panel), 5 nM si-Con or 5 nM p110 β (si-p110 β ; middle panel), or 10 nM si-Con or 5nM p110 α and 5nM p110 β (si-p110 α + β ; right panel). Forty-eight hours after transfection, myoblasts were treated with 125ng/ml IGF-I for 30-minutes in the indicated lanes. Some cells were then treated for an additional 4-hours with 400 μ M H₂O₂, as indicated. Controls for IGF-I and H₂O₂ were water. Western blotting was performed and the levels of Caspase-3, PARP, and GAPDH (as a loading control) analyzed. Intact Caspase-3 and PARP are labeled, and cleaved portions are indicated by the arrow (cleaved Caspase-3 – 17 kDa, cleaved PARP – 85 kDa). Numbers 1-8 along the bottom of images represent lane numbers. Blots are representative of three independent experiments. **(e)** Left panel: Approximately 95% confluent myoblasts were pre-treated with 0.1% DMSO or increasing concentrations of Akti for 1-hour before addition of IGF-I for 4.5 hours. Western blotting was performed using the indicated antibodies. Right panel: Cells were reverse-transfected with siRNA against p110 β as described above in **(d)**. Forty-eight hours following transfection, some cells were pre-incubated with 10 μ M Akti. Following the 1-hour incubation period, cells were treated with 400 μ M H₂O₂ for 4-hours before harvest and Western blotting with the indicated antibodies. Blots are representative of two independent experiments.

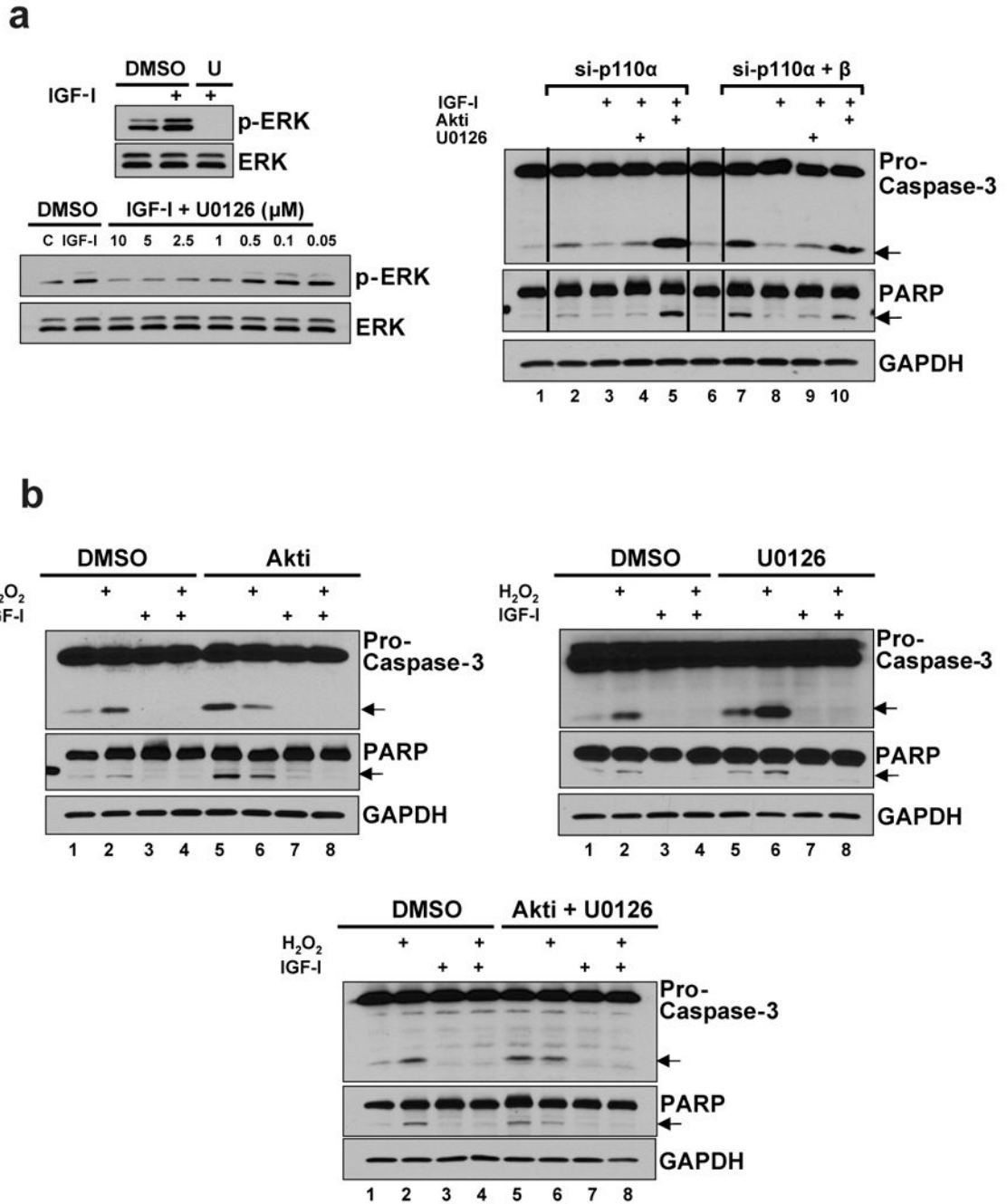


Figure 6. Pharmacological inhibition of Akt and MEK. (a) Upper panel: Cells were pre-incubated with 10 μM MEK inhibitor U0126 or 0.1% DMSO for one hour, and then treated with 125 ng/ml IGF-I for 30-minutes. Levels of phospho- and total ERK were analyzed by Western blot. Lower panel: Cells were pre-incubated with increasing concentrations of U0126 or 0.1% DMSO for one hour, and then treated with 125 ng/ml IGF-I for 4.5 hours. Levels of phospho- and total ERK were analyzed by Western blot. “C”- IGF-I control cells treated with water. Right panel: Cells were reverse-transfected and maintained as described in

