eLife

Genetics and Genomics
Immunology and Inflammation

Reviewed Preprint
v2 • January 6, 2025
Revised by authors

Reviewed Preprint
v1 • February 20, 2024

Paradoxical dominant negative activity of an immunodeficiency-associated activating *PIK3R1* variant

Patsy R Tomlinson, Rachel Knox, Olga Perisic, Helen C Su, Gemma V Brierley, Roger L Williams, Robert K Semple

The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK • MRC Metabolic Diseases Unit, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK • The National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge, UK • MRC Laboratory of Molecular Biology, Cambridge, UK • Laboratory of Clinical Immunology & Microbiology, Intramural Research Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, USA • Department of Comparative Biomedical Science, The Royal Veterinary College, London, UK • Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, UK • MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK

eLife Assessment

This **important** study reports on PI3KR mutations and a paradoxical mechanism of PI3KR signaling. The strength of evidence for the study is mostly **convincing**, as conclusions are supported by a variety of mutational strategies and cellular systems to look at interactions among signaling pathways.

https://doi.org/10.7554/eLife.94420.2.sa3

Abstract

PIK3R1 encodes three regulatory subunits of class IA phosphoinositide 3-kinase (PI3K), each associating with any of three catalytic subunits, namely p110α, p110β or p110δ. Constitutional *PIK3R1* mutations cause diseases with a genotype-phenotype relationship not yet fully explained: heterozygous loss-of-function mutations cause SHORT syndrome, featuring insulin resistance and short stature attributed to reduced p110α function, while heterozygous activating mutations cause immunodeficiency, attributed to p110δ activation and known as APDS2. Surprisingly, APDS2 patients do not show features of p110α hyperactivation, but do commonly have SHORT syndrome-like features, suggesting p110α hypofunction. We sought to investigate this. In dermal fibroblasts from an APDS2 patient, we found no increased PI3K signalling, with p110δ expression markedly reduced. In preadipocytes, the APDS2 variant was potently dominant negative, associating with Irs1 and Irs2 but failing to heterodimerise with p110α. This attenuation of p110α signalling by a p110δ-activating PIK3R1 variant potentially explains co-incidence of gain-of-function and loss-of-function *PIK3R1* phenotypes.

Introduction

PIK3R1 gene products (p85α, p55α and p50α) are essential for class IA phosphoinositide 3-kinase (PI3K) signalling. Each of the three protein products can bind any of three catalytic subunits, p110α, β and δ, encoded by *PIK3CA*, *PIK3CB*, and *PIK3CD* respectively. This stabilises and inhibits the catalytic subunits in the basal state and confers responsiveness to upstream stimuli including receptor tyrosine kinase activation, enabled by two phosphotyrosine-binding SH2 domains shared by all PIK3R1 products (Fruman *et al*, 2017).

PI3K signalling mediates responses to a myriad of cues, including growth factors, hormones such as insulin, and processed antigens, and so recent discovery that genetic perturbation of PIK3R1 in humans disrupts growth, insulin action and immunity is no surprise. Beyond this headline observation lies considerable complexity, however, and important questions about genotype-phenotype correlations in PIK3R1-related disorders are unresolved.

Some PIK3R1 mutations reduce basal inhibition of catalytic subunits, usually due to disruption of the inhibitory inter-SH2 domain, and are found in cancers (Philp *et al*, 2001) and vascular malformations with overgrowth(Cottrell *et al*, 2021). In both diseases, hyperactivated PI3Kα, composed of heterodimers of *PIK3R1* products and p110α, drives pathological growth. Distinct inter-SH2 domain *PIK3R1* mutations, mostly causing skipping of exon 11 and deletion of residues 434-475, hyperactivate PI3Kδ in immune cells, causing highly penetrant monogenic immunodeficiency (Deau *et al*, 2014; Lucas *et al*, 2014b). This phenocopies the immunodeficiency caused by genetic activation of p110δ itself, which is named Activated PI3K Delta Syndrome 1 (APDS1) (Angulo *et al*, 2013; Lucas *et al*, 2014a). The PIK3R1-related syndrome, discovered shortly afterwards, is thus named APDS2.

Despite ubiquitous *PIK3R1* expression, features attributable to PI3Kα activation have been described in only a single case of APDS2 to date (Wentink *et al*, 2017). In that case, the causal variant (N564K) was a constitutional activating mutation associated with cancer rather than one of the common APDS2 variants. Biochemical studies have suggested that apparently selective p110δ activation occurs because APDS2 mutations derepress p110δ, the predominant immune system catalytic subunit, more than p110α, due to differences in the inhibitory contacts between *PIK3R1* products and different catalytic subunits (Dornan *et al*, 2017).

More surprisingly, phenotypic overlap is reported between APDS2 and SHORT syndrome. SHORT syndrome, named for the characteristic developmental features (Short stature, Hyperextensibility, Hernia, Ocular depression, Rieger anomaly, and Teething delay) is caused by loss of PI3Kα function due to disruption of the phosphotyrosine-binding C-terminal SH2 domain (Chudasama *et al*, 2013; Dyment *et al*, 2013; Thauvin-Robinet *et al*, 2013). Like APDS2 it is stereotyped and highly penetrant. It features short stature, insulin resistance, and dysmorphic features (Avila *et al*, 2016). In recent years, both individual case reports (Bravo Garcia-Morato *et al*, 2017; Petrovski *et al*, 2016; Ramirez *et al*, 2020; Szczawinska-Poplonyk *et al*, 2022) and larger case series (Elkaim *et al*, 2016; Jamee *et al*, 2020; Maccari *et al*, 2023; Nguyen *et al*, 2023; Olbrich *et al*, 2016; Petrovski *et al*., 2016) have established that many people with APDS2 have overt features of SHORT syndrome, while, more generally, linear growth impairment is common in APDS2, but not in APDS1. These clinical observations are bolstered by the impaired linear growth and increased *in utero* mortality reported in mice with knock in of the common causal APDS2 mutation in Pik3r1(Nguyen *et al*., 2023). All people with SHORT-APDS2 overlap syndromes have well established activating PIK3R1 mutations in the inter-SH2 domain implicated in APDS2, but none have characteristic SHORT syndrome mutations, which are usually in the C-terminal SH2

domain. Conversely, no patient with a classical SHORT syndrome mutation has been shown to have immunodeficiency. There is thus now convincing evidence of a syndrome with features of both gain and loss of PI3K function. The mechanistic basis of this is unexplained.

PI3K activity is determined not just by activity of individual PI3K holoenzymes, but also by subunit stoichiometry. Regulatory and catalytic subunits stabilise each other upon binding (Brachmann *et al*, 2005; Yu *et al*, 1998), and only regulatory subunit monomers are stable enough to be detected in mammalian cells (Yu *et al*., 1998). Molar excess of regulatory subunits over catalytic subunits gives rise to free regulatory subunits that compete with holoenzyme for phosphotyrosine binding (Ueki *et al*, 2002). This has been invoked to explain increased insulin sensitivity on knockout or reduction of p85α alone (Mauvais-Jarvis *et al*, 2002), of p55α and p50α (Chen *et al*, 2004) or of all *Pik3r1* products (Fruman *et al*, 2000). The "free-p85" model has been contested by some reports suggesting no excess p85 (Geering *et al*, 2007; Zhao *et al*, 2006), however most observations, including some made recently using *in vivo* tagging and pulldown of PI3K components (Tsolakos *et al*, 2018) suggest that monomeric p85α can be seen *in vivo* in some tissues.

We now address mechanisms underlying human mixed gain- and loss-of function phenotypes observed in association with PIK3R1 mutations using primary cells, cell models and in vitro enzyme assay and suggest that they are explained by competing effects of APDS2 PIK3R1 mutations on PI3K activity and stability.

Results

PI3K subunit expression and signalling in APDS2 fibroblasts

Disorders in the *PIK3CA*-related overgrowth spectrum (PROS) feature soft tissue overgrowth and are caused by heterozygous, mosaic activating mutations in *PIK3CA*, encoding the p110α catalytic subunit of PI3Kα(Madsen *et al*, 2018). In this group, basal hyperactivation of PI3Kα signalling can be easily discerned in dermal fibroblasts from affected areas of the body(Lindhurst *et al*, 2012). *PIK3R1*, like *PIK3CA*, is ubiquitously expressed, yet overgrowth is not reported in APDS2 caused by heterozygous, constitutional activating mutations in *PIK3R1*. Dermal fibroblasts strongly express *PIK3R1*, but no studies to date have evaluated whether PI3K activity is increased in APDS2.

We began by assessing dermal fibroblasts cultured from a previously described woman with APDS2 due to the common causal PIK3R1 mutation. This affects a splice donor site that causes skipping of exon 11, leading to in-frame deletion of 42 amino acids (434-475 inclusive) in the inter-SH2 domain, which is shared by all PIK3R1 isoforms (Patient A.1 in (Lucas *et al*., 2014b))(Fig 1 figure supplement 1). These cells were compared to cells from healthy control volunteers, or from people with PROS. Confirmation of expression of all pathogenic mutations was undertaken by cDNA sequencing prior to further study (Fig 1 figure supplement 2A). We found that truncated p85α was expressed, but at a much lower protein level than full length wild-type p85α (**Fig 1A**, Fig 1 figure supplement 2B,C). Despite this, no increase in basal or insulin-stimulated AKT phosphorylation was seen in APDS2 cells compared to cells from wild-type volunteers or from people with PROS and activating *PIK3CA* mutations H1047L or H1047R (**Fig 1A-C**, Fig 1 figure supplement 3A,B). Although insulin-induced AKT phosphorylation was lower in fibroblasts from the one APDS2 patient studied compared to controls, we have previously reported extensive variability in insulin-responsiveness of primary dermal fibroblasts. Moreover even primary cells from a patient expressing high levels of the SHORT syndrome-associated p85α Y657X did not show attenuated insulin action. The reduced insulin action in APDS2 cells in the current study thus should not be overinterpreted until reproduced in further APDS2 cells.

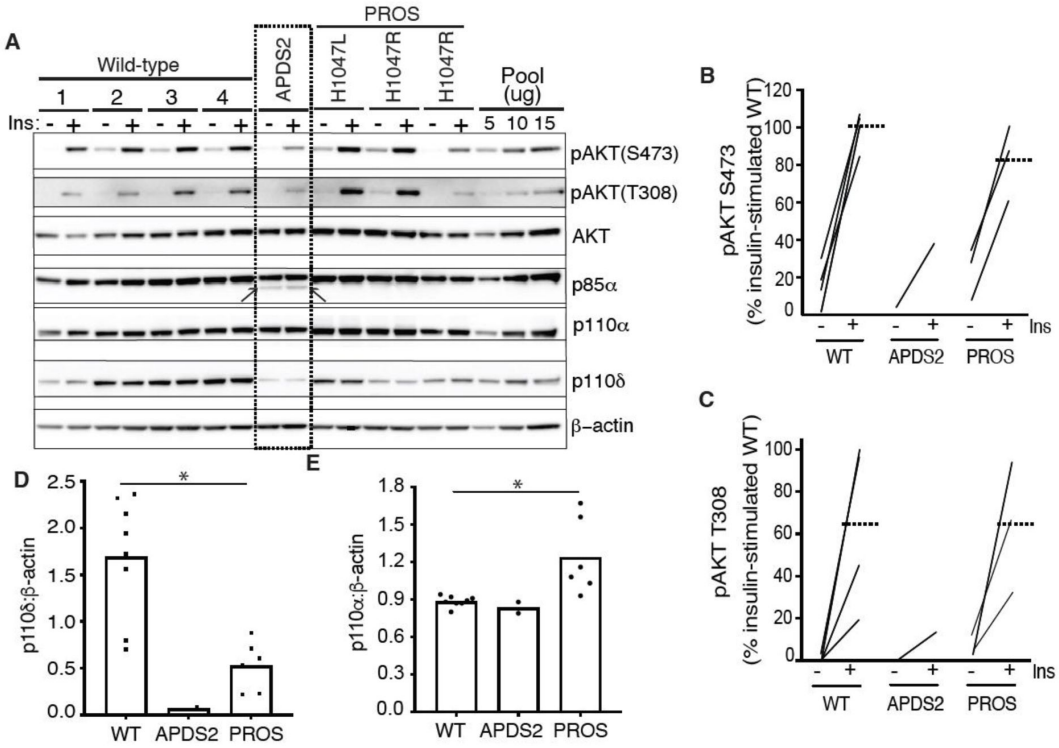

Figure 1.

PI3K subunit expression and signalling in primary dermal fibroblasts.

Immunoblotting of AKT, AKT phosphorylated at threonine 308 (T308) or serine 473 (S473), p85α, p110δ and p110α and are shown with and without stimulation by 100 nM insulin (Ins) for 10 minutes. β-actin is shown as a loading control, with different amounts of pooled lysate (Pool) used to demonstrate signal intensity in the linear range. Results from 4 healthy controls (WT; 1-4), 1 patient with Activating p110 Delta Syndrome 2 (APDS2) due to the p85α Δexon11 variant, and 3 patients with PIK3CA-Related Overgrowth Spectrum (PROS) caused by the activating *PIK3CA* mutations indicated. (A) Immunoblots, with the truncated p85α Δexon11 variant arrowed; (B)-(E) quantification of immunoblot bands from 3 independent experiments shown for phosphoAKT-S473, phosphoAKT-T308, p110δ and p110α respectively. Each point represents data from one of the patient cell lines in the immunoblots. Paired datapoints +/- insulin are shown in (B) and (C), and dotted lines mark means. Asterisks indicate a significant difference. More detailed statistical analysis including 95% confidence intervals for the paired mean differences for these comparisons are shown in Figure 1 figure supplement 2.

Previous studies have suggested that the truncated, APDS2-causal p85α variant exerts a much greater activating effect on p110δ, which has a more restricted tissue expression, including immune cells, than p110α, which is ubiquitous (Dornan *et al*., 2017). We found that both p110α and p110δ were easily detectable in control dermal fibroblasts, however p110δ was almost absent in APDS2 fibroblasts compared to controls, with lower levels also seen in PROS cells (**Fig 1A,D**, Fig 1 figure supplement 3D). p110α was unchanged in APDS2 cells but modestly increased in PROS cells (**Fig 1A,E**, Fig 1 figure supplement 3C). Collectively these findings suggest that any ability of the APDS2 PIK3R1 variant in skin cells to activate PI3K may be overcome by reduced protein levels of p110δ, likely through reduced binding and/or reduced stabilisation of p110δ by the mutant regulatory subunit.

