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Critical role for the p110α phosphoinositide-3-OH kinase in growth and metabolic regulation

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The eight catalytic subunits of the mammalian phosphoinositide-3-OH kinase (PI(3)K) family form the backbone of an evolutionarily conserved signalling pathway; however, the roles of most PI(3)K isoforms in organismal physiology and disease are unknown. To delineate the role of p110α, a ubiquitously expressed PI(3)K involved in tyrosine kinase and Ras signalling, here we generated mice carrying a knockin mutation (D933A) that abrogates p110α kinase activity. Homozygosity for this kinase-dead p110α led to embryonic lethality. Mice heterozygous for this mutation were viable and fertile, but displayed severely blunted signalling via insulin-receptor substrate (IRS) proteins, key mediators of insulin, insulin-like growth factor-1 and leptin action. Defective responsiveness to these hormones led to reduced somatic growth, hyperinsulinaemia, glucose intolerance, hyperphagia and increased adiposity in mice heterozygous for the D933A mutation. This signalling function of p110α derives from its highly selective recruitment and activation to IRS signalling complexes compared to p110β, the other broadly expressed PI(3)K isoform, which did not contribute to IRS-associated PI(3)K activity. p110α was the principal IRS-associated PI(3)K in cancer cell lines. These findings demonstrate a critical role for p110α in growth factor and metabolic signalling and also suggest an explanation for selective mutation or overexpression of p110α in a variety of cancers[1,2].

Class IA PI(3)Ks consist of a catalytic subunit (p110α, p110β or p110δ) in complex with one of five distinct regulatory subunits, collectively referred to as 'p85s' (ref. 3). The p85 SH2 domains recruit the cytosolic PI(3)Ks via tyrosine-phosphorylated protein complexes to the PI(3)K lipid substrates in the plasma membrane. p110δ is mainly found in leukocytes, and p110δ-null mice have a range of immunological defects[3]. In contrast, mice homozygous for knockout alleles of the broadly expressed p110α (encoded by *Pik3ca*) or p110β (encoded by *Pik3cb*) die as embryos[4,5], whereas heterozygote p110α and p110β knockout mice have no metabolic or growth phenotypes[6]. Therefore, the relative contributions of these PI(3)K isoforms to normal mammalian physiology remain unknown.

Deletion of PI(3)K genes in mice often alters expression of non-targeted PI(3)K family members[3], precluding accurate phenotypic analysis. For example, p85 is overexpressed in homozygous p110α knockout embryos[4], and expression of both p110α and p110β is reduced in p85α knockout mice[7]. We therefore created knockin mice (carrying a germline mutation in the DFG motif of the p110α ATP binding site) that express a kinase-dead $p110\alpha^{D933A}$ protein (Supplementary Fig. S1). In contrast to gene deletion, the knockin approach preserves signalling complex stoichiometry (illustrated in Fig. 1a). In heterozygous knockin mice, 50% of receptor–p110α complexes will be uncoupled from p110α activity, regardless of the stoichiometry of receptor to p85–p110α complexes. This contrasts with heterozygous knockout mice in which the capacity of any given receptor to signal via p110α will be reduced only if p110α expression is limiting relative to the receptor. Hence, heterozygous mice carrying the knockin mutation D933A (hereafter called $p110\alpha^{D933A/WT}$ mice) reveal the consequence of 50% loss of function of p110α activity in all tissues where it is expressed.

$p110\alpha^{D933A/WT}$ mice were viable whereas homozygous $p110\alpha^{D933A/D933A}$ embryos were growth-retarded from embryonic day 9 (E9) and died around E10–11 (data not shown). p110α, p110β and p110δ subunit expression in $p110\alpha^{D933A/D933A}$ embryos and in liver, muscle and fat from $p110\alpha^{D933A/WT}$ mice was similar to that in wild-type embryos and tissues (Fig. 1b and data not shown). In contrast to homozygous p110α knockout embryos[4], p85 was not overexpressed in $p110\alpha^{D933A/D933A}$ embryos (Fig. 1b). Selective immunoprecipitation of the $p110\alpha^{D933A}$ protein via its carboxy-terminal Myc epitope tag (Supplementary Fig. S1) confirmed that this protein had lost catalytic activity (Fig. 1c). PI(3)K activity in p110α immunoprecipitates was reduced by 50% and no compensatory alterations in the activities of p110β and p110δ were observed (Fig. 1c). Critically, $p110\alpha^{D933A/WT}$ mice displayed a 50% reduction in overall class IA PI(3)K activity in liver, muscle and fat, as assessed by either PDGF-receptor phosphopeptide pull down (Fig. 1d) or pan-p85 immunoprecipitations (data not shown). These data indicate that p110α accounts for most of the class IA PI(3)K activity in these tissues. Therefore, the $p110\alpha^{D933A/WT}$ mouse permitted the interrogation of the physiological effects of reduced p110α signalling at the organismal level.