Figure 1b. Myoblasts were pre-treated with Akti or U0126 for 1-hour as indicated. Following the pre-incubation period, cells were treated with 125 ng/ml IGF-I for 4-hours in the indicated lanes. Western blotting was performed and the levels of Caspase-3, PARP, and GAPDH analyzed. Intact Caspase-3 and PARP are labeled, and cleaved portions are indicated by the arrow (cleaved Caspase-3 – 17 kDa, cleaved PARP – 85 kDa). Numbers 1-10 along the bottom of images represent lane numbers. Blots are representative of three independent experiments. **(b)** Myoblasts were pre-treated with 10 μ M Akti (upper left panel), 10 μ M U0126 (upper right panel), or Akti and U0126 (lower panel) for 1-hour, as indicated. Following the pre-incubation period, cells were treated with 125 ng/mL IGF-I for 30-minutes before addition of 400 μ M H₂O₂ as indicated. Western blotting was performed as described in **(b)**. Representative blots of two independent experiments for each condition are shown.

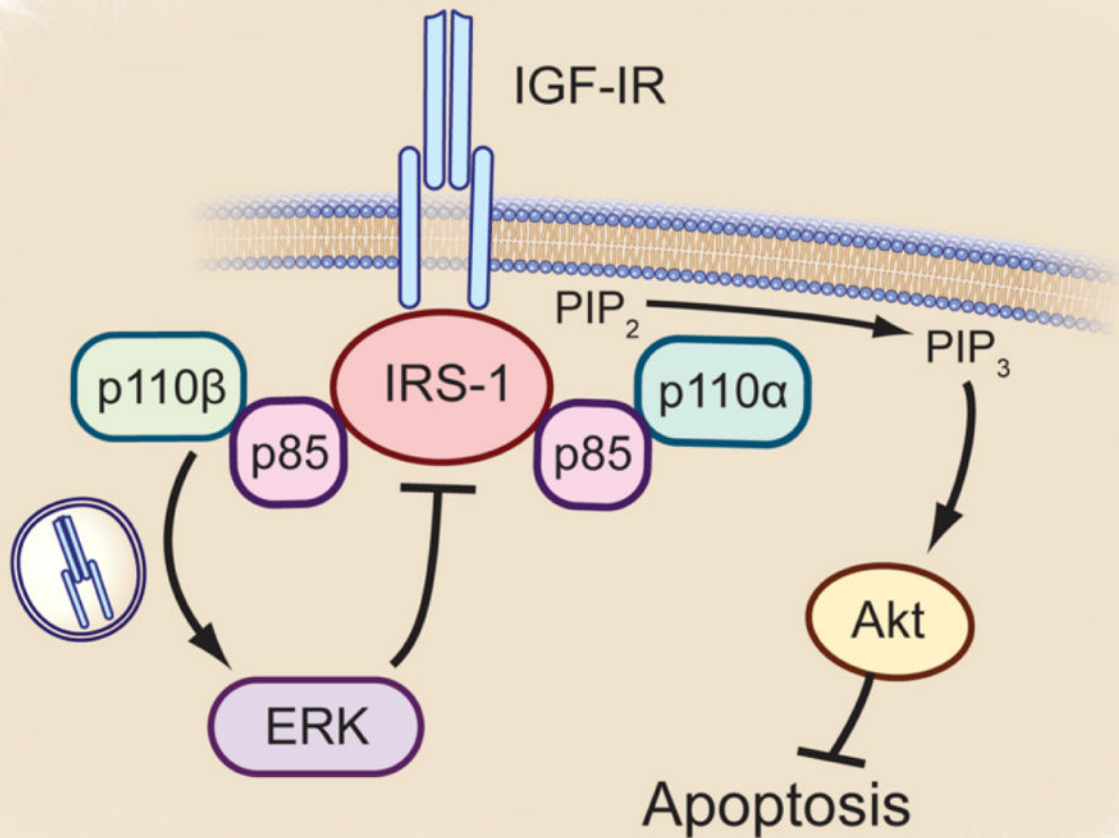


Figure 7.

Proposed mechanism of IGF-I signal transduction in myoblasts. Signaling through IGF-IR recruits adaptor molecules including IRS-1 to IGF-IR. This promotes binding of PI3K and generation of 3' lipid phosphoinositides that recruit other molecules, including Akt, to the membrane, ultimately resulting in their activation. PI3K p110 α catalytic subunit is required for Akt activation, but p110 β is not. A second pathway includes a non-catalytic action of p110 β that is associated with IGF-IR internalization and is required for activation of ERK. ERK may then phosphorylate IRS-1 at Ser 636/639, inhibiting PI3K binding and promoting IRS-1 degradation, thus attenuating IGF-IR signals.