Overexpressed PIK3R1 ΔExon11 is potently dominant negative in 3T3-L1 preadipocytes

We next turned to a well-established cellular system allowing conditional overexpression of *PK3R1* alleles of interest. We have previously shown that overexpression of two SHORT syndrome PIK3R1 variants – R649W and Y657X – impair insulin signalling and adipocyte differentiation of murine 3T3-L1 preadipocytes, consistent with impaired PI3Kα function and with the lipodystrophy and insulin resistance seen in SHORT syndrome (Huang-Doran *et al*, 2016). To assess the effect of the APDS2 ΔEx11 in the same, non-immunological, cell context, we used lentiviral vectors, as previously described (Huang-Doran *et al*., 2016), to generate clonal 3T3-L1 cell lines allowing conditional, tuneable overexpression of PIK3R1 variants in response to doxycycline (Figure 2 figure supplement 1). For signalling studies, we also generated cells conditionally overexpressing the PROS- and cancer-associated PIK3CA H1047R mutation as a positive control for increased PI3Kα signalling, while for differentiation studies we used previously reported lines conditionally expressing PIK3R1 R649W or Y657X (Huang-Doran *et al*., 2016) (Fig 1 figure supplement 1). In undifferentiated cells, we first confirmed doxycycline-dependent overexpression of p85α or p110α transgenes (**Fig 2A**), before assessing basal and insulin-stimulated Akt phosphorylation. As expected, overexpression of oncogenic H1047R p110α strongly increased basal Akt phosphorylation (**Fig 2A-C**, Fig 2 figure supplement 1A,C) with no additional increase on insulin stimulation (**Fig 2A-C**, Fig 2 figure supplement 1B,D). Surprisingly, however, not only did overexpression of the APDS2 ΔEx11 p85α not increase basal PI3Kα signalling, but it potently inhibited insulin-induced Akt phosphorylation, consistent with a strong dominant negative action on pathway activation (**Fig 2A-C**, Fig 2 figure supplement 1). Overexpressing wild-type p85α mildly reduced insulin-stimulated Akt phosphorylation (**Fig 2A-C**, Fig 2 figure supplement 1B,C), although WT p85α was not expressed to as high a level as ΔEx11 p85α. In keeping with impaired PI3Kα activity, overexpression of the ΔEx11 variant also severely impaired adipocyte differentiation, assessed by triglyceride accumulation in response to a standard differentiation protocol (**Fig 2D**). A similar effect was seen on overexpressing SHORT syndrome variants R649W and Y657X (**Fig 2D**).

To assess whether the inhibitory effect of ΔEx11 p85α might be an artefact caused by strong overexpression, doxycycline titration was used to assess whether a low level of overexpression might unmask signalling hyperactivation. However, no such hyperactivation was seen, and instead, a graded diminution of insulin-induced AKT phosphorylation was observed from the lowest to highest levels of p85α ΔEx11 overexpression (Fig 2 figure supplement 3).

Effect of PIK3R1 mutations on Phosphoinositide 3-kinase activity in vitro

Given this evidence that APDS2-associated PIK3R1 ΔEx11 potently inhibits PI3Kα signalling when overexpressed in 3T3-L1 preadipocytes, we next sought to investigate the biochemical basis of this observation. First, we assessed the effect of disease-causing PIK3R1 mutations on basal and phosphotyrosine-stimulated activity of purified PI3Kα, β and δ holoenzyme in a previously

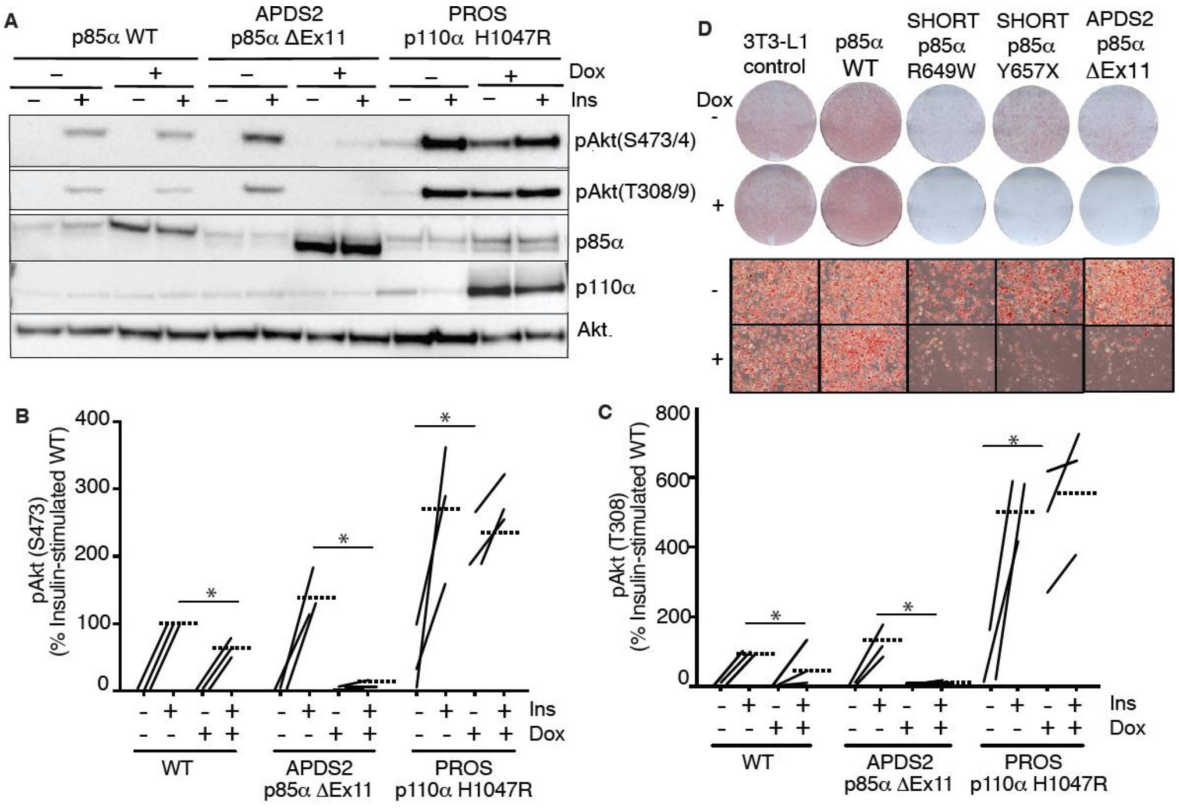

Figure 2.

Blunted insulin signaling in 3T3-L1 preadipocyte models of APDS2 and SHORT syndrome.

Immunoblotting of Akt, Akt phosphorylated at threonine 308 (T308) or serine 473 (S473), p85α, and p110α and are shown with and without stimulation with 100 nM insulin (Ins) for 10 minutes. Cells were treated with doxycycline (Dox) 1μg/mL for 72 hours prior to insulin stimulation as indicated. (A) One immunoblot representing 3 experiments is shown. (B)-(C) Quantification of immunoblot bands from all 3 independent experiments shown for phosphoAkt-S473 and phosphoAkt-T308 respectively. Paired datapoints +/- insulin are shown, and dotted lines mark means. Asterisks indicate a significant difference. More detailed statistical analysis including 95% confidence intervals for the paired mean differences for these comparisons are shown in Figure 2 figure supplement 2. (D) Staining for neutral lipid with Oil Red O of 3T3-L1 cells at day 10 of adipocyte differentiation. Induction of transgene expression by 1μg/mL doxycycline throughout differentiation is shown. Images of entire plates are shown above, with representative bright field microscopy images below.

described reconstituted *in vitro* system(Burke *et al*, 2012). As well as the APDS2 ΔEx11 mutation, we selected three SHORT syndrome-associated mutations to study. These were the most common causal mutation, R649W, which abolishes phosphotyrosine binding by the C-terminal SH2 (cSH2) domain (Chudasama *et al*., 2013), Y657X, which truncates the cSH2 domain (Huang-Doran *et al*., 2016; Kwok *et al*, 2020), and E489K which, atypically, lies in the inter-SH2 domain where most cancer, overgrowth and APDS2-associated mutations lie (Thauvin-Robinet *et al*., 2013). PIK3R1 E489K-containing primary cells were previously suggested to show basal hyperactivation (Thauvin-Robinet *et al*., 2013).

Wild-type and SHORT syndrome mutant holoenzymes were successfully purified for *in vitro* assay, but despite multiple attempts, ΔEx11 holoenzyme could only be made in small amounts under identical conditions, and moreover was unstable on storage, precluding further study. Such instability of *in vitro* synthesised ΔEx11 holoenzyme was previously reported (Dornan *et al*., 2017). Also in keeping with previous reports (Chudasama *et al*., 2013; Dornan *et al*., 2017; Dornan *et al*, 2020), p85α R649W showed severely reduced phosphotyrosine-stimulated activity in complex with p110α, with highly significant but lesser loss of function seen for Y657X and E489K (**Fig 3A**, Fig 3 figure supplement 1A). No increase in basal activity was seen for any variant.

We also assessed whether any of the SHORT syndrome variants affect function of PI3Kβ and found that all variants impaired phosphotyrosine-stimulated activity of PI3Kβ. Again, this impairment was less for p85α Y657X than for R649W, and in this case only very mild for E489K (**Fig 3B**, Fig 3 figure supplement 1B). For PI3Kδ, p85α R649W conferred severe loss of function, as for other isoforms (**Fig 3C**, Fig 3 figure supplement 1C), suggesting that the absence of immunodeficiency in SHORT syndrome is not accounted for by selective inhibition of PI3Kα function by causal mutations.

Binding of p110α by mutant p85α

Given the potent dominant negativity of APDS2-related p85α ΔEx11 in cells, and the instability of p85α ΔEx11-containing PI3K holoenzyme *in vitro*, we next used immunoprecipitation to assess binding of p110α by this and other p85α variants in cells. p85α was easily detected in anti-p110α immunoprecipitates from all cell lines at baseline (**Fig 4**). No increase in p110α levels was seen on conditional overexpression of wild-type or R649W p85α. Given the known instability of monomeric p110α, this suggests that all p110α is bound to p85α before overexpression. Although wild-type endogenous p85α may have been replaced by heterologously overexpressed p85α in these cells, this could not be detected without a size difference of the variant from wild-type. For cells overexpressing the SHORT syndrome-associated p85α Y657X, the truncated variant was strongly co-immunoprecipitated, accounting for nearly all of the p85α signal in anti-p110α immunoprecipitate. This demonstrates preserved binding of p110α by mutant p85α (**Fig 4**). In sharp contrast, although truncated p85α ΔEx11 was easily detected in cell lysates before immunoprecipitation and in supernatant after immunoprecipitation (arrowed in **Fig 4**), no truncated p85α ΔEx11 was seen in p110α immunoprecipitates, and no change in p110α expression was detected (**Fig 4**). This suggests that this truncated APDS2 causal variant does not supplant endogenous, full-length p85α binding to p110α, despite overexpression. This argues against destabilisation of p110α as the mechanism of the observed dominant negative activity.

Effect of PIK3R1 mutations on insulin-induced PI3K recruitment to IRS1/2

As APDS2 p85α ΔEx11 does not appear to displace wild-type p85α from p110α, despite strong overexpression, it is likely that there are high levels of truncated p85α unbound to p110α in the cell. This may be important, as p85α mediates recruitment of PI3K to activated tyrosine kinase receptors and their tyrosine phosphorylated substrates, including the insulin-receptor substrate

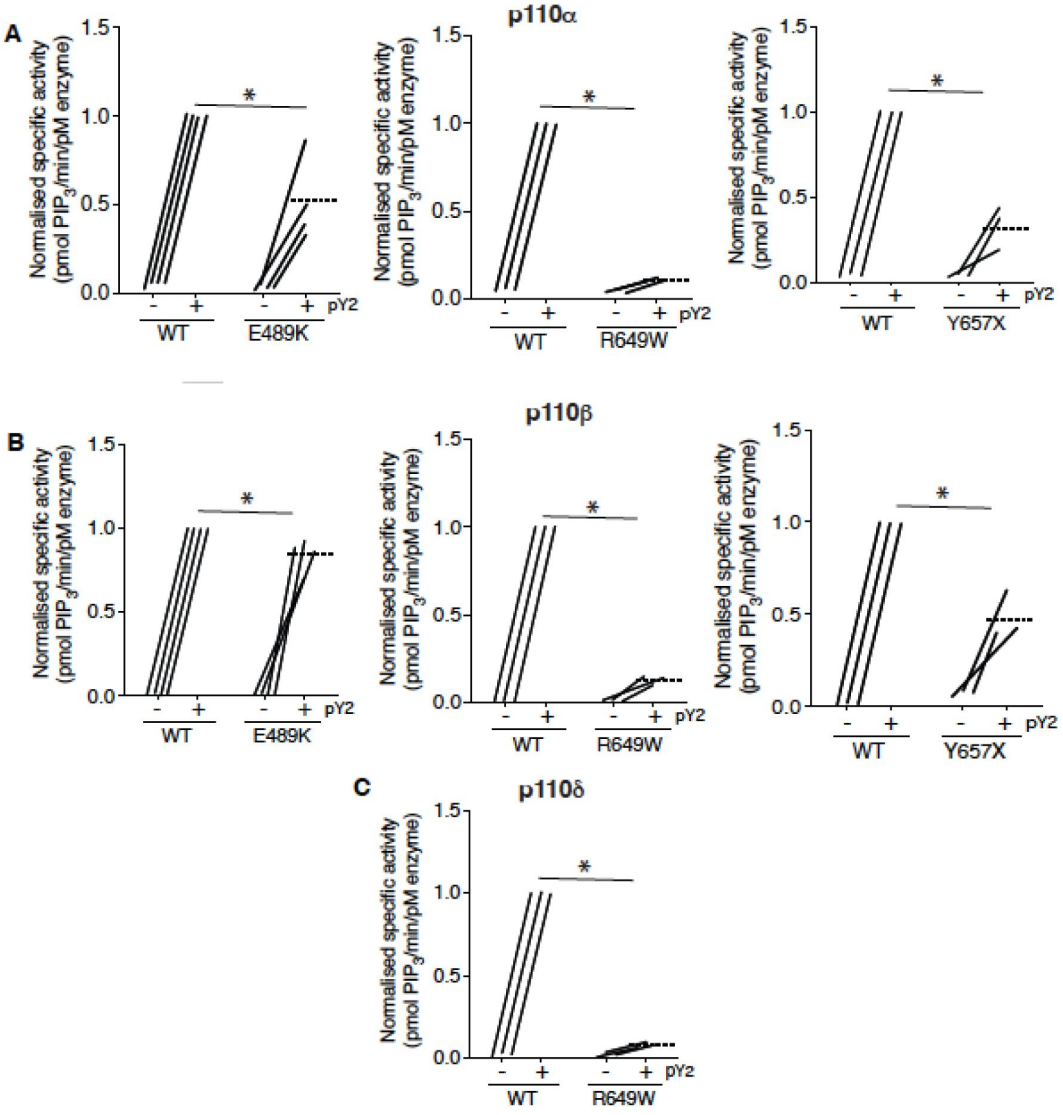

Figure 3.

SHORT syndrome p85α mutations impair phosphotyrosine-stimulated phosphoinositide 3-kinase activity.

Lipid kinase activity of purified recombinant PI3K complexes generated using baculoviral expression in *Sf9* cells was measured using a modified fluorescence polarisation assay. WT p85α or p85α SHORT syndrome mutations, E489K, R649W or Y657X bound to either (A) p110α (B) p110β or (C) p110δ were assayed for basal and bisphosphotyrosine (pY2)-stimulated lipid kinase activity. Dotted lines mark means, and asterisks indicate a significant difference between the bisphosphotyrosine (pY2)-stimulated state for WT and comparator mutant p85α. More detailed statistical analysis including 95% confidence intervals for the paired mean differences for these comparisons are shown in Figure 3 figure supplement 1.