$p110\alpha^{D933A/WT}$ mice showed reduced body weight (Fig. 1e) and length (Supplementary Fig. S2a). These parameters were also reduced in $p110\alpha^{D933A/WT}$ embryos just before birth (Fig. 1f), indicating that growth retardation starts during embryonic development and suggesting abnormalities in insulin-like growth factor-1 (IGF-1) receptor signalling[8]. Impaired IGF-1-stimulated proliferation of $p110\alpha^{D933A/WT}$ embryonic fibroblasts confirmed such a defect (Fig. 1g). The smaller size of adult $p110\alpha^{D933A/WT}$ mice was due to reduced lean mass (Supplementary Fig. S2b)—specifically, reduced skeletal muscle mass (Supplementary Fig. S2c). Major internal organ weight was not altered (Supplementary Fig. S3) and no gross pathological abnormalities were observed in tissues of 12-week-old $p110\alpha^{D933A/WT}$ mice (Supplementary Table 1). Although PI(3)K and downstream effectors such as PDK-1 and p70S6K have been implicated in growth regulation via alterations in cell size or number[9,10], the size of a range of cell types was unaffected in $p110\alpha^{D933A/WT}$ mice (data not shown).

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p110α$^{D933A/WT}$ mice displayed insulin resistance with increased insulin levels when fasted (Fig. 2a) or during a glucose tolerance test (Fig. 2b), and had an impaired hypoglycaemic response to insulin (Fig. 2c; Supplementary Fig. S4a). An increased pancreatic β-cell area also suggested β-cell compensation to insulin resistance (Supplementary Fig. S4b). Whereas fasting blood glucose levels were equivalent to those of wild-type mice (Fig. 2d), p110α$^{D933A/WT}$ mice displayed impaired glucose clearance (Fig. 2e; see also Supplementary Fig. S4c). These abnormalities were seen in both sexes and in pure C57BL/6 or mixed 129Sv–C57BL/6 genetic backgrounds (data not shown). These findings demonstrate that p110α regulates insulin sensitivity and glucose metabolism *in vivo*.

Despite their smaller size, young and 1-yr-old p110α$^{D933A/WT}$ mice were hyperphagic (Fig. 2f and data not shown) and displayed increased adiposity (Fig. 2g) with enlarged white adipose cells (Supplementary Fig. S4d). p110α$^{D933A/WT}$ mice showed elevated levels of plasma leptin (Fig. 2h), suggesting either primary CNS leptin resistance or adiposity-induced leptin resistance caused by defective hypothalamic insulin signalling[11] or other potential impairments in PI(3)K-dependent events in the hypothalamic circuits, such as neuronal development and maturation[12].

The receptors for insulin, IGF-1 and leptin directly or indirectly recruit IRS adaptor molecules to transmit their signals, mainly via IRS-bound PI(3)K[13–16]. There are four mammalian IRS proteins, of which IRS-1 and IRS-2 are widely expressed[17]. *Irs1*-null mice display growth retardation due to defective IGF-1 action, mild insulin resistance and glucose intolerance. In contrast, *Irs2* deletion results in diabetes due to insulin resistance and β-cell failure, and neuroendocrine dysfunction. p110α inactivation did not affect the expression levels of insulin receptor (IR) or IRS-1/2 under basal conditions (Fig. 3a). Insulin-stimulated tyrosine phosphorylation of IR and IRS and recruitment of p85 to IRS complexes were also equivalent in wild-type and p110α$^{D933A/WT}$ mice (Fig. 3a). In contrast, insulin-stimulated PI(3)K activity associated with IRS-1, IRS-2 or phosphotyrosine in skeletal muscle, liver and adipose tissue was reduced by approximately 50% in p110α$^{D933A/WT}$ mice (Fig. 3b and data not shown). Phosphorylation of Akt/protein kinase B (PKB), a key downstream target of PI(3)K implicated in insulin action and glucose homeostasis in mice *in vivo*[18], was also reduced (Fig. 3c). These observations suggest that the defects in glucose homeostasis are due to reduced insulin-stimulated p110α activity and diminished activation of effector pathways such as Akt/PKB in peripheral metabolic tissues.

