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Coordinatore: Prof. Alberto Gulino
Tutor: Prof.ssa Anna Coppa

The Phosphatidylinositol 3-Kinase p85alpha is the physical link between cAMP-PKA and retinoic receptor trasduction pathways.

Candidata: Caterina Francesca Donini

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Abstract

I membri della famiglia di proteine della fosfoinositide-3-OH chinasi (PI3K) sono spesso coinvolti nella regolazione di vari processi cellulari, come la sopravvivenza, la crescita, la proliferazione e la motilità, e rappresentano alcune fra le proteine più mutate nei tumori (Bunney T.D. e Katan M., 2010).

Numerosi studi sui meccanismi molecolari coinvolti nella trasduzione del segnale della PI3K hanno dimostrato che la subunità regolativa della PI3K, p85α, è responsabile di molte interazioni proteina-proteina. Attraverso il dominio inter-SH2 (iSH2), la $p85\alpha^{PI3K}$ interagisce con la subunità catalitica della PI3K, $p110^{PI3K}$ (Klippel A. et al, 1993), mentre con i domini SH3 e SH2 interagisce con tirosin-chinasi, proteine adattatrici, recettori nucleari e con la Protein-Chinasi A (PKA) (Hellyer N.J. et al, 1998; McGlade C.J. et al., 1992; Ciullo I. et al., 2001; Cosentino C. et al., 2007). Nonostante sia stato largamente studiato il ruolo del dominio SH2 della $p85\alpha^{PI3K}$ nella trasduzione del segnale, la funzione del dominio SH3 è ancora poco chiara.

L'Acido Retinoico (RA) è un derivato della vitamina A che regola la crescita cellulare, la differenziazione e l'apoptosi (DeLuca L.M., 1991; Sporn M.B e Roberts A.B., 1994), e per questo motivo si presenta come farmaco promettente per il trattamento e la prevenzione di numerosi tumori, compreso il carcinoma della mammella (Veronesi U. et al., 1999; Dragnev K.H. et al., 2000; Guruswamy et al., 2001; del Rincòn S.V. et al., 2003). Recentemente è stato dimostrato che il RA esercita un'azione rapida e non genomica sulla via di segnalazione della PI3K attraverso un meccanismo che prevede la formazione di un complesso stabile fra il recettore per l'acido retinoico (RARα) e $p85\alpha^{PI3K}$, che avviene anche in assenza di ligando (Masià S. et al., 2007).

È stato identificato un residuo (Ser83) adiacente al dominio SH3 N-terminale della $p85\alpha^{PI3K}$, critico per la progressione del ciclo cellulare e la sopravvivenza

delle cellule epiteliali normali. La Ser83 della $p85\alpha^{PI3K}$ è fosforilata dalla PKA *in vivo* e *in vitro*, e questa fosforilazione influenza la capacità del dominio SH3 di interagire con diversi partner, come il recettore per gli estrogeni, la subunità regolativa RIIβ della PKA e p21/Ras (Cosentino C. et al., 2007; De Gregorio G. et al., 2007). Potenzialmente, tutti i recettori in grado di legare la $p85\alpha^{PI3K}$ possono cooperare con la via del segnale della PKA attraverso la fosforilazione della Ser83 (Cosentino C. et al, 2007).

Questo studio dimostra l'esistenza di un complesso ternario costituito da RARα/PI3K/P-FAK nella linea cellulare tumorale di mammella MCF7, che è finemente regolato dalla fosforilazione della Ser83 della $p85\alpha^{PI3K}$ e dal trattamento con l'Acido Retinoico. La Ser83 rappresenta pertanto un interessante sito di convergenza fra diverse vie di trasduzione del segnale (cAMP-PKA, RA e FAK), ed è un importante regolatore della proliferazione e della capacità migratoria delle cellule MCF7.

INTRODUCTION

Tumorigenesis in humans is a multistep process that reflects genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. There are six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan D. and Weinberg R.A., 2000).