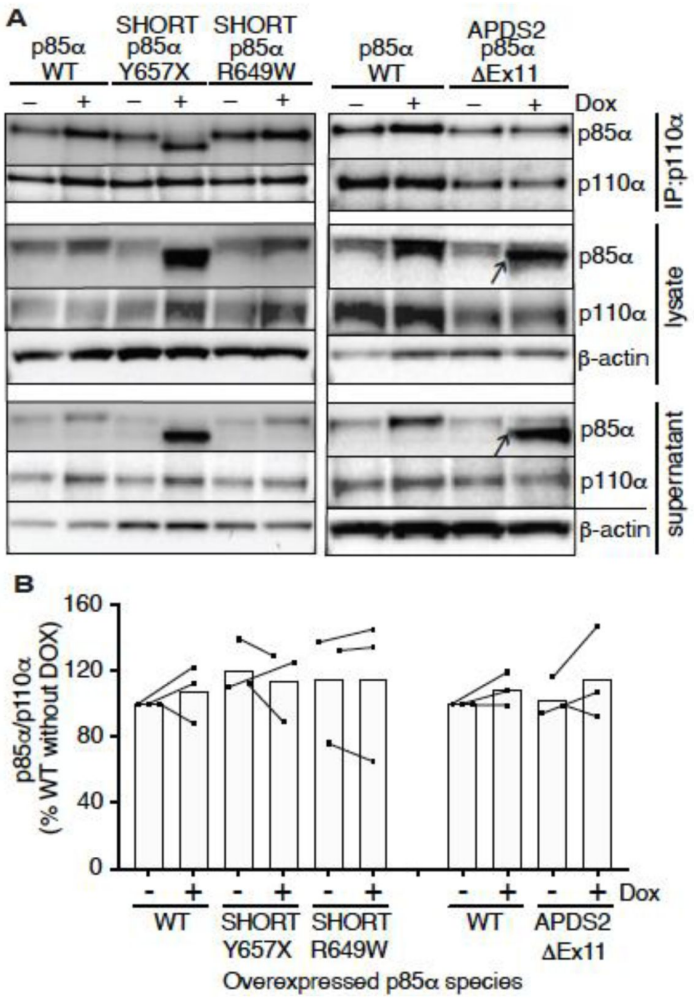

Figure 4.

Ability of pathogenic p85α variants to bind p110α, assessed by co-immunoprecipitation.

Results of immunoblotting of anti-p110α immunoprecipitates from 3T3-L1 cells expressing wild-type, APDS2-associated or SHORT syndrome-associated mutant p85α under the control of doxycycline (Dox) are shown. (A) One representative immunoblot of immunoprecipitate, cell lysate prior to immunoprecipitation, and post immunoprecipitation supernatant is shown. (B) Quantification of immunoblot bands from immunoprecipitates from 3 independent experiments, expressed as a percentage relative to the intensity of the band in WT cells without doxycycline exposure. Co-immunoprecipitated p85α is shown normalized to immunoprecipitated p110α from all 3 independent experiments. Datapoints from the same experiment +/- doxycline are connected by lines. No significant differences were found among conditions.

proteins Irs1 (Myers *et al*, 1992) and Irs2(Sun *et al*, 1995). Excess free regulatory subunits compete with heterodimeric PI3K holoenzyme for binding to these phosphotyrosines (Ueki *et al*., 2002), raising the possibility that excess free, truncated APDS2 p85α ΔEx11 may exert its inhibitory action similarly by outcompeting PI3K holoenzyme for phosphotyrosine binding.

To assess this possibility, we again used the 3T3-L1 cellular model to determine whether overexpression of disease-causing p85α variants impairs recruitment of p110α to Irs1 and Irs2. Irs1 was immunoprecipitated with or without conditional p85α overexpression and with or without insulin stimulation. Overexpression of wild-type p85α had no effect on basal or insulin-induced p110α recruitment to Irs1 (**Fig 5A,B**, Fig 5 supplement 1A,B). In contrast, overexpression of either p85α R649W or Y657X sharply reduced insulin-stimulated p110α recruitment to Irs1 on insulin stimulation (**Fig 5A,B**, Fig 5 supplement 1A,B). In keeping with the inability of the R649W cSH2 domain to bind phosphotyrosines, p85α R649W was also not recruited to Irs1, while overexpression of Y657X increased stimulated but not basal p85α recruitment (**Fig 5A,C**, Fig 5 supplement 1C,D). This suggests a pure defect in PI3K holoenzyme recruitment for R649W. For Y657X the signalling defect may have mixed mechanisms, with reduced activation by phosphotyrosine seen in *in vitro* studies coupled to increased abundance of monomeric mutant p85α, leading to recruitment of non p110α- bound mutant p85α to Irs1.

In keeping with our finding of severely attenuated insulin signalling upon p85α ΔEx11 overexpression (**Fig 2**), overexpression of this mutant p85α abolished p110α recruitment to Irs1 (**Fig 5A,B**, Fig 5 supplement 1A,B). However, non p110α-bound p85α ΔEx11 was strongly recruited to Irs1 even in the absence of insulin stimulation (**Fig 5A,C**, Fig 5 supplement 1C,D). This suggests that although p85α ΔEx11 does not effectively compete with wild-type p85α for binding to p110α, it has preserved or possibly enhanced ability to bind Irs1. This gives the mutant p85α properties that render it a more potent endogenous inhibitor of PI3K signalling than free wild-type p85α. Experiments conducted with immunoprecipitation of Irs2 instead of Irs1 yielded closely similar findings for all p85α species (**Fig 6** and Fig 6 supplement 1).

Discussion

Murine genetic studies of Pik3r1 have proved complicated to interpret, due in part to functional redundancy among PI3K regulatory subunits, and in part to unanticipated effects of perturbing PI3K subunit stoichiometry. This has left several important questions about *in vivo* functions of Pik3r1 unresolved. Identification of a series of human genetic disorders caused by constitutional PIK3R1 mutations over the past 10 years has given fresh impetus to the field and has been mechanistically illuminating.

Homozygous truncating PIK3R1 mutations abolishing p85α expression while preserving p55α and p50α produce agammaglobulinaemia (Conley *et al*, 2012; Tang *et al*, 2018) (Figure 1 figure supplement 1). This resembles the immunodeficiency reported in *Pik3r1* knockout mice (Fruman *et al*, 1999; Suzuki *et al*, 1999) and suggests an essential, non-redundant function of p85α in B cell development in humans. Thereafter, human genetics has provided more novel insights. *PIK3R1* mutations were identified in SHORT syndrome in 2013 (Chudasama *et al*., 2013; Dyment *et al*., 2013; Thauvin-Robinet *et al*., 2013), nearly all in the C-terminal SH2 domain, which together with the N-terminal SH2 domain enables PI3K recruitment to activated RTKs (Rordorf-Nikolic *et al*, 1995). SHORT syndrome features short stature and insulin resistance consistent with impaired ligand-induced p110α action, a phenotype distinct from the enhanced insulin sensitivity produced by genetic ablation of one or more *Pik3r1* products in mice (Chen *et al*., 2004; Fruman *et al*., 2000; Mauvais-Jarvis *et al*., 2002). Mice with SHORT syndrome mutations knocked in were generated after human findings, and faithfully reproduce the human phenotype (Kwok *et al*., 2020; Solheim *et al*, 2018; Winnay *et al*, 2016), confirming that expression of a signalling-impaired PIK3R1 has different consequences to Pik3r1 knockout. No

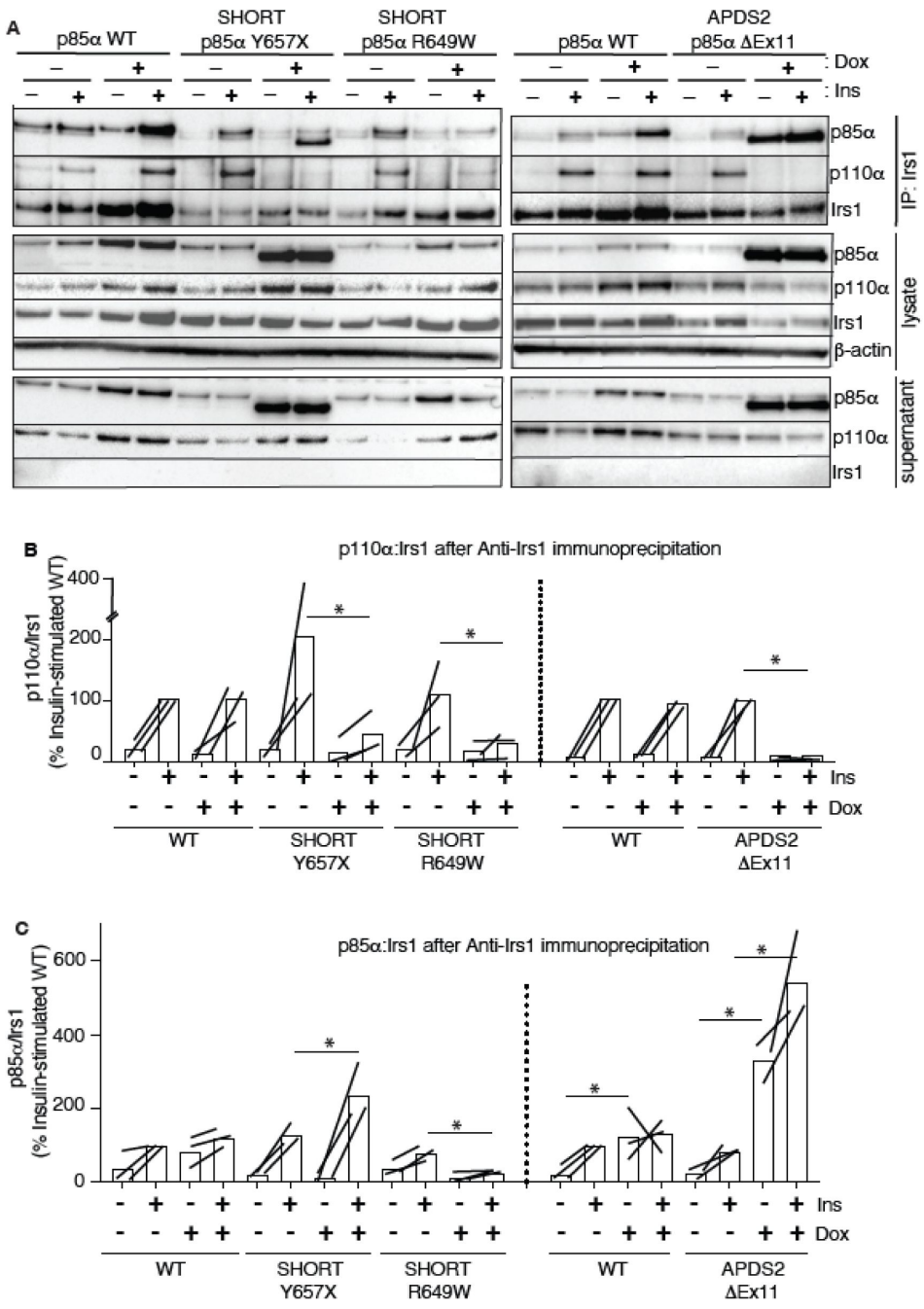

Figure 5.

Attenuated insulin-induced association of p110α with Irs1 in the presence of APDS2 and SHORT syndrome mutant p85α.

Results of immunoblotting of anti-Irs1 immunoprecipitates from 3T3-L1 cells expressing wild-type, APDS2-associated or SHORT syndrome-associated mutant p85α under the control of doxycycline (Dox) are shown. Treatment with 100nM insulin (Ins) is indicated. (A) One representative immunoblot of immunoprecipitate, cell lysate prior to immunoprecipitation, and post immunoprecipitation supernatant is shown. Two separate sets of gels, including independent wild-type controls are shown on left and right. (B-C) Quantification of immunoblot bands from immunoprecipitates from 3 independent experiments. Immunoprecipitated p110α is shown normalized to immunoprecipitated Irs1 from all 3 independent experiments in (B), and immunoprecipitated p85α similarly in (C). Datapoints from the same experiment +/- insulin are connected by lines. Asterisks indicate significant differences induced by transgene overexpression (i.e. plus versus minus doxycycline). More detailed statistical analysis including 95% confidence intervals for the paired mean differences for these comparisons are shown in Figure 5 figure supplement 1.

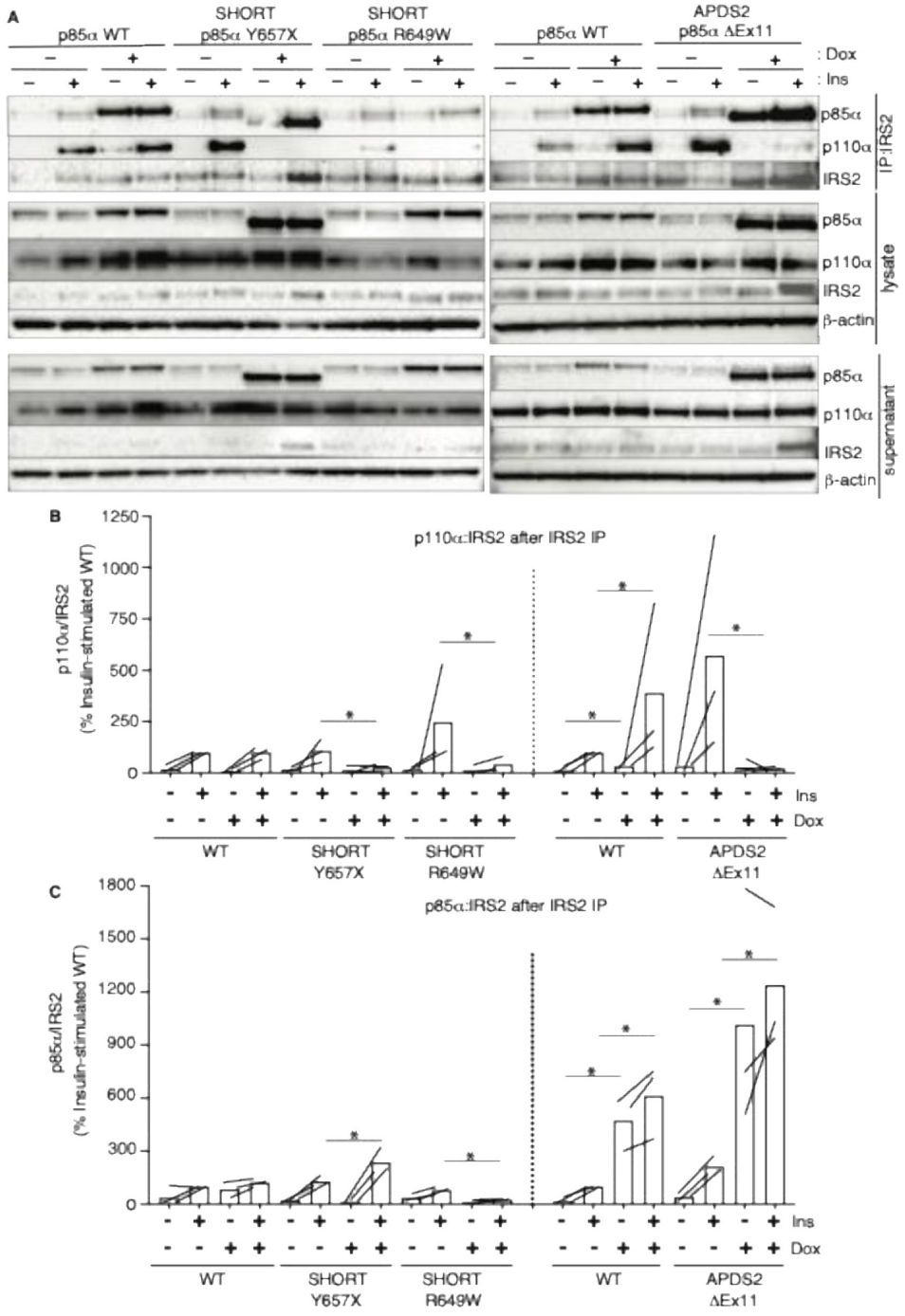

Figure 6.

Attenuated insulin-induced association of p110α with Irs2 in the presence of APDS2 and SHORT syndrome mutant p85α.