Studies relying on the use of broad-spectrum pharmacological PI(3)K inhibition have suggested that food intake and adiposity are controlled by PI(3)K-dependent insulin and leptin signalling pathways in the CNS[14–16]. IRS-1/2-associated PI(3)K activity in hypothalamic extracts of insulin- or leptin-treated p110α$^{D933A/WT}$ mice was severely reduced or absent (Fig. 3d), demonstrating that IRS-associated p110α has either a direct role in insulin and leptin signalling in hypothalamic neurons or is required for the integrity of the hypothalamic circuits that respond to these hormones.

This predominant role of p110α in insulin signalling *in vivo* contrasts with cell-based studies implicating p110β in insulin-stimulated glucose uptake and cell proliferation[19–22]. To examine how p110α exerts its apparently selective role in IRS-mediated signalling, we performed quantitative immunoblot analysis of p110α and p110β expression, recruitment and activation in wild-type mice. p110α

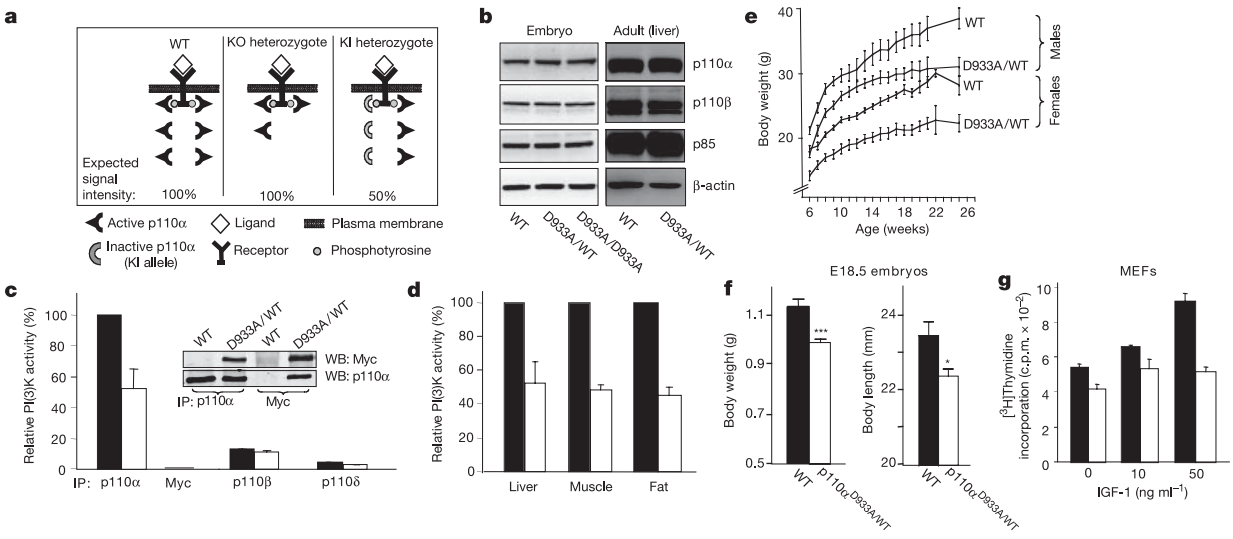

Figure 1 | Mechanism of action of p110α D933A knockin strategy and effect on signalling and growth in p110α$^{D933A/D933A}$ embryos and p110α$^{D933A/WT}$ mice. a, Representation of the potential differential effect of p110α knockout (KO, gene deletion) and knockin (KI, inactivation of gene product by point mutation) on the intensity of tyrosine kinase receptor-stimulated PI(3)K signalling. Binding of a growth factor to its cognate tyrosine kinase receptor results in tyrosine phosphorylation of the receptor and intracellular adaptor molecules (such as IRS proteins), leading to recruitment of class IA PI(3)Ks. If p110α expression is not limiting, the remaining wild-type p110α allele in cells heterozygous for the p110α knockout allele will still give rise to sufficient p110α protein to maintain a normal PI(3)K signalling output. In contrast, in mice heterozygous for the p110α knockin allele, kinase-dead p110α will become recruited to the receptor–adaptor complexes in the same way as wild-type p110α (given that this occurs via p85) and reduce p110α-dependent signalling by 50%, regardless of the stoichiometry of receptor to p85–p110α. **b**, PI(3)K subunit expression. Homogenates of E9.5 embryos or adult liver were analysed by SDS–PAGE and immunoblotting using the indicated antibodies. **c**, PI(3)K activity associated with distinct p110 isoforms. Homogenates of livers were immunoprecipitated using the indicated antibodies, followed by PI(3)K activity assay. The p110α^{D933A} protein was immunoprecipitated via its C-terminal Myc epitope tag. Immunoprecipitation of p110α^{D933A} was confirmed by immunoblotting (insert). **d**, PI(3)K activity associated with class IA PI(3)K regulatory subunits. Homogenates of the indicated tissues were absorbed onto Sepharose-immobilized phosphopeptide, which binds all class IA PI(3)K regulatory subunits, followed by *in vitro* lipid kinase assay. **e**, Reduced body weight of p110α$^{D933A/WT}$ mice. Body weight of a cohort of 11 male (wild type, $n = 6$; p110α$^{D933A/WT}$, $n = 5$) and 16 female ($n = 8$ per genotype) littermates was recorded over a period of 6 months. **f**, Weight and crown-to-rump length of E18.5 embryos isolated from pregnant females from ten litters (p110α$^{D933A/WT}$ ($n = 25$) and wild-type ($n = 15$) littermates). **g**, Impaired IGF-1-induced proliferation in MEFs from E13.5 p110α$^{D933A/WT}$ mice. For **c**, **d**, **f**, **g**, filled bars indicate wild-type mice and open bars indicate p110α$^{D933A/WT}$ mice. Error bars in all figures show standard error of the mean, except for Fig. 1e, which shows standard deviation. Asterisks indicate statistical significance (see Methods).