Members of the mammalian phosphoinositide-3-OH kinase (PI3K) family of proteins are critical regulators of various cellular process including cell survival, growth, proliferation and motility, and they are among the most frequently mutated proteins in cancer (Bunney T.D. and Katan M., 2010). Recent studies have demonstrated that somatic mutations in the PI3K regulatory subunit $p85\alpha^{PI3K}$ promote tumorigenesis (Jaiswal B.S. et al., 2009). It has been identified a serine (at codon 83) adjacent to N-terminus SH3 domain in the PI3K regulatory subunit $p85\alpha^{PI3K}$, that is critical for cell cycle progression and cell survival in normal epithelium. $p85\alpha^{PI3K}$ Ser83 is phosphorylated by Protein Kinase A in vivo and in vitro, influencing the ability of SH3 domain to interact with different partners, such as estrogen receptor, PKA regulatory subunit and p21 Ras (Cosentino C. et al., 2007; De Gregorio G. et al., 2007).

Retinoic Acid exerts rapid, nongenomic effects on PI3K signalling pathway, through a novel mechanism in which a stable complex including RARα and $p85\alpha^{PI3K}$ occurs also in the absence of RA (Masià S. et al., 2007).

The aim of this study is to investigate if $p85\alpha^{PI3K}$ Ser83 is a critical site for binding of PI3K to RARα and/or other effectors, and if this site links cAMP-PKA signal to other signalling pathways.

Moreover this study characterizes the role of $p85\alpha^{PI3K}$ Ser83 in the regulation of motility, growth and survival, of the human tumour cell line MCF7.

PI3K

Classification of PI3Ks

PI3Ks are a family of intracellular lipid kinases that phosphorylate the head group of phosphoinositides at the 3' position, generating lipid second messengers; the effects of PI3Ks are transmitted through these lipid products, which bind to and regulate downstream protein effectors (Cain R.J. and Ridley A.J., 2009). The multiple isoforms of PI3Ks can be divided into three distinct groups on the basis of their sequence homology, structure and substrate specificity: class I (A and B), class II and class III.

Class I PI3Ks primarily phosphorylate in vivo phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3); class II enzymes preferentially phosphorylate in vitro PtdIns and PtdIns-4-P. There is only one class III PI3K in mammals, VPS34 (vacuolar protein sorting 34), whose preferential substrate is PtdIns, and that represents the only mammalian PI3K that is also conserved in yeast (Chalchoub N. and Baker S.J., 2009).

From a structural point of view, all PI3Ks catalytic subunits share an homologous region that consists of a catalytic kinase domain (HR1; homology region 1) linked to HR2 (also known as the PIK or PI kinase homology domain) and a C2-like domain (HR3) (Vanhaesebroeck B. et al., 2001).

Class IA and class IB PI3Ks are closely related, both existing as stable heterodimers, comprising a highly homologous 110 kDa catalytic subunit and a smaller tightly associated regulatory subunit. Class IB is primarily activated by G-protein-coupled receptor (GCPRs) signalling and Ras, while class IA PI3Ks

are activated by growth factor tyrosine kinases (RTKs), as well as Ras signalling. There are three class IA catalytic isoforms: α and β, which have a broad tissue distribution, and δ, which has a more restricted expression pattern; these catalytic subunits bind to one of five regulatory subunits. Both class IA and IB are characterized by an N-terminal Ras Binding Domain (RBD), implying that Ras can activate them (Engelman J.A. et al., 2006).

Class II PI3Ks are large proteins (170-210 KDa) of which there are three isoforms in mammals (PI3K-C2α, PI3K-C2β, and PI3K-C2γ) that are largely associated with membranes and are not thought to require a regulatory subunit. This class is characterized by a PIK domain, quite similar to the one of class I PI3Ks; a large coiled-coil proline-rich motif near the N-terminus (CC) that may mediate as yet uncharacterized protein-protein interactions, and a C2 C-terminus domain. Another characteristic domain of class II PI3Ks is the PX, common to molecule such as NADPH-oxidase, phox-40 and phox-47. The large N-terminal regions of class II PI3Ks contain a Ras binding domain fold but otherwise show no homology to any known protein, and there are no indications for binding of these PI3Ks to adaptor proteins or Ras (Arcaro A. et al., 1998).