Results of immunoblotting of anti-Irs2 immunoprecipitates from 3T3-L1 cells expressing wild-type, APDS2-associated or SHORT syndrome-associated mutant p85α under the control of doxycycline (Dox) are shown. Treatment with 100nM insulin (Ins) is indicated. (A) One representative immunoblot of immunoprecipitate, cell lysate prior to immunoprecipitation, and post immunoprecipitation supernatant is shown. Two separate sets of gels, including independent wild-type controls are shown on left and right. (B-C) Quantification of immunoblot bands from immunoprecipitates from 3 independent experiments. Immunoprecipitated p110α is shown normalized to immunoprecipitated Irs2 from all 3 independent experiments in (B), and immunoprecipitated p85α similarly in (C). Datapoints from the same experiment +/- insulin are connected by lines.

immunodeficiency has been described associated with classic SHORT syndrome mutations, however, suggesting a selective effect on PI3Kα, but the basis of this selectivity has not previously been investigated.

In contrast to SHORT syndrome, mutations in the inter SH2 domain of PIK3R1, mostly leading to skipping of exon 11, were shown in 2014 to activate PI3K *in vitro* and to cause immunodeficiency (APDS2) (Deau *et al*., 2014; Lucas *et al*., 2014b) similar to that caused by activating mutations in p110δ (APDS1) (Angulo *et al*., 2013; Lucas *et al*., 2014a). Neither overgrowth nor metabolic features of APDS2 have been described to suggest p110α hyperactivation, and indeed short stature is common in APDS2 (Elkaim *et al*., 2016; Jamee *et al*., 2020; Maccari *et al*., 2023; Olbrich *et al*., 2016; Petrovski *et al*., 2016), with a growing number of APDS2 patients described with features of SHORT syndrome (Bravo Garcia-Morato *et al*., 2017; Maccari *et al*., 2023; Nguyen *et al*., 2023; Petrovski *et al*., 2016; Ramirez *et al*., 2020; Szczawinska-Poplonyk *et al*., 2022). Moreover mice with the common APDS2 causal Pik3r1 variant knocked in show impaired growth and *in utero* survival, unlike APDS2 murine models(Nguyen *et al*., 2023).

Thus, study of distinct PIK3R1-related syndromes shows that established loss-of-function PIK3R1 mutations produce phenotypes attributable to selectively impaired PI3Kα hypofunction, while activating mutations produce phenotypes attributable to selectively increased PI3Kδ signalling. Indeed, not only do such activating mutations not produce phenotypes attributable to PI3Kα activation, but they surprisingly have features characteristic of impaired PI3Kα function.

Lack of overgrowth in APDS2 has been attributed to greater ability of APDS2 PIK3R1 variants to activate p110δ than p110α (Dornan *et al*., 2017), but the co-occurence of gain- and loss- of function phenotypes has not been explained to date. Our findings suggest that the explanation may lie in competing effects of APDS2 PIK3R1 variants to activate PI3Kδ on one hand, as previously shown (Angulo *et al*., 2013; Dornan *et al*., 2017; Lucas *et al*., 2014b), while on the other hand interfering with PI3Kα through the dominant negative effect of non p110α-bound mutant p85α.

The low expression of truncated p85α ΔEx11 we described in dermal fibroblasts is similar to observations made in lymphocytes, however, in lymphocytes this is associated with increased basal AKT phosphorylation (Deau *et al*., 2014; Lucas *et al*., 2014b) that is abolished by p110δ inhibition (Lucas *et al*., 2014b). P110δ is also expressed in fibroblasts, but protein levels were reduced in the cells studied, consistent with the previously reported observation that when expressed in insect cells the PI3K holoenzyme containing p85α ΔEx11 has a low yield and is unstable (Dornan *et al*., 2017). We speculate that in cells with low endogenous p110δ protein expression, the destabilising effect of mutant PIK3R1 predominates over its activating effect.

The balance between expression and signalling in different cells may be a fine one, however, as transient overexpression of FLAG-tagged p85α ΔEx11 did increase AKT phosphorylation in 3T3 fibroblasts in a previous study, although expression of p110α, β and δ was not determined (Deau *et al*., 2014). We find, in contrast, that overexpression of untagged p85α ΔEx11 has a strong inhibitory effect on insulin signalling, and we were unable to identify a window of overexpression where increased AKT phosphorylation could be observed. We further demonstrated that most or all of the mutant p85α expressed is not bound to p110α, while unbound mutant p85α still binds to Irs1/2, effectively competing with PI3K holoenzyme. The observation that p85α ΔEx11 can associate with Irs1/2 is in agreement with reports that p85α ΔEx11-containing PI3Kα and PI3Kδ can be stimulated by pY2-peptides (Dornan *et al*., 2017; Dornan *et al*., 2020) and that p85α ΔEx11 is recruited to tyrosine-phosphorylated LAT in T cells(Lucas *et al*., 2014b). The competition we suggest between unbound mutant p85α and PI3K holoenzyme for binding to the activated RTKs is in keeping with longstanding evidence that free p85 downregulates PI3K signalling through the

same competitive mechanism (Thorpe *et al*, 2017; Ueki *et al*., 2002), with a single study suggesting in addition that overexpression of tagged p85α leads to Irs1 sequestration with free p85α in cytosolic foci where PI(3,4,5)P3 production does not occur (Luo *et al*, 2005).

The current study has limitations. We have studied primary cells from only a single APDS2 patient, and in the 3T3-L1 cell model, we did not determine whether p110δ protein could be detected. If not, this could explain the lack of detectable AKT phosphorylation with induction of Pik3r1 ΔEx11. Indeed, previous pharmacological studies in 3T3-L1 adipocytes has shown that selective inhibition of p110δ or p110β does not alter insulin-induced phosphorylation of any protein studied in the PI3-K pathway, attesting to the dominance of p110α in insulin action in this cell model (Knight *et al*, 2006). Our study moreover raises further questions. Full length p85α subunits can homodimerize (Cheung *et al*, 2015; Harpur *et al*, 1999; LoPiccolo *et al*, 2015), and it is speculated that homodimers may outcompete p85α/p110 heterodimers for binding to activated Irs1 due to configuration of the four SH2 domains. In the 3T3-L1 preadipocyte APDS2 models, p85α ΔEx11 expression was high despite impaired p110α association, but whether this is composed of monomeric or homodimeric p85α, and whether mutant p85α expression leads to sequestration of Irs1 remote from the insulin receptor, as previously suggested for tagged wild-type p85α(Luo *et al*., 2005), is undetermined.

In summary, it is already established that: **A.** genetic activation of PIK3CD causes immunodeficiency without disordered growth, while **B.** inhibition of PIK3R1 recruitment to RTKs and their substrates impairs growth and insulin action, without immunodeficiency, despite all catalytic subunits being affected and **C.** loss of p85α alone causes immunodeficiency. The current study, coupled with prior reports, suggests that the common APDS2 mutation in PIK3R1 has mixed consequences, producing greater hyperactivation of p110δ than p110α, based on subtle differences in the inhibitory interactions of regulatory and catalytic subunits, while also destabilising PI3K holoenzyme and exerting dominant negative activity on wild-type PIK3R1 function. We suggest that these competing activating and inhibitory consequences are finely balanced, potentially differing among tissues, leading to mixed clinical profiles of gain- and loss-of function features.

Materials and Methods

Ethics

The patient studied gave written informed consent for NIH IRB-approved research protocols 06-I-0015 and 09-I-0133 and was reported previously(Lucas *et al*., 2014b). Dermal fibroblasts were isolated from a skin punch biopsy and cultured as previously described(Huang-Doran *et al*., 2016).

Baculovirus Generation

p85α point mutation expression constructs were created by site-directed mutagenesis of a Hsp85α_pACEBac1 plasmid using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, 200521). In-Fusion cloning (Takara, 638909) was used to generate Hsp85α_pACEBac1_p85α ΔEx11. In brief, Hsp85α_pACEBac1 backbone was digested (BamHI/NotI) and purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, D4001), while the cDNA insert was purified using NucleoSpin® Clean-up Kit (Takara, 740609.10). Insert generation was performed by High-Fidelity PCR using primers designed to yield fragments containing exons 1-10 or 12-16 with the necessary overlap, with inserts verified by electrophoresis and purified using NucleoSpin® Clean-up kit. In-Fusion reactions were performed according to manufacturer's guideline using 100ng each of linearised plasmid and both inserts in 10μL. Products were transformed into Stellar Competent cells (Takara, 636763). Purified plasmid inserts were sequenced and verified by restriction enzyme digest, exploiting loss of a DpnI site within excised exon 11.

Hsp85α_pACEBac1, and Hsp110α_pACEBac1, Hsp110β_pACEBac1 and Hsp110δ_pFastBac™HT B plasmids encoding N terminally tandem His-tagged p110 subunits were used to generate bacmid DNA. MAX Efficiency DH10Bac™ Competent (Invitrogen, 10361-012) or EMBacY cells (Thermofisher, 10361012) were transformed with 40ng plasmid DNA and turbid cultures plated onto agar containing 50μg/mL kanamycin, 10μg/mL gentamicin, 10μg/mL tetracycline, 100μg/mL X-Gal and 40μg/mL IPTG. Single colonies were picked, expanded and purified at 2 days and bacmid concentrations quantified by Nanodrop 1000 Spectrophotometer (Thermo Scientific). 2-4μg bacmid was transfected into Sf9 cells in 6 well plates using Insect-XPRESS (Promega E2311). Cells were incubated at 2°C for 5 days and bacmid YFP expression assessed by Leica DM IL LED Fluo microscope using a green fluorescent protein filter cube. Pooled virus from 2 wells of supernatant (P1 stock) was used to produce high titre P2 stock by transfection of 1.5x106 Sf9 cells/mL (Thermofisher, 11496015) in 450mL using Insect-XPRESS with L-Glutamine (Lonza LZBE12-730Q). For catalytic subunits, 1-2mL P2 stock was used for a further round of Sf9 transfection and expansion to generate P3 stock.

Purification of PI3K holoenzymes

1.5-2L of 1.5x106 Sf9 cells/mL were co-infected with 18mL P3 catalytic subunit and 4mL P2 regulatory subunit baculovirus. Non infected Sf9 cells were negative controls, and a prior protein preparation of 320 kDa was the positive control. Cells were cultured at 27°C, harvested 48h post-infection, pelleted, washed and stored at -80°C. Pellets were later lysed by sonication in buffer containing 20 mM TrisHCl (pH 8.0), 300 mM NaCl, 20 mM imidazole (pH 8.0) and 1 mM TCEP (pH 7.0) at 4 °C. Universal Nuclease (Thermofisher, 88702) was added to lysates before ultracentrifugation at 35,000 rpm for 35min at 4°C. p85α/p110 heterodimers were pulled down via 6 tandem p110 N-terminal His tags, preventing purification of monomeric p85α, using tandem Ni2+HisTrap Fast Flow columns (GE Healthcare) (equilibration buffer 20 mM TrisHCl (pH 8.0), 100 mM NaCl, 20 mM imidazole and 1 mM TCEP (pH 7.0); elution buffer 20 mM TrisHCl (pH 8.0), 100 mM NaCl, 200 mM imidazole and 1 mM TCEP (pH 7.0)). Further purification utilised a heparin HiTrap Q HP column (GE Healthcare) equilibrated with 20mM TrisHCl (pH 8.0) and 1mM TCEP (pH 7.0), with proteins eluted in 20mM TrisHCl (pH 8.0), 1 M NaCl and 1mM TCEP (pH 7.0). Eluted fractions were concentrated to ≥1mg/mL using Millipore Amicon Ultra-15 Centrifugal Filter Units with Ultracel-50 membrane, and 1mL concentrated fractions were gel filtered on Superdex 200 16/60 columns (GE Healthcare) equilibrated in 20mM HEPES (pH 7.5), 100mM NaCl and 2mM TCEP (pH 7.0). The p110 6-His tag was retained for proteins used in functional analyses. KTA Protein Purification Systems (GE Healthcare) and UNICORN Control Software version 5.11 (GE Healthcare) were used for all purifications. Purity of eluted complexes was verified by SDS-PAGE, and purified proteins were quantified using a Nanodrop 1000 Spectrophotometer (Thermofisher). Protein concentration was calculated using the molecular extinction coefficient (assuming full cysteine reduction) determined by heterodimer sequence input to the ProtParam tool (ExPASy). Purified proteins were stored at –80°C in single-use aliquots.

Total injection volume onto gel filtration columns was kept at 1 mL. Yields of PI3K complexes were determined using the area under the curve (280nm mAU per mL eluted protein) from gel filtration chromatograms, and normalised to the volume of Sf9 cells infected.

Liposome preparation

Chloroform/methanol solutions of sphingomyelin, cholesterol, porcine brain phosphatidylcholine, porcine brain phosphatidylethanolamine, porcine brain phosphatidylserine and porcine brain PI(4,5)P2 (Avanti Polar Lipids) were mixed to generate a preparation containing 5/10/15/45/20/5% (wt/vol) of each lipid, with a total lipid concentration of 5 mg/mL and final PI(4,5)P2 concentration 250 μg/mL. Lipid preparations were dessicated under argon and then in a vacuum desiccator. Lipids were rehydrated in buffer containing 20mM HEPES (pH 7.5), 100 mM KCl and 1 mM EGTA (pH 8.0) using vortexing for 3 min, waterbath sonication for 15 min, and 10 cycles between liquid

nitrogen and a 43°C water bath. Unilamellar vesicles were generated by extrusion through polycarbonate filters with 100nm pores, using a glass-tight syringe. Single use aliquots were stored at -80 °C.

Fluorescence Polarisation Assay

PI(3,4,5)P3 production was measured by modified PI3-Kinase activity fluorescence polarisation assay (Echelon Biosciences, Salt Lake City, UT, USA). 10µL reactions in 384-well black microtitre plates used 1mM liposomes containing 50µM PI(4,5)P2, optimised concentrations of purified PI3K proteins, 100µM ATP, 2mM MgCl2, with or without 1µM tyrosine bisphosphorylated 33-mer peptide derived from mouse PDGFRβ residues 735-767, including phosphotyrosine at positions 740 and 751 ("pY2"; 735-ESDGG**Y**MDMSKDESID**Y**VPMLDMKGDIKYADIE-767; Cambridge peptides). Reactions were quenched with 5µL of solution containing 20mM HEPES (pH 7.5), 150mM NaCl, 30mM EDTA (pH 7.4) and 400nM GST–Grp1-PH, followed by addition of 5µL 40nM TAMRA Red Fluorescent Probe in HNT buffer. Identity of the lipid group coupled to the TAMRA probe was not disclosed by Echelon Biosciences, but as Grp1 recognises specific lipid head groups in competition with PI(3,4,5)P3, it is likely to be a lipid with a similar head group such as inositol 1,3,4,5-tetrakisphosphate. After 1h equilibration, fluorescence polarisation was measured using a PHERAstar spectrofluorometer (BMG Labtech, Ortenberg, Germany) with the FP/540-20/590-20/590-20 optical module. The concentrations of wild-type protein complexes used in the assay were: 20-40nM p85α/p110α, 80-160nM p85α/p110β and 80-160nM p85α/p110δ for basal activities, and 1-2nM p85α/p110α, 2-4 nM p85α/p110β and 2-6nM p85α/p110δ for pY2-stimulated activities. Concentrations of thawed protein aliquots were assessed as 280 nm absorbance by Nanodrop 1000 Spectrophotometer, and samples of preparations used in assays were resolved by SDS-PAGE to confirm equal amounts of each complex. Three technical replicates were used per assay on at least three different occasions. Relevant wild-type p85α/p110 catalytic isoforms were included in each assay as controls. Other controls were 1. Stop mix + probe without lipids, to assess maximum fluorescence polarisation. 2. Stop mix + probe with lipids, to determine the effect of lipids on maximum fluorescence polarisation. 3. Probe without stop mix or lipids to determine minimum fluorescence polarisation and 4. Probe with lipids but no stop mix to determine the effect of lipids on minimum fluorescence polarisation. Standard curves of diC8-PI(3,4,5)P3 in the presence of liposomes were included in every experiment and used to infer PIP3 generated by purified PI3K. Graphpad Prism v6.0 was used to generate sigmoidal standard curves by plotting log transformed diC8-PI(3,4,5)P3 concentrations against fluorescence polarisation. Standard curves for each experiment provided the linear range within which PIP3 could be accurately determined. Preliminary studies found only trivial differences in measured fluorescence polarisation in the presence of pY2, which was thus omitted from subsequent standard curves. Quadruplicate interpolated PIP3 concentrations were averaged and normalised by reaction time (30 min) and enzyme concentration in pM to yield PI3K activity in pmol PIP3/min/pM enzyme. Specific activities were then further normalised to activity of p85α wild-type controls in the presence of pY2 in each experiment.