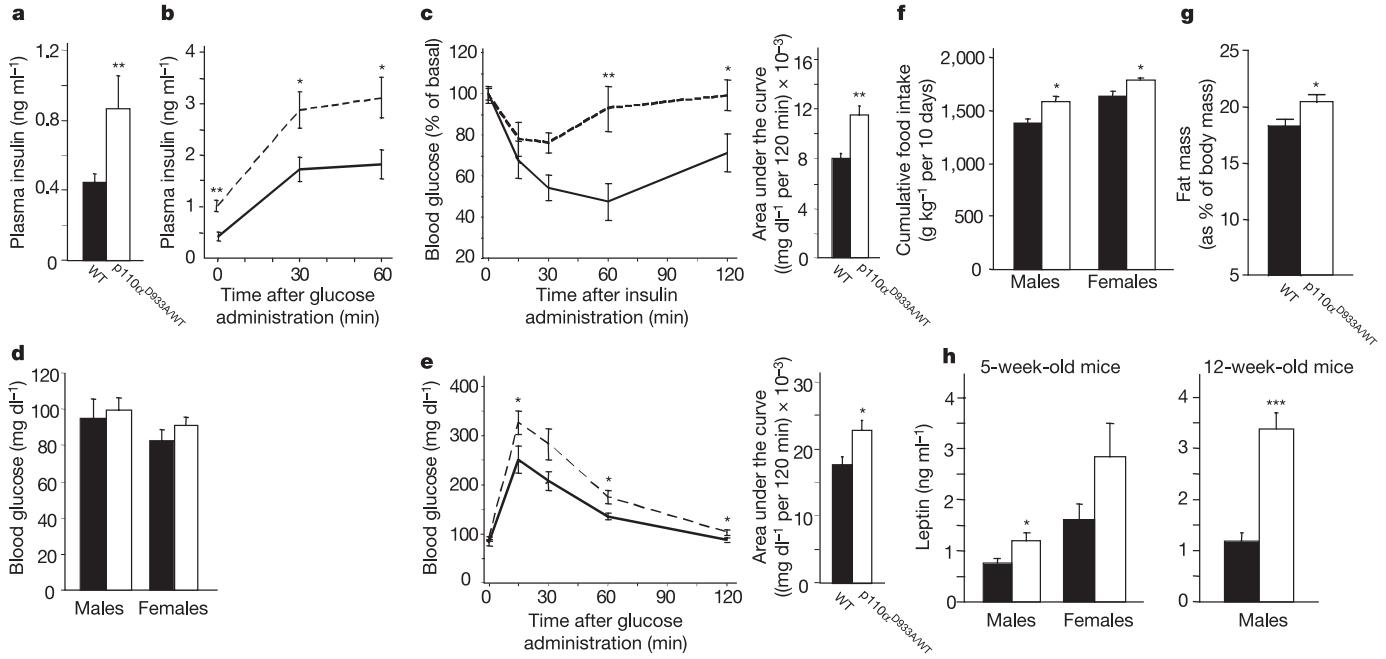

Figure 2 | Hyperinsulinaemia, impaired glucose and insulin tolerance, increased food intake, increased adiposity and hyperleptinaemia in p110α$^{D933A/WT}$ mice. Solid lines/filled bars, wild-type mice; dashed lines/open bars, p110α$^{D933A/WT}$ mice. **a**, Plasma insulin levels in fasted 8–9-week-old male mice ($n = 12$ per genotype). **b**, Insulin levels in the plasma of p110α$^{D933A/WT}$ male mice and wild-type littermates ($n = 7$ per genotype) subjected to a glucose tolerance test. **c**, Insulin tolerance test in female p110α$^{D933A/WT}$ ($n = 9$) and wild-type ($n = 9$) littermates. Calculated areas under the curves are shown. **d**, Blood glucose levels in fasted 8–9-week-old male and female mice ($n = 18$ per sex and genotype). **e**, Glucose tolerance test in female p110α$^{D933A/WT}$ ($n = 19$) mice and wild-type ($n = 17$) littermate mice. Calculated areas under the curves are shown. **f**, Food intake in a cohort of 12-week-old wild-type (n = 6 for males; n = 7 for females) and p110α$^{D933A/WT}$ ($n = 5$ for males; $n = 6$ for females) littermates. **g**, Fat mass of p110α$^{D933A/WT}$ mice ($n = 11$) and wild-type littermates ($n = 11$) was determined by DEXA analysis and expressed as per cent of total body mass. **h**, Plasma leptin levels in p110α$^{D933A/WT}$ mice and wild-type littermates (5-week-old mice: males, $n = 16$ per genotype; females, $n = 9$ per genotype; 12-week-old mice: males, $n = 11$ per genotype). Asterisks indicate statistical significance (see Methods).