The prototype of class III enzymes, Vps34 was first identified in yeast in a screening for mutants defective in protein sorting. This catalytic subunit exists in complex with a Ser/Thr protein kinase (Vps15p in yeast, p150 in mammals), which is essential for the intracellular trafficking. This class of PI3Ks lacks the Ras Binding Domain (**Fig. 1**) (Walker E.H. et al., 1999).

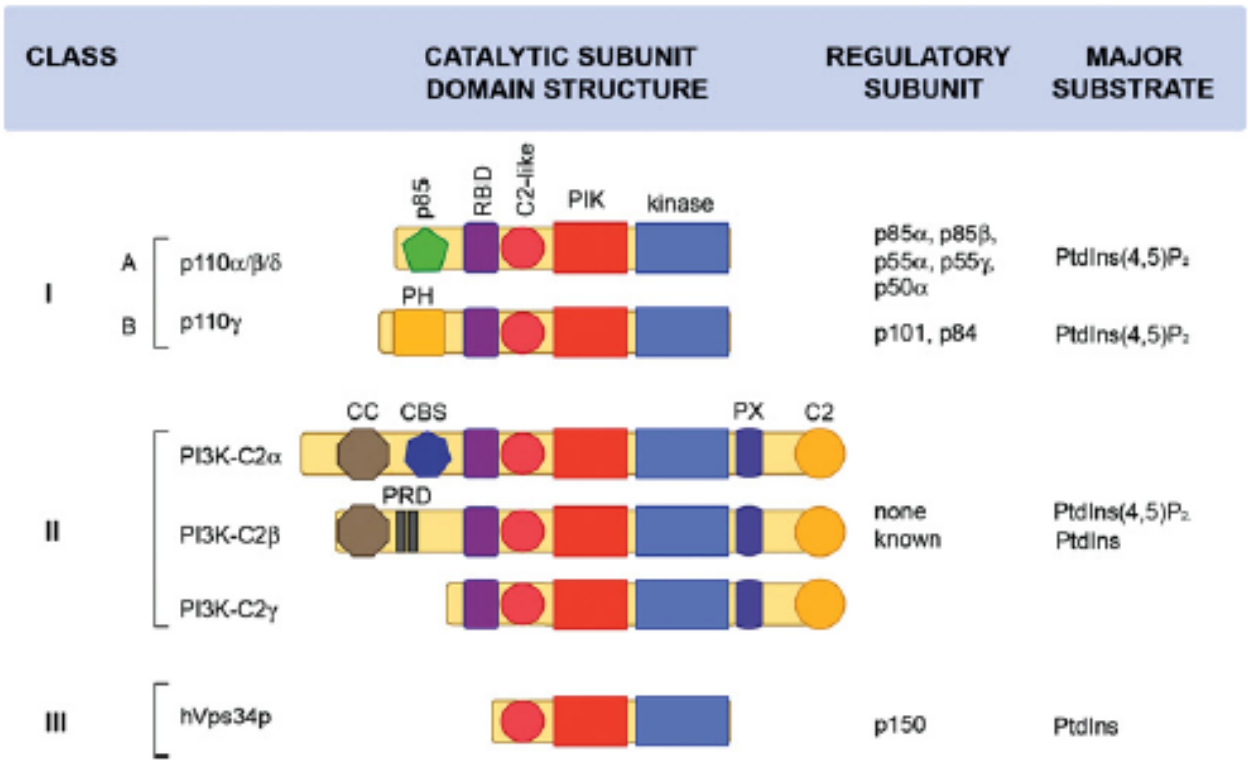

Figure 1 | Domain organization of the PI3K family of lipid kinases
The three classes of the PI3K family, the domain structure of the catalytic subunits, their main tissue distribution and their preferred substrate in vivo, if known. Abbreviations: C2(-like), PKC homology 2(-like) domain; CBS, clathrin-binding site; CC, coiled-coil domain; p85, p85-binding domain; PRD, proline-rich domain; PX, phox domain; RBD, Ras-binding domain (Cain J. and Ridley A.J., 2009).