Generation of 3T3-L1 cells conditionally expressing p85α or p110α

3T3-L1 preadipocytes (Zenbio, lot 3T3062104, passage 8) were cultured, differentiated and stained for neutral lipid with Oil Red O as previously described(Huang-Doran *et al*, 2011). Generation of 3T3-L1 sublines inducibly expressing WT, Y657X, or R649W p85α was also previously reported(Huang-Doran *et al*., 2016; Hussain *et al*, 2011). The 3T3-L1 lines inducibly expressing p85α ΔEx11 or p110α H1047R were generated using essentially the same procedure, starting with In-Fusion subcloning of the p85α ΔEx11 cDNA insert from the pACEBac1 p85α ΔEx11 plasmid described above, and of PIK3CA H1047R cDNA (Based on Uniprot P42336) derived by PCR of a pre-existing expression vector, into the pEN_Tmcs entry vector. After sequence verification these inserts were integrated into a pSLIK-Hygro lentiviral vector, packaged into VSV-G–pseudotyped lentiviral particles, using packaging plasmids pMDLg/pRRE and pRSVREV, and VSV-G envelope

plasmid pVSV, and used to infect 3T3-L1 preadipocytes (Plasmids listed in **Table 2**). Transgene expression was induced with 1 µg/mL doxycycline for 72 hours, or with variable concentrations of doxycycline as indicated.

Insulin signalling studies

3T3-L1 cells serum starved in DMEM containing 0.5 % BSA for 16h were stimulated with 100nM Actrapid insulin (Novo Nordisk) for 10min. Monolayers were snap frozen in liquid N2 and stored at -80°C. 200-600µL co-IP lysis (20mM HEPES, 150mM NaCl, 1.5mM MgCl2, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1mM EGTA pH 7.4, 1mM PMSF, 2mM activated sodium orthovanadate, Complete Mini EDTA-free protease inhibitor cocktail and PhosSTOP (Roche 04906837001), in Milli-Q Ultrapure water (Millipore)) or RIPA buffer (50mM Tris HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with added Complete Mini EDTA-free protease inhibitor cocktail and PhosSTOP, in Milli-Q Ultrapure Water (Millipore)) was added to frozen cells before scraping of lysate into pre-chilled tubes, incubation on ice for 30min and clearing by centrifugation. Protein was quantified using the Bio-Rad DC assay.

Lysates were mixed with NuPAGE® LDS Loading buffer (Life Technologies, NP0008) supplemented with 5% β-mercaptoethanol and boiled before SDS-PAGE. For co-immunoprecipitation (Co-IP) lysates (150-300µg protein) were incubated in 500µL Co-IP buffer with immunoprecipitating antibody overnight at 4°C. 10µg lysate mixed with NuPAGE® SDS Loading buffer and 5% β-mercaptoethanol was stored to represent Co-IP input. Co-IP samples were incubated for 2h with 1.5mg PBST-washed Protein G Dynabeads® (Life Technologies, 10003D) at 4°C before centrifugation and bead removal using a DynaMag™-2 Magnet (Invitrogen). Supernatants mixed 1:1 with Co-IP elution buffer (2X NuPAGE® LDS, 100 mM NuPAGE® Sample Reducing Agent (Invitrogen, NP0009), in Co-IP lysis buffer) were boiled for 10 min. Beads were washed 5 times in 50µL Co-IP lysis buffer, before protein elution with 25µL Co-IP Elution buffer and boiling for 10min. Input, supernatant and co-IP samples underwent SDS-PAGE using NuPAGE® 4-12% gradient Bis-Tris gels (ThermoFisher, NP0321BOX) in NuPAGE® MOPS SDS Running Buffer (Life Technologies, NP0001). Transfer to nitrocellulose was performed using the iBlot™ Dry Blotting System (Invitrogen), with preincubation in Equilibration Transfer Buffer for proteins above 150kDa.

For immunoblotting, blocked membranes were incubated overnight at 4°C in primary antibody, washed and incubated with HRP-linked anti-rabbit or anti-mouse IgG secondary antibody diluted 1:5000 in blocking buffer. Proteins were visualized using the ChemiDoc™ MP System (Bio-Rad) and band intensities quantified using Image Lab software (Bio-Rad).

Statistical Analysis

For quantitative data, all biological replicates (i.e. results of independent experiments on different days) are represented in figures, with paired points (e.g. with/without insulin in the same experiment) connected by lines. For fluorescence polarisation assays, each biological replicate shown is the mean of 3 technical replicates. Sample size for individual experiments was not pre-determined. To avoid the pitfalls of dichotomous significance testing on low-throughput biological datasets, we used estimation statistics (Data Analysis using Bootstrap-Coupled ESTimation) with default settings (5000 resamples, BCa boostrap)(Ho *et al*, 2019). This focuses on effect sizes and derives confidence intervals derived from bootstrapping for differences in means; the small (but typical) sample size of the experiments analysed limits reliable bootstrapping, but it offers additional confidence to the consistent patterns seen in independent replicates. We have indicated significance in main text figures with an asterisk, and show the 95% confidence intervals for mean differences in extended view figures.

Table 1

Antibodies Used

Antibody Target	Company	Catalogue No.	Species	Dilution used (immunoblot/IP)
p85α	CST	4257	Rabbit	1:1000
Phospho-AKT/Akt (T308)	CST	9275	Rabbit	1:1000
Phospho-AKT/Akt (S473)	CST	9271	Rabbit	1:1000
AKT/Akt (1,2,3)	CST	2920	Mouse	1:1000
p110α	CST	4249	Rabbit	1:1000/1:50
p110δ	CST	34050	Rabbit	1:1000
Irs1	Millipore	06-248	Rabbit	1:500
Irs1	CST	2382	Rabbit	1:1000/1:50
Irs2 clone 9.52	Millipore	MABS15	Mouse	1:1000/1:50
β-actin	CST	4967	Rabbit	1:5000
Anti-rabbit IgG; HRP-linked	CST	7074	Goat	1:5000
Anti-mouse IgG; HRP-linked	CST	7076	Horse	1:5000

Table 2

Plasmids Used

Plasmid Name	Insert	Comment
Hsp85a_pACEBac1	Human p85α (UniProtKB P27986.2)	Generated in Williams Lab, MRC-LMB
Hsp85a-Y657*_pACEBac1	Human p85α-Y657*	Based on Hsp85a_pACEBac1
Hsp85a-R649W_pACEBac1	Human p85α-R649W	Based on Hsp85a_pACEBac1
Hsp85a-E489K_pACEBac1	Human p85α-E489K	Based on Hsp85a_pACEBac1
Hsp85a-dEx11_pACEBac1	Human p85α-dEx11	Based on Hsp85a_pACEBac1
Hsp110a_pFastBacHT B	Human p110α (UniProtKB P42336.2)	Generated in Williams Lab, MRC-LMB
Hsp110b_pACEBac1	Human p110β (UniProtKB P42338.1)	Generated in Williams Lab, MRC-LMB
Hsp110d_pFastBacHT B	Human p110δ (UniProtKB O00329.2)	Generated in Williams Lab, MRC-LMB
pEN_Tmcs		Addgene #25751
pSLIK-Hygro		Addgene #25737
Hsp85a-dEx11_pSLIK-Hygro	Human p85α-dEx11	
pMDLg/pRRE		Addgene #12251
pRSV-Rev		Addgene #12253
pMD2.G		Addgene #12259

Data and Resource Availability

This study includes no data deposited in external repositories. Uncut Western blot and other source images are available on the Open Science Framework project webpage: *https://osf.io/eyc4h/?view_only=9af8645c8fe5408790ce19920d0084c6*. All antibodies used are listed in **Table 1**, and plasmids in **Table 2**.

Acknowledgements

This work was supported by the Wellcome Trust through a grant to RKS [210752/Z/18/Z] and a studentship to PRT [102356/Z/13/Z]. Additional support was from the UK Medical Research Council (MRC) [MC_UU_12012/5 and MC_U105184308 (to RLW)] and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (to HCS). We thank Dr. Koneti Rao, Debra Long-Priel, and Angela Wang for clinical, technical, and regulatory assistance.

Additional information

Author Contributions

PRT: conceptualisation, methodology, formal analysis, investigation, visualization, writing - original draft. RK: formal analysis, investigation, visualization. OP: supervision, methodology, investigation, writing - review & editing. HCS: resources, funding acquisition, writing - review & editing. GVB: supervision, methodology, writing - review & editing. RLW: supervision, methodology, investigation, funding acquisition, writing - review & editing. RKS: conceptualisation, supervision, methodology, funding acquisition, writing - original draft, writing - review & editing, funding acquisition.

Disclosure and competing interests statement

RKS has consulted for Novartis on clinical aspects of PIK3CA-related overgrowth, and for Alnylam, Amryt and AstraZeneca on clinical aspects of monogenic insulin resistance and lipodystrophy

References

Angulo I *et al.* (2013) **Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage** *Science* **342**:866–871

Avila M *et al.* (2016) **Clinical reappraisal of SHORT syndrome with PIK3R1 mutations: toward recommendation for molecular testing and management** *Clin Genet* **89**:501–506

Brachmann SM, Ueki K, Engelman JA, Kahn RC, Cantley LC (2005) **Phosphoinositide 3-kinase catalytic subunit deletion and regulatory subunit deletion have opposite effects on insulin sensitivity in mice** *Mol Cell Biol* **25**:1596–1607

Bravo Garcia-Morato M, Garcia-Minaur S, Molina Garicano J, Santos Simarro F, Del Pino Molina L, Lopez-Granados E, Ferreira Cerdan A, Rodriguez Pena R (2017) **Mutations in PIK3R1 can lead to APDS2, SHORT syndrome or a combination of the two** *Clin Immunol* **179**:77–80

Burke JE, Perisic O, Masson GR, Vadas O, Williams RL (2012) **Oncogenic mutations mimic and enhance dynamic events in the natural activation of phosphoinositide 3-kinase p110alpha (PIK3CA)** *Proc Natl Acad Sci U S A* **109**:15259–15264

Chen D, Mauvais-Jarvis F, Bluher M, Fisher SJ, Jozsi A, Goodyear LJ, Ueki K, Kahn CR (2004) **p50alpha/p55alpha phosphoinositide 3-kinase knockout mice exhibit enhanced insulin sensitivity** *Mol Cell Biol* **24**:320–329

Cheung LW, Walkiewicz KW, Besong TM, Guo H, Hawke DH, Arold ST, Mills GB (2015) **Regulation of the PI3K pathway through a p85alpha monomer-homodimer equilibrium** *Elife* **4**

Chudasama KK *et al.* (2013) **SHORT syndrome with partial lipodystrophy due to impaired phosphatidylinositol 3 kinase signaling** *Am J Hum Genet* **93**:150–157

Conley ME, Dobbs AK, Quintana AM, Bosompem A, Wang YD, Coustan-Smith E, Smith AM, Perez EE, Murray PJ (2012) **Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K** *J Exp Med* **209**:463–470

Cottrell CE *et al.* (2021) **Somatic PIK3R1 variation as a cause of vascular malformations and overgrowth** *Genet Med*

Deau MC *et al.* (2014) **A human immunodeficiency caused by mutations in the PIK3R1 gene** *J Clin Invest* **124**:3923–3928

Dornan GL, Siempelkamp BD, Jenkins ML, Vadas O, Lucas CL, Burke JE (2017) **Conformational disruption of PI3Kdelta regulation by immunodeficiency mutations in PIK3CD and PIK3R1** *Proc Natl Acad Sci U S A* **114**:1982–1987

Dornan GL, Stariha JTB, Rathinaswamy MK, Powell CJ, Boulanger MJ, Burke JE (2020) **Defining How Oncogenic and Developmental Mutations of PIK3R1 Alter the Regulation of Class IA Phosphoinositide 3-Kinases** *Structure* **28**:145–156

Dyment DA *et al.* (2013) **Mutations in PIK3R1 cause SHORT syndrome** *Am J Hum Genet* **93**:158–166

Elkaim E *et al.* (2016) **Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study** *J Allergy Clin Immunol* **138**:210–218

Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT (2017) **The PI3K Pathway in Human Disease** *Cell* **170**:605–635

Fruman DA, Mauvais-Jarvis F, Pollard DA, Yballe CM, Brazil D, Bronson RT, Kahn CR, Cantley LC (2000) **Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha** *Nat Genet* **26**:379–382

Fruman DA, Snapper SB, Yballe CM, Davidson L, Yu JY, Alt FW, Cantley LC (1999) **Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha** *Science* **283**:393–397

Geering B, Cutillas PR, Nock G, Gharbi SI, Vanhaesebroeck B (2007) **Class IA phosphoinositide 3-kinases are obligate p85-p110 heterodimers** *Proc Natl Acad Sci U S A* **104**:7809–7814

Harpur AG, Layton MJ, Das P, Bottomley MJ, Panayotou G, Driscoll PC, Waterfield MD (1999) **Intermolecular interactions of the p85alpha regulatory subunit of phosphatidylinositol 3-kinase** *J Biol Chem* **274**:12323–12332

Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A (2019) **Moving beyond P values: data analysis with estimation graphics** *Nat Methods* **16**:565–566

Huang-Doran I *et al.* (2011) **Genetic defects in human pericentrin are associated with severe insulin resistance and diabetes** *Diabetes* **60**:925–935

Huang-Doran I *et al.* (2016) **Insulin resistance uncoupled from dyslipidemia due to C-terminal PIK3R1 mutations** *JCI Insight* **1**

Hussain K *et al.* (2011) **An activating mutation of AKT2 and human hypoglycemia** *Science* **334**

Jamee M *et al.* (2020) **Clinical, Immunological, and Genetic Features in Patients with Activated PI3Kdelta Syndrome (APDS): a Systematic Review** *Clin Rev Allergy Immunol* **59**:323–333

Knight ZA *et al.* (2006) **A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling** *Cell* **125**:733–747

Kwok A *et al.* (2020) **Truncation of Pik3r1 causes severe insulin resistance uncoupled from obesity and dyslipidaemia by increased energy expenditure** *Mol Metab* **40**

Lindhurst MJ *et al.* (2012) **Mosaic overgrowth with fibroadipose hyperplasia is caused by somatic activating mutations in PIK3CA** *Nat Genet* **44**:928–933