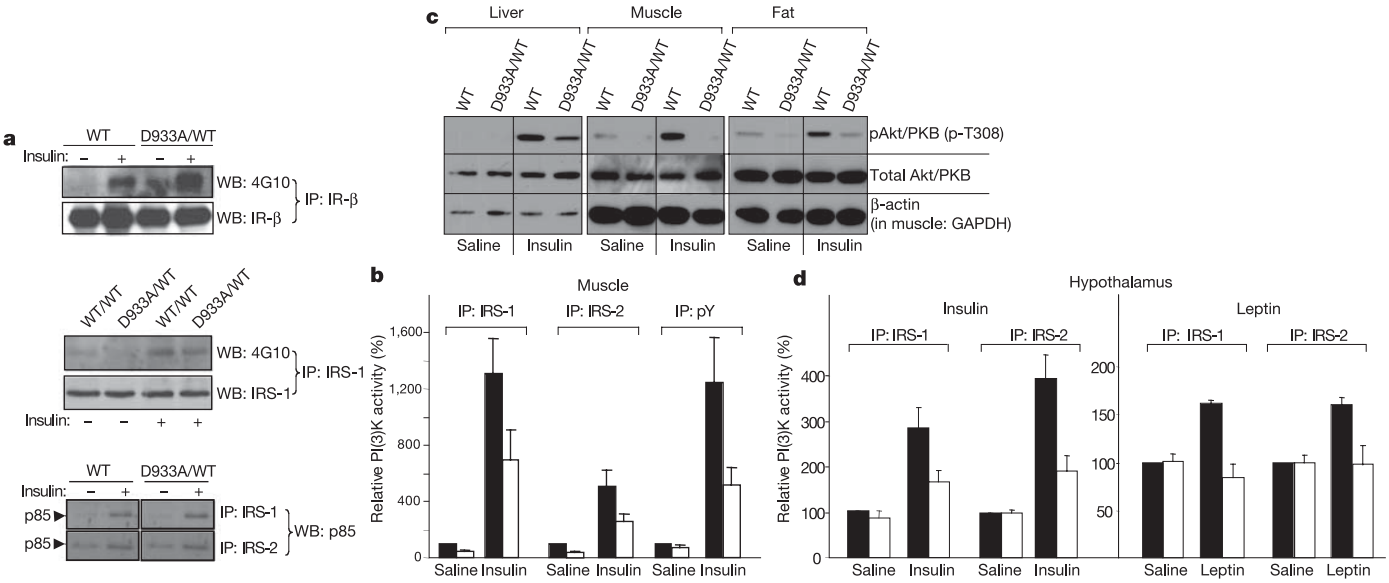

Figure 3 | Normal proximal insulin receptor signalling events but impaired PI(3)K pathway activation in p110α$^{D933A/WT}$ mice. **a**, IR or IRS-1/2 were immunoprecipitated from livers of insulin-treated p110α$^{D933A/WT}$ mice, immunoblotted with phosphotyrosine antibodies, followed by stripping and re-probing with antibodies to IR-β or IRS-1. For assessment of PI(3)K recruitment to IRS, IRS-1/2 immunoprecipitates were probed with antibodies to p85. **b**, **c**, p110α$^{D933A/WT}$ mice were injected in the inferior vena cava with saline or 0.1 mU g^{-1} insulin. Liver, muscle (gastrocnemius) and perigenital fat pad homogenates were either immunoprecipitated using the indicated antibodies followed by PI(3)K lipid kinase assay (**b**) or analysed by SDS–PAGE and immunoblotting using the indicated antibodies (**c**). **d**, Defective hypothalamic IRS signalling in p110α$^{D933A/WT}$ mice. IRS-1/2-associated PI(3)K activity in hypothalamic homogenates from mice injected with 5 U per mouse insulin or 1 μg g^{-1} leptin.