Phosphoinositide signalling

The phosphoinositide signalling system is a crucial regulator of most cellular processes. Phosphatidylinositol (PtdIns) is a membrane phospholipid consisting of a glycerol backbone that is esterified at its sn-1 and sn-2 positions to two fatty acids, and linked via phosphate at its sn-3 position to the D1 position of a D-myo-inositol head group (Sasaki T. et al., 2009).

In mammals, the hydroxyl groups at the D3, 4 and 5 positions of the inositol ring can be phosphorylated to generate seven phosphoinositides: PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P2, PtdIns(4,5)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3 (**Fig. 2**) (Bunney T.D and Katan M., 2010).

Phosphorylation and dephosphorylation by lipid kinases and phosphatases can rapidly interconvert phosphoinositide species, while Phospholipase C (PLC)-dependent hydrolysis of PtdIns(4,5)P2 generates the second messenger molecules diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1, 4,5)P3), which in turn stimulate several DAG effectors and Ins(1,4,5)P3-mediated calcium mobilization (**Fig. 3**) (Sasaki T. et al., 2009; Katan M., 2005).

Through this second-messenger system and the big amount of phosphoinositide-protein interactions, phosphoinositides have a major role in recruiting and regulating proteins at the membrane interface, and so control a wide range of processes, including the assembly and activity of signalling cascades, membrane budding and fusion, actin and microtubule dynamics (Di Paolo G. and De Camilli P., 2006).