LoPiccolo J *et al.* (2015) **Assembly and Molecular Architecture of the Phosphoinositide 3-Kinase p85alpha Homodimer** *J Biol Chem* **290**:30390–30405

Lucas CL *et al.* (2014) **Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency** *Nat Immunol* **15**:88–97

Lucas CL *et al.* (2014) **Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K** *J Exp Med* **211**:2537–2547

Luo J, Field SJ, Lee JY, Engelman JA, Cantley LC (2005) **The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex** *J Cell Biol* **170**:455–464

Maccari ME *et al.* (2023) **Activated phosphoinositide 3-kinase delta syndrome: Update from the ESID Registry and comparison with other autoimmune-lymphoproliferative inborn errors of immunity** *J Allergy Clin Immunol* **152**:984–996

Madsen RR, Vanhaesebroeck B, Semple RK (2018) **Cancer-Associated PIK3CA Mutations in Overgrowth Disorders** *Trends Mol Med* **24**:856–870

Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC, Kahn CR (2002) **Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes** *J Clin Invest* **109**:141–149

Myers MG, Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF (1992) **IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85** *Proc Natl Acad Sci U S A* **89**:10350–10354

Nguyen T *et al.* (2023) **Human PIK3R1 mutations disrupt lymphocyte differentiation to cause activated PI3Kdelta syndrome 2** *J Exp Med*

Olbrich P *et al.* (2016) **Activated PI3Kdelta syndrome type 2: Two patients, a novel mutation, and review of the literature** *Pediatr Allergy Immunol* **27**:640–644

Petrovski S *et al.* (2016) **Dominant Splice Site Mutations in PIK3R1 Cause Hyper IgM Syndrome, Lymphadenopathy and Short Stature** *J Clin Immunol* **36**:462–471

Philp AJ, Campbell IG, Leet C, Vincan E, Rockman SP, Whitehead RH, Thomas RJ, Phillips WA (2001) **The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors** *Cancer Res* **61**:7426–7429

Ramirez L, Tamayo W, Ale H (2020) **APDS2 and SHORT Syndrome in a Teenager with PIK3R1 Pathogenic Variant** *J Clin Immunol* **40**:1020–1025

Rordorf-Nikolic T, Van Horn DJ, Chen D, White MF, Backer JM (1995) **Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit** *J Biol Chem* **270**:3662–3666

Solheim MH, Winnay JN, Batista TM, Molven A, Njolstad PR, Kahn CR (2018) **Mice Carrying a Dominant-Negative Human PI3K Mutation Are Protected From Obesity and Hepatic Steatosis but Not Diabetes** *Diabetes* **67**:1297–1309

Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG, Glasheen E, Lane WS, Pierce JH, White MF (1995) **Role of IRS-2 in insulin and cytokine signalling** *Nature* **377**:173–177

Suzuki H, Terauchi Y, Fujiwara M, Aizawa S, Yazaki Y, Kadowaki T, Koyasu S (1999) **Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase** *Science* **283**:390–392

Szczawinska-Poplonyk A, Bernat-Sitarz K, Schwartzmann E, Piechota M, Badura-Stronka M (2022) **Clinical and immunological assessment of APDS2 with features of the SHORT syndrome related to a novel mutation in PIK3R1 with reduced penetrance** *Allergol Immunopathol (Madr* **50**:1–9

Tang P, Upton JEM, Barton-Forbes MA, Salvadori MI, Clynick MP, Price AK, Goobie SL (2018) **Autosomal Recessive Agammaglobulinemia Due to a Homozygous Mutation in PIK3R1** *J Clin Immunol* **38**:88–95

Thauvin-Robinet C *et al.* (2013) **PIK3R1 mutations cause syndromic insulin resistance with lipoatrophy** *Am J Hum Genet* **93**:141–149

Thorpe LM, Spangle JM, Ohlson CE, Cheng H, Roberts TM, Cantley LC, Zhao JJ (2017) **PI3K-p110alpha mediates the oncogenic activity induced by loss of the novel tumor suppressor PI3K-p85alpha** *Proc Natl Acad Sci U S A* **114**:7095–7100

Tsolakos N *et al.* (2018) **Quantitation of class IA PI3Ks in mice reveals p110-free-p85s and isoform-selective subunit associations and recruitment to receptors** *Proc Natl Acad Sci U S A* **115**:12176–12181

Ueki K, Fruman DA, Brachmann SM, Tseng YH, Cantley LC, Kahn CR (2002) **Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival** *Mol Cell Biol* **22**:965–977

Wentink M *et al.* (2017) **Genetic defects in PI3Kdelta affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infections** *Clin Immunol* **176**:77–86

Winnay JN *et al.* (2016) **PI3-kinase mutation linked to insulin and growth factor resistance in vivo** *J Clin Invest* **126**:1401–1412

Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM (1998) **Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit** *Mol Cell Biol* **18**:1379–1387

Zhao JJ, Cheng H, Jia S, Wang L, Gjoerup OV, Mikami A, Roberts TM (2006) **The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation** *Proc Natl Acad Sci U S A* **103**:16296–16300

Author information

Patsy R Tomlinson

The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, MRC Metabolic Diseases Unit, Wellcome Trust-MRC

Institute of Metabolic Science, Cambridge, UK

Rachel Knox
The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, MRC Metabolic Diseases Unit, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, The National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge, UK

Olga Perisic
MRC Laboratory of Molecular Biology, Cambridge, UK

Helen C Su
Laboratory of Clinical Immunology & Microbiology, Intramural Research Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, USA
ORCID iD: 0000-0002-5582-9110

Gemma V Brierley
Department of Comparative Biomedical Science, The Royal Veterinary College, London, UK, The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK
ORCID iD: 0000-0002-4600-9970

Roger L Williams
MRC Laboratory of Molecular Biology, Cambridge, UK
ORCID iD: 0000-0001-7754-4207

Robert K Semple
The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, UK, Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, UK, MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh, UK, The National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge, UK
ORCID iD: 0000-0001-6539-3069

For correspondence: rsemple@ed.ac.uk

Editors

Reviewing Editor
Marcus Seldin
University of California, Irvine, Irvine, United States of America

Senior Editor
Jonathan Cooper
Fred Hutchinson Cancer Research Center, Seattle, United States of America

Reviewer #1 (Public review):

Summary:

This study provides convincing data showing that expression of the PIK3R1(deltaExon11) dominant negative mutation in Activated PI3K Delta Syndrome 1/2 (APDS1/2) patient-derived cells reduces AKT activation and p110δ protein levels. Using a 3T3-L1 model cell system, the

authors show that overexpressed p85α(deltaExon 11) displays reduced association with the p110α catalytic subunit but strongly interacts with Irs1/2. Overexpression of PIK3R1 dominant negative mutants inhibit AKT phosphorylation and reduce cellular differentiation of preadipocytes. The experimental design, interpretation, and quantification broadly support the authors' conclusions, which establishes a new paradigm that warrants future studies.

Strengths:

The strength of this study is the clear results derived from Western blots analysis of cell signaling markers (e.g. pAKT1), and co-immunoprecipitation of PI3K holoenzyme complexes and associated regulatory factors (e.g. Irs1/2). The authors analyze a variety of PIK3R1 mutants (i.e. deltaExon11, E489K, R649W, and Y657X), which reveals a range of phenotypes that support the proposed model for dominant negative activity. The use of clonal cell lines with doxycycline induced expression of the PIK3R1 mutants (deltaExon 11, R649W, and Y657X) provides convincing experimental data concerning the relationship between p85α mutant expression and AKT phosphorylation in vivo. This approach for overexpression is excellent and should be utilized more broadly by cell biologists. The authors convincingly show that p85α(deltaExon11, R649W, or Y657X) is unable to associate with p110α but instead more strongly associates with Irs1/2 compared to wild type p85α. Overall, this article does a great job of motivating future studies of SHORT and APDS2 PIK3R1 mutants expressed from their endogenous loci (e.g. knock-in mice).

Weaknesses:

The limitations for this study lie in the complexity of the cell signaling pathway under investigation, rather than a lack of rigor by the authors. Future experimentation will help reconcile the cell type specific differences (e.g. APDS2 patient derived cells vs. the 3T3-L1 cell model system) in PIK3R1 mutant behavior reported by the authors. This is also intimately linked to variable expression of PIK3R1 mutants and cell-type specific regulatory factors. Although beyond the scope of this work, an unbiased proteomic study that broadly evaluates the cell signaling landscape could provide a more holistic understanding of the APDS2 and SHORT mutants compared to a candidate-based approach. Additional structural biochemistry of the p110α/p85α(deltaExon 11) complex is needed to explain why PIK3R1 mutant regulatory subunits do not strongly associate with the p110 catalytic subunit. A more comprehensive biochemical analysis of p110α/p85α, p110β/p85α, and p110δ/p85α mutant protein complexes will also be necessary to explain various cell signaling phenotypes. A minor limitation of this study is the use of single end point assays to measure PI3K lipid kinase activity in the presence of one regulatory input (i.e. RTK-derived pY peptide). An expanded biochemical analysis of purified mutant PI3K complexes across the canonical membrane signaling landscape will be important for deciphering how competition between wild-type and mutant regulatory subunits are regulated in different cell signaling contexts.

https://doi.org/10.7554/eLife.94420.2.sa2

Reviewer #2 (Public review):

Patsy R. Tomlinson et al; investigated the impact of different p85 alpha variants associated with SHORT syndrome or APDS2 on insulin mediated signaling in dermal fibroblasts and preadipocytes. They perform this study as APDS2 patients oftern present with features of SHORT syndrome. They found no evidence of hyperactive PI3K signalling monitored by pAKT in a APDS2 patient-derived dermal fibroblast cells. In these cells p110 alpha protein levels were comparable to levels in control cells, however, p110 delta protein levels were strongly reduced. Remarkably, the truncated APDS2-causal p85 alpha variant was less abundant in these cells than p85 alpha wildtype. Afterwards they studied the impact of ectopically

expressed p85 alpha variants on insulin mediated PI3K signaling in 3T3-L1 preadipocytes. Interestingly they found that the truncated APDS2-causal p85 alpha variant impaired insulin induced signaling. Using immunoprecipitation of p110 alpha they did not find truncated APDS2-causal p85 alpha variant in p110 alpha precipitates. Furthermore, by immunoprecipitating IRS1 and IRS2 they observed that the truncated APDS2-causal p85 alpha variant was very abundant in IRS1 and IRS2 precipitates, even in the absence of insulin stimulation. These important findings add in an interesting way possible mechanistic explanation for the growing number of APDS2 patients described with features of SHORT syndrome.

Strengths:

Based on state-of-the-art functional studies, the authors show that the p85 alpha variant responsible for APDS2, known to be associated with increased PI3K-delta signaling, can attenuate PI3K-alpha signalling in preadipocytes, providing a possible mechanistic explanation for the growing number of APDS2 patients with features of SHORT syndrome.

Weaknesses:

The proposed paradigm is based on one cell line derived from an APDS2 patient and an overexpressing system. The investigation of a larger number of cell lines derived from APDS2 patients would further substantiate the conclusion.

https://doi.org/10.7554/eLife.94420.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *The authors identify new mechanisms that link a PIK3R1 mutant to cellular signaling and division in Activated PI3 Kinase Delta Syndrome 1 and 2 (APDS1/2). The conclusion that this mutant serves as a dominant negative form of the protein, impacting PI3K complex assembly and IRS/AKT signaling, is important, and the evidence from constitutive and inducible systems in cultured cells is convincing. Nevertheless, there are several limitations relating to differences between cell lines and expression systems, as well as more global characterization of the protein interaction landscape, which would further enhance the work.*

We are pleased by this fair assessment, while noting that this work relates to APDS2 (PIK3R1-related) rather than APDS1 (PIK3CD-related). Our findings we believe are clear, but the observation that studies including more global proteomics/phosphoproteomics in cells expressing mutants at endogenous levels would add further insight is well made. We hope that this report may motivate such studies by laboratories with wider access to primary cells from patients and knock-in mice.

> ***Public Reviews***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This study provides convincing data showing that expression of the PIK3R1(delta Exon11) dominant negative mutation in Activated PI3K Delta Syndrome 1/2 (APDS1/2) patient-derived cells reduces AKT activation and p110δ protein levels. Using a 3T3-L1 model cell*

> *system, the authors show that overexpressed p85α delta Exon 11) displays reduced association with the p110α catalytic subunit but strongly interacts with Irs1/2. Overexpression of PIK3R1 dominant negative mutants inhibits AKT phosphorylation and reduces cellular differentiation of preadipocytes. The strength of this article is the clear results derived from Western blots analysis of cell signaling markers (e.g. pAKT1), and co-immunoprecipitation of PI3K holoenzyme complexes and associated regulatory factors (e.g. Irs1/2). The experimental design, interpretation, and quantification broadly support the authors' conclusions.*
>
> *Strengths:*
>
> *The authors analyze a variety of PIK3R1 mutants (i.e. delta Exon11, E489K, R649W, and Y657X), which reveals a range of phenotypes that support the proposed model for dominant negative activity. The use of clonal cell lines with doxycycline-induced expression of the PIK3R1 mutants (DExon 11, R649W, and Y657X) provides convincing experimental data concerning the relationship between p85α mutant expression and AKT phosphorylation in vivo. The authors convincingly show that p85α delta Exon11, R649W, or Y657X) is unable to associate with p110α but instead more strongly associates with Irs1/2 compared to wild type p85α. This helps explain why the authors were unable to purify the recombinant p110α/p85α delta Exon 11) heterodimeric complex from insect cells.*
>
> *Weaknesses:*
>
> *Future experimentation will be needed to reconcile the cell type specific differences (e.g. APDS2 patient-derived cells vs. the 3T3-L1 cell model system) in PIK3R1 mutant behavior reported by the authors.*

This is a fair comment. It has been established for many years that relative protein levels even of wild type PIK3CA and PIK3R1 gene products influence sensitivity of PI3K to growth factor stimulation. Such issues of stoichiometry become exponentially more complicated when the numerous potential interactions among the full repertoire of Class 1 PI3K regulatory subunits (3 splice variants of PIK3R1, and also PIK3R2 and PIK3R3) and corresponding catalytic subunits (PIK3CA, PIK3CB, PIK3CD) are considered, and when different activities and stabilities of PIK3R1 mutants are added to the mix. It thus seems obvious to us that different levels of expression of different mutants in different cellular contexts will have different signalling consequences. We establish a paradigm in this paper using an overexpression system, and we strongly agree that this merits further investigation in a wider variety of primary cells (or cells with knock in at the endogenous locus), where available.

> *An unbiased proteomic study that broadly evaluates the cell signaling landscape could provide a more holistic understanding of the APDS2 and SHORT mutants compared to a candidate-based approach.*

We agree. This would be highly informative, but we think would best be carried out in both “metabolic” and “immune” cells with endogenous levels of expression of SHORT or APDS2 PIK3R1 mutants. These are not all currently available to us, and require follow up studies.

> *Additional biochemical analysis of p110α/p85α delta Exon 11 complex is needed to explain why this mutant regulatory subunit does not strongly associate with the p110 catalytic subunit.*

We agree. We present this observation in our overexpression system, which is clear and reproducible, even though somewhat surprising. The failure to bind p110a is likely not

absolute, as sufficient p110a-p85a^{DEx11} was synthesised in vitro in a prior study to permit structural and biochemical studies, although a series of technical workarounds were required to generate enough heterodimeric PI3K to study in vitro given the manifest instability of the complex, particularly when concentrated (PMID 28167755). We already note in discussion that p85a can homodimerize and bind PTEN, likely among other partners, and it may be that the APDS2 deletion strongly favours binding to proteins that effectively compete with p110a. However this requires further study of the wider interactome of the mutant PIK3R1, which, as noted above, are beyond the scope of the current study.