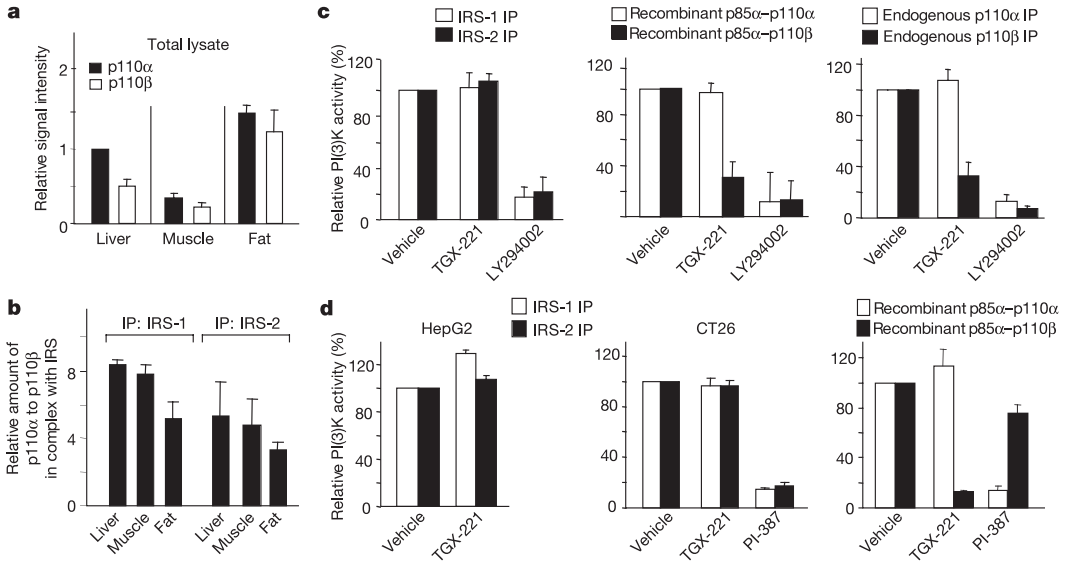

Figure 4 | Selective engagement of p110α in IRS signalling. **a**, Relative expression of p110α and p110β in insulin-sensitive tissues of wild-type mice under basal conditions, as determined by two-colour immunoblot. The graph shows data acquired from six independent experiments. **b**, Association of p110α and p110β with IRS-1/2 in tissues of insulin-treated wild-type mice. IRS-1 or IRS-2 was immunoprecipitated from homogenates of organs isolated from wild-type mice injected with 5 U per mouse insulin, and associated p110α and p110β quantified as described in **a**. Data were pooled from two independent experiments. **c**, Effect of PI(3)K inhibitors on IRS-associated PI(3)K activity in liver. Left panel: sensitivity of IRS-1/2-associated PI(3)K activity to treatment with TGX-221 (p110β-selective inhibitor) or LY294002 (pan-PI(3)K inhibitor). IRS-1/2 immunoprecipitates from liver homogenates of wild-type mice injected with insulin were treated with vehicle or inhibitors (LY294002 at 5 μM or TGX-221 at 100 nM), followed by an *in vitro* lipid kinase assay. Middle and right panels: effect of inhibitors on lipid kinase activity of recombinant p85α–p110s (middle panel) or p110α and p110β immunoprecipitates from liver homogenates (right panel). **d**, Sensitivity of IRS-1/2-associated PI(3)K activity to treatment with TGX-221 and PI-387 in cancer cell lines. IRS-1/2 immunoprecipitates from the indicated cell lines (HepG2 human liver hepatocarcinoma; CT26 murine colonic adenocarcinoma), which had been stimulated with 100 ng ml^{-1} IGF-1 for 15 min, were treated with vehicle, TGX-221 (100 nM) or PI-387 (300 nM), followed by an *in vitro* PI(3)K lipid kinase assay. The effect of TGX-221 and PI-387 on recombinant p85α–p110s was tested in parallel (right panel).

levels were twofold higher than p110β in liver and muscle, but similar in adipose tissue, which had the highest levels of p110α and p110β (Fig. 4a). Upon insulin stimulation, p110α was significantly enriched compared with p110β in IRS-1 and IRS-2 complexes in all three tissues (Fig. 4b). We next assessed the relative contribution of p110α and p110β to the insulin-stimulated PI(3)K lipid kinase activity associated with IRS proteins. TGX-221, a p110β-selective ATP-competitive inhibitor[23], had no effect on IRS-associated lipid kinase activity at doses that inhibit recombinant or immunoprecipitated p110β (Fig. 4c). In contrast, the pan-PI(3)K inhibitor LY294002 completely inhibited PI(3)K activity associated with IRS complexes (Fig. 4c). These data suggest a minimal