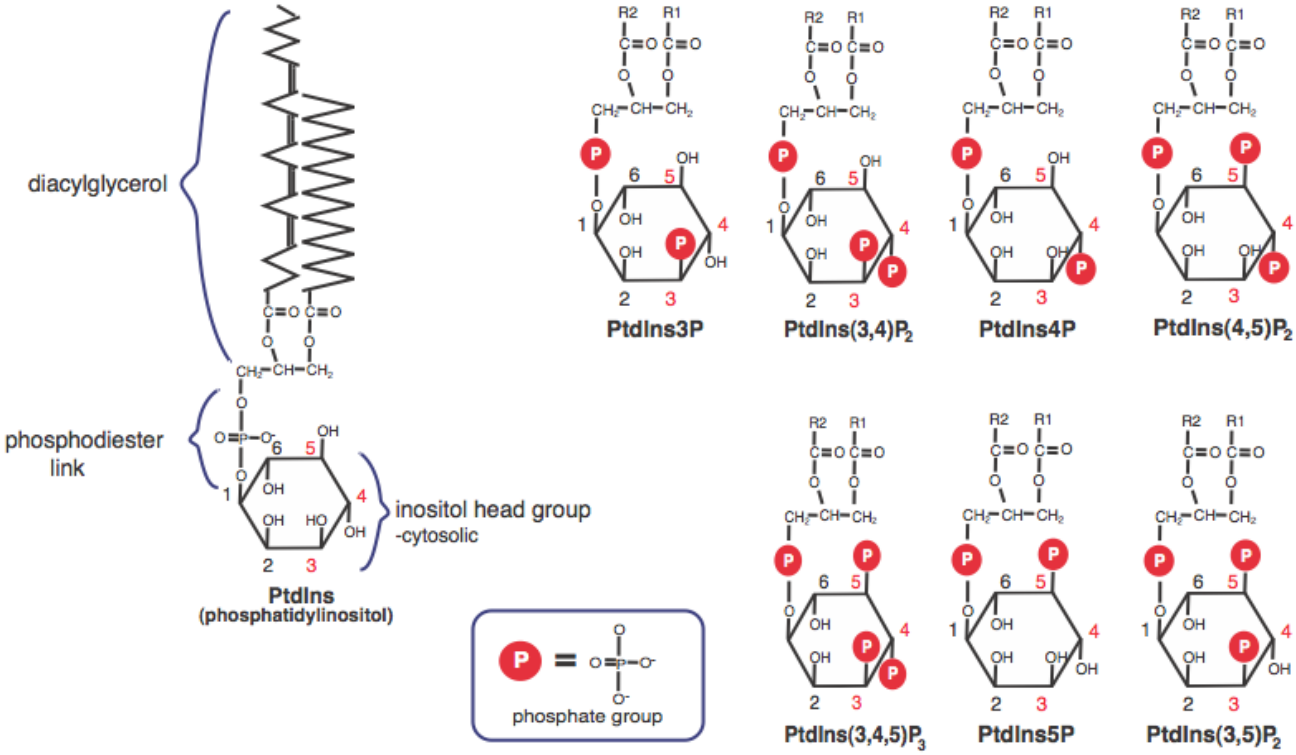

Fig. 2 | Structures of phosphatidylinositol and its phosphoinositide derivatives

Phosphatidylinositol consists of a glycerol backbone that is esterified at its sn-1 and sn-2 positions to two fatty acids, and linked via phosphate at its sn-3 position to the D1 position of a D-myo-inositol head group. In mammals, the hydroxyl groups at the D3, 4 and 5 positions of the myo-inositol head group can be phosphorylated to yield the indicated mono-phosphate, bis-phosphate and tris-phosphate phosphoinositide derivatives. (Adapted from Sasaki T. et al., 2009; Vanhaesebroeck B. et al., 2001).