> *It remains unclear why p85α delta Exon 11 expression reduces p110δ protein levels in APDS2 patient-derived dermal fibroblasts.*

We caution that we only had the opportunity to study dermal fibroblasts cultured from a single APDS2 patient, as noted in the paper, and so replication of this finding in future will be of interest. Nevertheless the observation is robust and reproducible in these cells, and we agree that this apparently selective effect on p110d is not fully explained. Having said that, it has been observed previously that heterodimers of the DEx11 p85a variant with either p110a or p110d are unstable, and when the unstable complexes were eventually synthesised, p110a and p110d were demonstrated to show differences in engagement with the mutant p85, with greater disruption of inhibitory interactions observed for p110d (PMID 28167755). It is thus not a great stretch to imagine that as well as disinhibiting p110d more, the DEx11 p85a variant also destabilises the p85a-p110d complex more, potentially explaining its near disappearance in cells with low baseline p110d expression. Following on from the preceding question and response, however, is an alternative explanation, based on the 3T3-L1 overexpression studies in this paper, wherein we were unable to demonstrate binding of p110a by DEx11 p85a. If, in any given cellular context, the mutant p85 could bind p110d but not p110a, then the destabilising effect would be observed only for p110d. So in summary, we believe the selective effect on p110d is explained by differences in binding kinetics and heterodimer stability for different DEx11 p85a-containing complexes. The net effect of these differences may vary among cell types depending on relative levels of subunit expression.

> *This study would benefit from a more comprehensive biochemical analysis of the described p110α/p85α, p110β/p85α, and p110δ/p85α mutant protein complexes. The current limitation of this study to the use of a single endpoint assay to measure PI3K lipid kinase activity in the presence of a single regulatory input (i.e. RTK-derived pY peptide). A broader biochemical analysis of the mutant PI3K complexes across the canonical signaling landscape will be important for establishing how competition between wild-type and mutant regulatory subunits is regulated in different cell signaling pathways.*

We agree that a wider analysis of upstream inputs and downstream network would be of interest, though as noted above the ultimate functional consequences of mutants will be an amalgam of any differential signalling effects of complexes that are stable enough to function, and differential effects of mutant p85a on the kinetics of distinct heterodimer assembly and stability. In this paper we seek to suggest a paradigm worthy of further, deeper assessment. We note that the search space here is large indeed (A. different cell types with differing profiles of PI3K subunit expression B. Multiple upstream stimuli and C. Multiple downstream outputs, with timecourse of responses an additional important factor to consider). These studies are realistically beyond the scope of the current work, but we hope that further studies, as suggested by the reviewer, follow.

> ***Reviewer #2 (Public Review)***
>
> *Summary:*

> *Patsy R. Tomlinson et al; investigated the impact of different p85alpha variants associated with SHORT syndrome or APDS2 on insulin-mediated signaling in dermal fibroblasts and preadipocytes. They find no evidence of hyperactive PI3K signalling monitored by pAKT in APDS2 patient-derived dermal fibroblast cells. In these cells p110alpha protein levels were comparable to levels in control cells, however, the p110delta protein levels were strongly reduced. Remarkably, the truncated APDS2-causal p85alpha variant was less abundant in these cells than p85alpha wildtype. Afterwards, they studied the impact of ectopically expressed p85alpha variants on insulin-mediated PI3K signaling in 3T3-L1 preadipocytes. Interestingly they found that the truncated APDS2-causal p85alpha variant impaired insulin-induced signaling. Using immunoprecipitation of p110alpha they did not find truncated APDS2-causal p85alpha variant in p110alpha precipitates. Furthermore, by immunoprecipitating IRS1 and IRS2, they observed that the truncated APDS2-causal p85alpha variant was very abundant in IRS1 and IRS2 precipitates, even in the absence of insulin stimulation. These important findings add in an interesting way possible mechanistic explanation for the growing number of APDS2 patients described with features of SHORT syndrome.*
>
> *Strengths:*
>
> *Based on state-of-the-art functional investigation the authors propose indicating a loss-of-function activity of the APDS2-disease causing p85alpha variant in preadipocytes providing a possible mechanistic explanation for the growing number of APDS2 patients described with features of SHORT syndrome.*
>
> *Weaknesses:*
>
> *Related to Figure 1: PIK3R1 expression not only by Western blotting but also by quantifying the RNA transcripts, e.g. mutant and wildtype transcripts, was not performed. RNA expression analysis would further strengthen the suggested impaired stabilization/binding.*

It is not completely clear to us how further PIK3R1 mRNA analysis would enhance the points we seek to make. Perhaps the reviewer's point is that changes in protein expression could be explained by reduced transcription rather than having anything to do with altered protein turnover? As shown in Figure 1 supplemental figure 1, sequencing cDNA from each of the primary cell lines studied indicates that both mutant and WT alleles are expressed at or close to 50% of the total mRNA for PIK3CA or PIK3R1 as relevant. While this is not strictly quantitative, allied to prior evidence that these are dominant alleles which require to be expressed to exert their effect, with no evidence for altered mRNA expression of these variants in prior studies, we don't believe any further quantification of mRNA expression would add value.

> *Related to Figure 2*
>
> *As mentioned by the authors in the manuscript the expression of p110delta but also p110beta in 3T3-L1 preadipocytes ectopically expressing p85alpha variants has not been analyzed.*

We agree that such determination would have been a useful addition to the study, but regretfully it was not undertaken in these modified 3T3-L1 cells at the time of study. However independent bulk RNAseq studies of the founder 3T3-L1 cells from which the stably transduced cells were generated, undertaken as part of an unrelated study, revealed the following relative levels of endogenous expression of PI3K subunit mRNA:

Author response table 1.

ENSEMBL_ID	Gene	Description	logCPM	CPM
ENSMUSG00000041417	Pik3r1	p85/p55/p50alpha	7.65	201
ENSMUSG00000027665	Pik3ca	P110alpha	7.09	137
ENSMUSG00000032462	Pik3cb	P110beta	3.54	12
ENSMUSG00000039936	Pik3cd	P110delta	-0.14	1

We have not determined endogenous protein expression, and so have left the text of the discussion unchanged, simply noting that we have not formally assessed protein expression of p110d/p110b. However these transcriptomic findings suggest that p110d protein is likely either undetectable, or else present at extremely low levels compared to endogenous p110a. p110b also appears to be expressed at a much lower level than p110a. In our studies overexpressing mutant PIK3R1 and assessing insulin action, we believe we are largely or perhaps entirely assaying the effect of the mutants on p110a, in keeping with the fact that genetic and pharmacological studies have firmly established that it is p110a that is responsible for mediating the metabolic actions of insulin in adipose tissue and preadipocytes including 3T3-L1 (e.g. PMID 16647110). Indeed, to quote from this study, in 3T3-L1 "… inhibitors of p110b (TGX-115 and TGX-286) and p110d (IC87114 and PIK-23) had no effect on the insulin-stimulated phosphorylation of any protein in the PI3-K pathway."

We have added the following sentence to the discussion:

"The current study has limitations. We have studied primary cells from only a single APDS2 patient, and in the 3T3-L1 cell model, we did not determine whether p110d protein could be detected. If not, this could explain the lack of detectable AKT phosphorylation with induction of Pik3r1 DEx11. Indeed, previous pharmacological studies in 3T3-L1 adipocytes has shown that selective inhibition of p110d or p110b does not alter insulin-induced phosphorylation of any protein studied in the PI3-K pathway, attesting to the dominance of p110a in insulin action in this cell model (Knight et al, 2006)."

> *Furthermore, a direct comparison of the truncated APDS2-causal p85alpha variant with SHORT syndrome-causal p85alpha variants in regard to pAKT level, and p85alpha expression level has not been performed.*
>
> *These investigations would further strengthen the data.*

The cell lines conditionally expressing SHORT syndrome variants have been reported already, as cited (PMID: 27766312). Remarkably, the degree of inhibition of insulin-stimulated signalling is actually less pronounced for the SHORT syndrome variants than for the overexpressed APDS2 variant, as seen in the excerpt from the prior paper below. In this prior paper the maximum insulin concentration used, 100nM, was the concentration used in the current study. While overexpression of the APDS2 p85a variant ablated the response to insulin entirely, it is still seen in the prior study, albeit at a clearly reduced level.

> *Related to Figure 3*
>
> *The E489K and Y657X p85alpha variants should be also tested in combination with p110delta in the PI3K activity in vitro assay. This would help to further decipher the overall impact, especially of the E489K variant.*

We agree that this would make our data more complete, but for logistical reasons (primarily available personnel) we were compelled to constrain the number of p85-p110 combinations we studied. We elected to prioritise the PIK3R1 R649W variant as by far the most common causal SHORT syndrome variant, and as the variant showing the "cleanest" functional

perturbation, namely severely impaired or absent ability to dock to phosphotyrosines in cognate proteins. The paradox that we sought to explain in this paper, namely the phenotypic combination of gain-of-function APDS2 with loss-of-function SHORT syndrome features holds only for APDS2 PIK3R1 variants, and so while it is interesting to document that the canonical SHORT syndrome variant also inhibits PI3Kb and PI3Kd activation in vitro, this was not the main purpose of our study.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Points of clarification and suggestions for improving the manuscript:*
>
> *(1) Explain whether there are any PIK3R1-independent genetic alterations in the APDS2 and PROS-derived cell lines. For example, are there differences in the karyotype of mutant cell lines compared to wild-type cells?*

Karyotypic abnormalities are not an established feature of either PROS or APDS2, and the patients from whom cells were derived were documented to be of normal karyotype. Karyotypic abnormalities acquired during cell culture would not be unprecedented, but confirming normal karyotypes in primary cell lines where there is no specific reason to suppose any alteration exceeds normal expectations for primary cell studies, and so this has not been undertaken.

> *(2) When introducing the APDS2-associated PIK3R1 mutation (lines 126-128), the authors describe both the exon 11 skipping and in-frame deletions. I recommend rewording this sentence to say exon 11 skipping results in an in-frame deletion of PIK3R1. The current wording makes it seem like APDS2-derived cells contain two genetic perturbations: (1) exon 11 skipping and (2) in-frame deletion. Include a diagram in Figure 1 to help explain the location of the mutations being studied in relationship to the PIK3R1 gene sequence and domains (i.e. nSH2, iSH2, cSH2). The description of the exon 11 skipping and in-frame deletions (lines 126-128) would benefit from having a complementary figure that diagrams the location of these mutations in the PIK3R1 gene.*

On review we agree that clarity of description could be enhanced. We have now edited these lines as follows:

“We began by assessing dermal fibroblasts cultured from a previously described woman with APDS2 due to the common causal PIK3R1 mutation. This affects a splice donor site and causes skipping of exon 11, leading to an in-frame deletion of 42 amino acids (434-475 inclusive) in the inter-SH2 domain, which is shared by all PIK3R1 isoforms (Patient A.1 in (Lucas et al., 2014b))(Figure 1 figure supplement 1).”

We have moreover introduced a further figure element including a schematic of all PIK3R1 mutations reported in the current study (new Figure 1 figure supplement 1)

> *(3) For Figure 2, I recommend including a cartoon that illustrates the experimental design showing the induced expression of PIK3R1 mutants, R649W and Y657X, in the background of the wild-type endogenous gene expression.*

Such a figure element has now been generated and included as Figure 2 figure supplement 1, duly called out in the results section where appropriate.

> *(4) For the data plotted in Figure 1B-1C, please clarify whether the experiments represent a single patient or all 3-4 patients shown in Figure 1A.*

Each datapoint shown represents one of the patients in the immunoblots, with all patients included. Each point in turn is the mean from 3 independent experiments. We have added the following to the Figure legend:

“(B)-(E) quantification of immunoblot bands from 3 independent experiments shown for phosphoAKT-S473, phosphoAKT-T308, p110d and p110a respectively. Each point represents data from one of the patient cell lines in the immunoblots. Paired datapoints +/- insulin are shown in (B) and (C), and dotted lines mark means.”

> *(5) I recommend rewording the following sentence: "Given this evidence that APDS2-associated PIK3R1 delta Exon 11 potently inhibits PI3Kα when overexpressed in 3T3-L1 preadipocytes," to say "... potently inhibits PI3Kα signaling when overexpressed in 3T3-L1 preadipocytes." The data shown in Figures 1 and 2 do not support a direct biochemical inhibition of PI3Kα lipid kinase activity by p85α (delta Exon 11).*

This edit has been made.

> *(6) Provide more discussion concerning the percentage of humans with APDS2 or SHORT syndrome that contain the mutations discussed in this paper. How strong is the genotype-phenotype link for these diseases? Are these diseases inherited or acquired through environmental stresses?*

Both APDS2 and SHORT syndrome are very well established, highly penetrant and stereotyped monogenic disease. APDS is defined by the presence of activating PIK3R1 mutations such as the one studied here (by far the commonest causal mutation). SHORT syndrome clinically has some superficial resemblance to other human genetic syndrome including short stature, but when careful attention is paid to characteristic features it is nearly universally attributable to loss-of-function PIK3R1 mutations with the single exception of one case in which a putatively pathogenic PKCE mutation was described (PMID: 28934384). Although both syndromes are monogenic it is often not accurate to refer to them as inherited, as, particularly in SHORT syndrome, de novo mutations (i.e. not found in either parent) are common. Environmental modifiers of phenotypes have not been described. To the introduction has now been added the comment that both conditions are highly penetrant and monogenic.

> *(7) The data presented in Figure 5 would benefit from additional discussion and citations that describe the molecular basis of the interaction between PI3K and Irs1/2. What studies have previously established this is a direct protein-protein interactions? Are there PI3K mutants that don't interact with Irs1/2 that can be included as a negative control? Alternatively, the authors can simply reference other papers to support the mechanism of interaction.*

There is a voluminous literature dating back to the early 1990s documenting the mode of interaction of PI3K with Irs1/2. Relevant papers have now been cited as requested:

p85-Irs1 binding: PMID 1332046 (White lab, PNAS 1992)

p85-Irs2 binding: PMID 7675087 (White lab, Nature 1995)

“This may be important, as p85a mediates recruitment of PI3K to activated tyrosine kinase receptors and their tyrosine phosphorylated substrates, including the insulin-receptor substrate proteins Irs1 (PMID 1332046) and Irs2 (PMID 7675087).”

Regarding PI3K mutants that don't interact with Irs1/2, the SHORT syndrome mutant R649W which we include in this study is perhaps the best example of this, so it is both disease-

causing and functions as such a negative control.