contribution from p110β towards IRS-associated lipid kinase activity. The intrinsically lower specific activity of p110β compared to p110α (ref. 24), or differences in the mechanism to fully activate p110β (refs 25, 26), may underlie these findings. Taken together, our results demonstrate an unexpected specificity in IRS signalling, with selective recruitment and activation of p110α to IRS complexes in metabolic tissues.

Selective functional association of p110α with IRS complexes was also found in IGF-1-stimulated cancer cell lines. Indeed, IRS-associated PI(3)K activity was resistant to TGX-221 but sensitive to PI-387, an inhibitor with selectivity for p110α over p110β (Fig. 4d). IRS-mediated signalling is critical for IGF-1 receptor-driven cancer development and progression[27]. The existence of a major signalling cassette composed of p110α and IRS proteins in both growth factor and metabolic signalling may account for the observation that, of the eight distinct PI(3)K catalytic subunits in mammals, only p110α is commonly mutated or overexpressed in cancer cells[1,2], possibly leading to selective activation of pathways involved in cell growth and metabolism.

Our knockin mouse gene targeting strategy has demonstrated that p110α is a key intermediate in IGF-1, insulin and leptin signalling, revealing a clearly defined role for p110α at the organismal level. We have demonstrated specificity for p110α in the relationship between PI(3)K catalytic subunits and IRS proteins. The observation that insulin resistance in p110α$^{D933A/WT}$ mice does not progress to overt diabetes even at old age indicates a level of plasticity in metabolic control mechanisms after perturbation of the PI(3)K signalling axis. This suggests that small-molecule inhibitors of p110α, currently under development as anticancer agents, may not lead to unmanageable metabolic disturbances[28,29].

METHODS

Mice. Mice were kept in individually ventilated cages and cared for according to UK Home Office regulations. Unless otherwise mentioned, the mice used were backcrossed to the C57BL/6 background for ten generations, and wild-type littermates were used as controls. Embryos were obtained from timed pregnant females. The day of presence of a copulation plug was considered day E0.5.

Reagents. A mouse monoclonal antibody specific for p110α (clone U3A)[30] was used for two-colour immunoblotting. Anti-Akt/PKB (pThr308, pSer473 and total) and anti-Myc tag monoclonal antibody (clone 9B11) were from Cell Signalling Technologies. Antibodies to IRS-1, IRS-2 and phosphotyrosine (4G10) were from Upstate.

***In vivo* stimulation with insulin or leptin.** Overnight-fasted mice (9–12 weeks old) were terminally anaesthetized by intraperitoneal injection of 150 mg kg^{-1} sodium pentobarbital. Recombinant human insulin at doses varying from 0.01 mU g^{-1} to 250 mU g^{-1} or 5 U per mouse (Actrapid, Novo Nordisk) or recombinant mouse leptin (1 μg g^{-1}; R&D Systems) were injected into the inferior vena cava, followed 5 min (for insulin) or 10 min (for leptin) later by harvesting and freezing of the tissues in liquid nitrogen.

Quantitative immunoblot analysis of p110α and p110β. Recombinant p110α and p110β in complex with p85α were produced by baculovirus infection of Sf9 insect cells and purified by affinity chromatography using a Sepharose-immobilized phosphopeptide corresponding to Tyr 751 of PDGF receptor β. Purified PI(3)K proteins and a titration series of bovine serum albumin (BSA) were resolved by SDS–polyacrylamide gel electrophoresis (PAGE), followed by

Coomassie-blue staining and densitometric analysis of the gel using a Bio-Rad GS-800 densitometer, in order to determine the absolute amounts of p110α and p110β from the BSA protein standard curve. Equimolar amounts of p110α and p110β were then resolved by SDS–PAGE along with test samples (such as total protein from tissue homogenates), followed by simultaneous immunoblotting using antibodies specific for p110α (mouse monoclonal; clone U3A) and p110β (rabbit polyclonal; Santa-Cruz, S-19). These primary antibodies were detected using fluorescently labelled species-selective secondary antibodies (anti-mouse IRDye 800-conjugated (Rockland) and anti-rabbit Alexa-Fluor 680-conjugated (Molecular Probes)). Detection and quantification were performed using an Odyssey infrared scanner (LICOR) using the manufacturer's software. All signal intensities were normalized to those of the recombinant p110α and p110β standards in order to account for different avidity/affinity of the p110α and p110β antibodies.

Cell size analysis. Determination of diameter or volume of various cell types derived from $p110\alpha^{D933A/WT}$ or wild-type mice was performed with a CASY Model TT cell counter and analyser (Schärfe System).

MEF proliferation assay. E13.5 embryos were minced, dissociated with trypsin and cells allowed to adhere on gelatin-coated tissue culture dishes. For proliferation assays, early passage (P2–P4) mouse embryonic fibroblasts (MEFs) were incubated at 10^5 cells per well in 96-well plates, deprived of fetal calf serum for 16 h followed by stimulation with or without IGF-1 in 200 μl medium (DMEM, 10% FCS, penicillin/streptomycin). [^{3}H]Thymidine was added for the last 4 h of a 24-h culture, followed by harvesting of the cells and quantification of incorporated [^{3}H]thymidine by scintillation counting.

Metabolic studies and morphometric analysis. Measurements of body weight and length, glucose tolerance tests, plasma insulin and leptin level determination, and pancreatic morphometric analysis were performed as described elsewhere[11]. Body composition was analysed by dual energy X-ray absorptiometry (DEXA) using a PIXImus 2 densitometer (Lunar). Insulin tolerance tests were performed on randomly fed mice by intraperitoneal injection of $0.75\ U\ kg^{-1}$ of recombinant human insulin, followed by measurement of blood glucose at various time points. For adipocyte morphometric analysis, epididymal fat pads were fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into 8-μm-thick sections. Adipose tissue sections were stained with haematoxylin and eosin, following standard protocols. In each mouse, adipocyte size was analysed in four sections (150 μm apart) using Simple PCI software. At least 300 cells from each mouse were measured.

Food intake analysis. Mice were singly housed and allowed to acclimatize for a week before study. Food intake and body weight were measured for ten consecutive days. Results were expressed as cumulative food intake (grams of chow) per kg of body weight per 10 days. Experiments were performed on 12-week-old and 1-yr-old mice.

Statistical analysis. Values are presented as mean ± standard error of the mean. *P*-values were calculated using the non-parametric two-tailed Mann–Whitney *U*-test (comparisons with wild-type mice). *P*-values ≤0.05 were considered to be statistically significant (designated by a single asterisk; double asterisk, $P \leq 0.01$; triple asterisk, $P \leq 0.001$). The number of animals in each group is indicated by *n*.

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