> *(8) To see the effect of the dominant negative delta Exon 11, the truncated p85α needs to be super stoichiometric to the full-length p85α (Figure 2 - Supplemental Figure 2). This is distinct from the results in Figure 1 showing the ADPS2-derived dermal fibroblast express 5-10x lower levels of p85α delta Exon 11 compared to full-length p85α (Figure 1A), but still strongly inhibits pAKT S473 and T308 (Figure 1B-1C). The manuscript would benefit from more discussion concerning the cell type specific differences in phenotypes. Alternatively, do the APDS2-derived dermal fibroblasts have other genetic perturbations that are not accounted for that potentially modulate cell signaling differently compared to 3T3-L1 preadipocytes?*

The reviewer is astute to point out this apparent contrast. First of all, we have no reason to suppose there is any specific, PI3K-modifying genetic perturbation present in the primary dermal fibroblasts studied, although of course the genetic background of these cells is very distinct to that of 3T3-L1 mouse embryo fibroblasts. Related to such background differences, however, substantial variability is usually apparent in insulin-responsiveness even of healthy control dermal fibroblasts. This means that caution should be exercised in extrapolating from studies of the primary cells of a single individual. To illustrate this, we point the reviewer to our 2016 study in which we extensively studied the dermal fibroblasts of a proband with SHORT syndrome due to PIK3R1 Y657X:

From this study we conclude that A. WT controls show quite substantial variation in insulin-stimulated AKT phosphorylation and B. even the SHORT syndrome p85a Y657X variant, expressed at higher levels that WT p85a in dermal fibroblasts, does not produce an obvious decrease in insulin-stimulated AKT phosphorylation, despite extensive evidence from other human cell studies and knock-in mice that it does indeed impaired insulin action in metabolic tissues. For both these reasons we are not convinced that the lower insulin-induced AKT phosphorylation we described in Figure 1 should be overinterpreted until reproduced in other studies with primary cells from further APDS2 patients. This is why we did not comment more extensively on this. We now add the following qualifier in results:

“Despite this, no increase in basal or insulin-stimulated AKT phosphorylation was seen in APDS2 cells compared to cells from wild-type volunteers or from people with PROS and activating PIK3CA mutations H1047L or H1047R (Fig 1A-C, Fig 1 figure supplement 3A,B). Although insulin-induced AKT phosphorylation was lower in fibroblasts from the one APDS2 patient studied compared to controls, we have previously reported extensive variability in insulin-responsiveness of primary dermal fibroblasts from WT controls. Moreover even primary cells from a patient expressing high levels of the SHORT syndrome-associated p85a Y657X did not show attenuated insulin action, so we do not believe the reduced insulin action in APDS2 cells in the current study should be overinterpreted until reproduced in further APDS2 cells.”

Nevertheless we remind the reviewer that the main purpose of our primary cell experiment was to determine if there were any INCREASE in basal PI3K activity, or any difference in p110a or p110d protein levels, and we regard our findings in these regards to be clear.

> *The manuscript would benefit from additional explanation concerning why the E489K, R649W, and Y657X are equivalent substitutes for the characterization of p110α/p85α delta Exon 11). Perhaps a more explicit description of these mutations in relationship to the location of p85α delta Exon 11) mutation would help. I recommend including a diagram in Figure 3 showing the position of the delta Exon 11, E489K, R649W, and Y657X mutations in the PIK3R1 coding sequence. B. Also, please clarify whether all three holoenzyme complexes were biochemically unstable (i.e. p110α/p85α, p110β/p85α, p110δ/p85α) when p85α delta Exon 11) was expressed in insect cells.*

A. Whether or not E489K, R649W and Y657X are “equivalent” to the APDS2 mutant is not really a meaningful issue here. These mutants are being studied because they cause SHORT syndrome without immunodeficiency, while the APDS2 mutant causes APDS2 often with features of SHORT syndrome. That is, it is naturally occurring mutations and the associated genotype-phenotype correlation that we seek to understand. Of the 3 SHORT syndrome causal mutations chosen, R649W is by far the commonest, effectively preventing phosphotyrosine binding, Y657X has the interesting attribute that it can be discriminated from full length p85 on immunoblots due to its truncation, and is moreover a variant that we have studied in cells and mice before, while the rarer E489K is an interesting SHORT syndrome variant as it is positioned more proximally in the p85a protein than most SHORT syndrome causal variants. All variants studied are now illustrated in the new Figure 1 figure supplement 1. B. Regarding stability of PI3K heterodimers containing the APDS2 p85a variant, we tried extensively to purify p110a and p110d complexes without success despite several approaches to optimise production. We did not try to synthesise the p110b-containing complex.

> *(10) I recommend presenting the results in Figure 4 before Figure 3 because it provides a good rationale for why it's difficult to purify the p110α/p85α delta Exon 11) holoenzyme from insect cells.*

This would be true of p110d were studied in Figure 4 but it is not. Figure 4 looks instead at effects on p110a of heterologous overexpression of mutant p85, is a natural lead in to the ensuing figures 5 and 6, and we do not agree it would add value or enhance flow to swap Figures 3 and 4.

> *(11) The authors show that overexpression of the p85α delta Exon 11) did not result in p110α/p85α delta Exon 11) complex formation based on co-immunoprecipitation. Do the authors get the same result when they co-immunoprecipitation p110α/p85α and p110δ/p85α in the APDS2-derived dermal fibroblasts used in Figure 1A?*

This is an interesting question but not an experiment we have done. It is not unfeasible, but generating enough cells to undertake IP experiments of this nature in dermal fibroblasts is a significant undertaking, and with finite resources available and only one primary cell line to study we elected not to pursue this.

> *Details in Methods section:*
>
> *(1) Include catalog numbers and vendors for reagents (e.g. lipids, PhosSTOP, G-Dynabeads, etc.). There is not enough information provided to reproduce this work.*

We have now added all vendors and catalogue numbers where relevant.

> *(2) Concerning the stated lipid composition (5/10/15/45/20/5 %) in the liposome preparation protocol. Please clarify whether these numbers represent molar percentages or mg/mL percentages.*

We have now added that this is expressed as “(wt/vol)”

> *(3) What is the amino acid sequence of the PDGFR (pY2) peptide used for the PI3K activity assay?*

This assay has been published and references with detailed methods are cited. For clarity, however we now say:

“PI(3,4,5)P3 production was measured by modified PI3-Kinase activity fluorescence polarisation assay (Echelon Biosciences, Salt Lake City, UT, USA). 10µL reactions in 384-well black microtitre plates used 1mM liposomes containing 50µM PI(4,5)P2, optimised concentrations of purified PI3K proteins, 100µM ATP, 2mM MgCl2, with or without 1µM tyrosine bisphosphorylated 33-mer peptide derived from mouse PDGFRβ residues 735-767, including phosphotyrosine at positions 740 and 751 (“pY2”; 735-ESDGGYMDMSKDESIDYVPMLDMKGDIKYADIE-767; Cambridge peptides).”

> *(4) Include a Supplemental file containing a comprehensive description of the plasmids and coding sequencing used in this study.*

Such a supplemental file has been created and is included as Table 2

> *Minor points of clarification, citations, and typos:*
>
> *(1) Clarify why Activated PI3K Delta Syndrome 1 (APDS1) is thus named APDS2. See lines 71-72 of the introduction. Also see line 89: "...is common in APDS2, but not in APDS1." Briefly describe the difference between APDS1 and APDS2?*

This is described in the introduction, but we apologise if our wording was not sufficiently clear. We have tried now to remove any ambiguity:

“Some PIK3R1 mutations reduce basal inhibition of catalytic subunits, usually due to disruption of the inhibitory inter-SH2 domain, and are found in cancers (Philp et al, 2001) and vascular malformations with overgrowth(Cottrell et al, 2021). In both diseases, hyperactivated PI3Ka, composed of heterodimers of PIK3R1 products and p110a, drives pathological growth. Distinct inter-SH2 domain PIK3R1 mutations, mostly causing skipping of exon 11 and deletion of residues 434-475, hyperactivate PI3Kd in immune cells, causing highly penetrant monogenic immunodeficiency (Deau et al, 2014; Lucas et al, 2014b). This phenocopies the immunodeficiency caused by genetic activation of p110d itself, which is named Activated PI3K Delta Syndrome 1 (APDS1) (Angulo et al, 2013; Lucas et al, 2014a). The PIK3R1-related syndrome, discovered shortly afterwards, is thus named APDS2.”

> *(2) Figure legend 1. Clarify reference to "Figure EV2".*
>
> *(3) Figure legend 2. Clarify reference to "Figure EV3".*
>
> *(4) Figure legend 3. Clarify reference to "Figure EV5".*

Thank you for pointing out this oversight, arising from failure to update nomenclature fully between versions. “EV” figures actually are the figure supplements in the submission. All labels have now been updated.

> *(5) For Figure 1 - supplemental figure 1C, indicate experimental conditions on the blot (e.g. -/+ insulin).*

This is now added

> *(6) Figure 4B, y-axis. Clarify how data was quantified. Perhaps reword "(% WT without DOX)" for clarity.*

We have left the Y axis label as it is, but have added the following to the figure legend:

“(B) Quantification of immunoblot bands from immunoprecipitates from 3 independent experiments, expressed as a percentage relative to the intensity of the band in WT cells

without doxycycline exposure."

> *(7) In the results section (lines 117-124), please explicitly state whether the described mutations are homo- or heterozygous.*

All mutations are heterozygous, as now explicitly stated

> *(8) I recommend spelling out the SHORT and APDS2 acronyms in the abstract to make this study more accessible.*

We respectfully disagree that such spelling out in the abstract would improve accessibility. Both acronyms are clunky and wordy and are more likely to obscure meaning by squeezing out other words in the abstract. APDS is already spelled out in the introduction, and we now add the following for SHORT syndrome:

"More surprisingly, phenotypic overlap is reported between APDS2 and SHORT syndrome. SHORT syndrome, named for the characteristic developmental features (Short stature, Hyperextensibility, Hernia, Ocular depression, Rieger anomaly, and Teething delay) is caused by loss of PI3Ka function due to disruption of the phosphotyrosine-binding C-terminal SH2 domain (Chudasama et al, 2013; Dyment et al, 2013; Thauvin-Robinet et al, 2013)."

> *(9) I recommend explaining in more detail or rewording the following jargon/terms to make the writing more accessible to a broad audience: "reduced linear growth" (line 83) and "larger series" (line 86). I assume "reduced linear growth" is height.*

Edited as follows:

"It features short stature, insulin resistance, and dysmorphic features (Avila et al, 2016). In recent years, both individual case reports (Bravo Garcia-Morato et al, 2017; Petrovski et al, 2016; Ramirez et al, 2020; Szczawinska-Poplonyk et al, 2022) and larger case series (Elkaim et al, 2016; Jamee et al, 2020; Maccari et al, 2023; Nguyen et al, 2023; Olbrich et al, 2016; Petrovski et al., 2016) have established that many people with APDS2 have overt features of SHORT syndrome, while, more generally, linear growth impairment is common in APDS2, but not in APDS1."

> *(10) For clarity, reword lines 214-215 to read, "No increase in p110α levels was seen on conditional overexpression of wild-type or R649W p85α."*

Change made, thank you

> *(11) Figure 6A - Western blot label says, "657X" instead of "Y657X."*

Now corrected

> *(12) Lines 214-215: For clarity, reword the sentence to say, "No increase in p110α was seen on conditional overexpression...".*

REPEAT OF POINT 10 ABOVE

> *(13) Clarify what interactions are being competed for in the following statement: "... delta Ex11 may exert its inhibitory action by competing with PI3K holoenzyme" (lines 237-238). Are you referring to the interaction between p110α and p85α or the interaction between p110α/p85α and another protein?*

We have endeavoured to clarify by editing as follows:

“As APDS2 p85a DEx11 does not appear to displace wild-type p85a from p110a despite strong overexpression, it is likely that there are high levels of truncated p85a unbound to p110a in the cell. This may be important, as p85a mediates recruitment of PI3K to activated tyrosine kinase receptors and their tyrosine phosphorylated substrates, including the insulin-receptor substrate proteins Irs1 and Irs2. Excess free regulatory subunits compete with heterodimeric PI3K holoenzyme for binding to these phosphotyrosines (Ueki et al., 2002), raising the possibility that excess free, truncated APDS2 p85a DEx11 may exert its inhibitory action similarly by outcompeting PI3K holoenzyme for phosphotyrosine binding.”

> *(14) Provide more information about the following statement and how it relates to the mutations in this study: "Homozygous truncating PIK3R1 mutations abolishing p85α expression while preserving p55α and p50α produce agammaglobulinaemia" (lines 271-272). The manuscript would benefit from a more explicit description of the nature of these mutations.*

This wording seems to us to be explicit, however we agree that a schematic of PIK3R1 genotype-phenotype correlation, as requested elsewhere, would help readers. Such a schematic is now included as Figure 1 figure supplement 1.

> *(15) Typo on line 299: "unclike".*

Corrected.

> *(16) The data presented in this study support a model in which p85α (DExon 11) expression functions as a dominant negative. Please clarify why in the discussion section you explain that p85α (DExon 11) activates PI3K. For example, "...skipping of exon 11, were shown in 2014 to activate PI3K..." (lines 290-291), "...activate PI3Kδ on one hand..." (line 309); "...APDS2 mutations in PIK3R1 has mixed consequences, producing greater hyperactivation of p110δ than p110α" (lines 354-355).*

We do not entirely understand the reviewer’s question and thus request here. p85α (DExon 11) activates PI3Kd in immune cells and in vitro, and this is accepted, based on numerous reports, to be the mechanism underlying immunodeficiency. We do not challenge this, and cite evidence for any such claims in our report. The dominant negative activity we describe here towards PI3Ka activation is based not on inhibition of mutant-containing heterodimer, but rather on destabilisation of and/or competition with heterodimeric WT holoenzyme. This is the basis of the model we present; that is, a finely balanced competition between enzymic activation and mutant holoenzyme destabilisation and competition of mutant free p85a with WT holoenzyme, whose net effect likely differs among cells and tissues, most likely based on the repertoire and proportions of PI3K subunit expression. If the reviewer has specific suggestions for us that will make this point clearer still we should be happy to consider them.

> *(17) Provide references for the statements in lines 349-353 of the discussion.*

This brief closing paragraph is a succinct recap and summary of the key points made throughout the manuscript and thoroughly referenced therein. We prefer to keep this section clean to maximise clarity, but are happy to copy references from the various other places in the manuscript to back up these assertions if this is preferred by the editorial team. Current text:

“In summary, it is already established that: A. genetic activation of PIK3CD causes immunodeficiency without disordered growth, while B. inhibition of PIK3R1 recruitment to RTKs and their substrates impairs growth and insulin action, without immunodeficiency,

despite all catalytic subunits being affected and C. loss of p85 alone causes immunodeficiency."

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *In the abstract line 42 I would rather talk from SHORT syndrome like features.*

Some patients do indeed meet the criteria for SHORT syndrome, but there is a spectrum. We have thus added this qualification and removed "short stature" to maintain the word count, as this is itself a SHORT syndrome-like feature.

> *Line 74 It would be helpful for the reader to give the amino-acid exchange and affected position of this single case.*

We agree. Now added.

> *Furthermore, an illustration indicating the location of the different PIK3R1 variants on the p85 alpha level would be helpful for the reader.*

As noted above such a figure element is now included as Figure 1 figure supplement 1 and duly called out in the text

> *The sentence in lines 298-300 makes no sense to me. Do you mean, unlike APDS1 murine models?*

We agree, on review, that this paragraph is convoluted and makes a simple observation complex. We have rewritten now in what we hope is a more accessible style:

"Thus, study of distinct PIK3R1-related syndromes shows that established loss-of-function PIK3R1 mutations produce phenotypes attributable selectively to impaired PI3Ka hypofunction, while activating mutations produce phenotypes attributable to selectively increased PI3Kd signalling. Indeed, not only do such activating mutations not produce phenotypes attributable to PI3Ka activation, but they surprisingly have features characteristic of impaired PI3Ka function."

> *Line 321 I propose including the notion of different cells: "The balance between expression and signalling in different cells may be a fine one …"*

This change has been made

> *Line 352 C. loss replace with complete loss.*

"C." actually denotes the last in a list after "A." and "B.". We have now used bold to emphasise this, but we imagine house style may dictate how we approach this.

https://doi.org/10.7554/eLife.94420.2.sa0