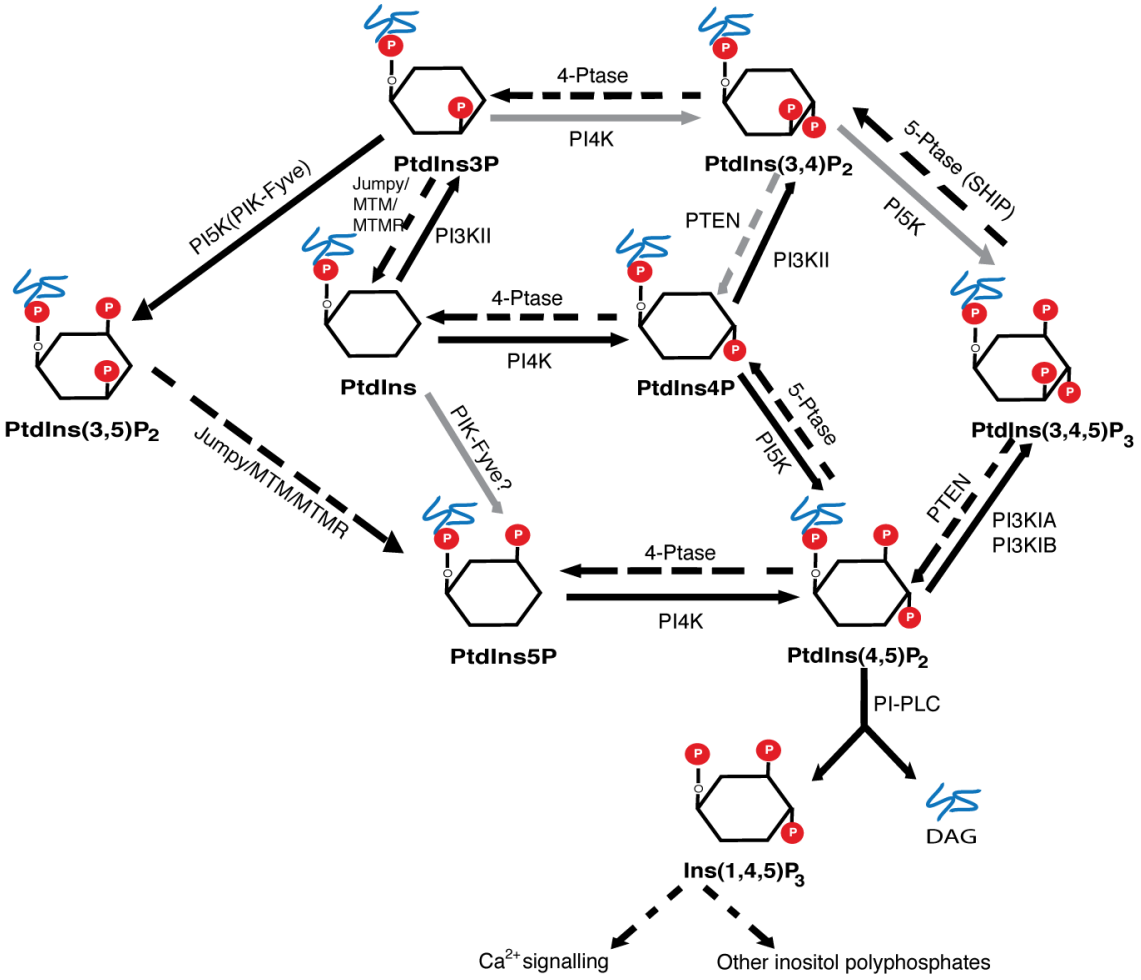

Figure 3 | The phosphoinositide metabolic cycle
Schematic representation of the phosphoinositide metabolic cycle. The parent lipid phosphatidylinositol (PtdIns) represents the core structure of phosphoinositides, and phosphate groups can be attached to any permutation of 3-, 4- and 5-hydroxyl groups. Phosphorylation (solid arrows) and dephosphorylation (dashed arrows) reactions and enzymes involved in the interconversion of phosphoinositide species are indicated. Also shown is phospholipase C (PLC)-catalysed production of the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P3). Grey arrows represent less well-characterized pathways, or pathways through which a minority of the phosphoinositide is generated (Adapted from Bunney T. D. and Katan M., 2010).

Signalling through the PI3K pathway

Upstream: cAMP/PKA

Cyclic adenosine 3'5' monophosphate (cAMP) is a second messenger that plays a role in intracellular signal transduction of various stimuli, regulating the growth of many cells types (Pastan I.H. et al., 1975).

Adenylyl cyclase is the enzyme that converts AMP in cAMP whose major function in eukaryotes is the activation of cAMP-dependent protein kinase (PKA) (Naviglio S. et al., 2009). PKA is a holoenzyme composed by two regulatory (R) and two catalytic subunits (cPKA) (Feliciello A. et al., 2005). cAMP binds to the regulatory subunits leading to the release of the catalytic ones, which in turn phosphorylate many nuclear and cytoplasmic substrates controlling multiple cell functions, including motility, metabolism, differentiation, synaptic transmission, ion channel activities, growth and gene transcription (Edelman A.M. et al., 1987; Haynes J. et al., 1992; Meinkoth J.L et al., 1993; Feliciello A. et al., 2001). There are two different types of PKA:

1. PKA I: it binds the regulatory subunit RIα that has an high affinity for cAMP, and therefore it is activated by low level of cAMP;
2. PKA II: binds the regulatory subunit RII that has a low affinity for cAMP; this implies that it is activated by high level of cAMP, typically upon Gs activation. Class II of PKA can be further divided into two subtypes depending on the kind of RII present in the holoenzyme: RIIα or RIIβ. RIIα is ubiquitous (as PKA I), while RIIβ is expressed mainly in endocrine, brain, fat and reproductive tissues (Edelman A.M. et al., 1987; Haynes J. et al., 1992). Moreover, RIIβ has a binding affinity to cAMP lower than RIIα.

Stimulation of growth by cAMP–PKA in selected cell types, as thyroid cells, is tightly dependent on Ras and PI3K (Ciullo I. et al., 2001). Also, cAMP and PKA are powerful survival signals in several cell types (Affaitati A. et al.,

2003). To date the mechanisms and the relevant players mediating cAMP–PKA effects on growth and survival are not completely known.

Downstream: AKT

Activated PI3Ks catalyze the formation of PIP3 from PIP2, and the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) directly opposes the activity of PI3Ks by dephosphorylating PIP3 into PIP2, thus acting as central negative regulator of PI3K (Chalchoub N. and Backer S.J., 2009). PIP3 may act on different molecules involved in vesicle trafficking and budding, cell survival and proliferation and protein synthesis. PIP3 transduces activating signals by binding to the pleckstrin homology (PH) domains characteristic of all PI3K's downstream effectors, thereby recruiting them to the membrane (Engelman J.A. et al., 2006). The serine-threonine kinase AKT, also known as PKB because of its homology to PKA and PKC, is a centrally important downstream effector of PIP3 (**Fig. 4**).

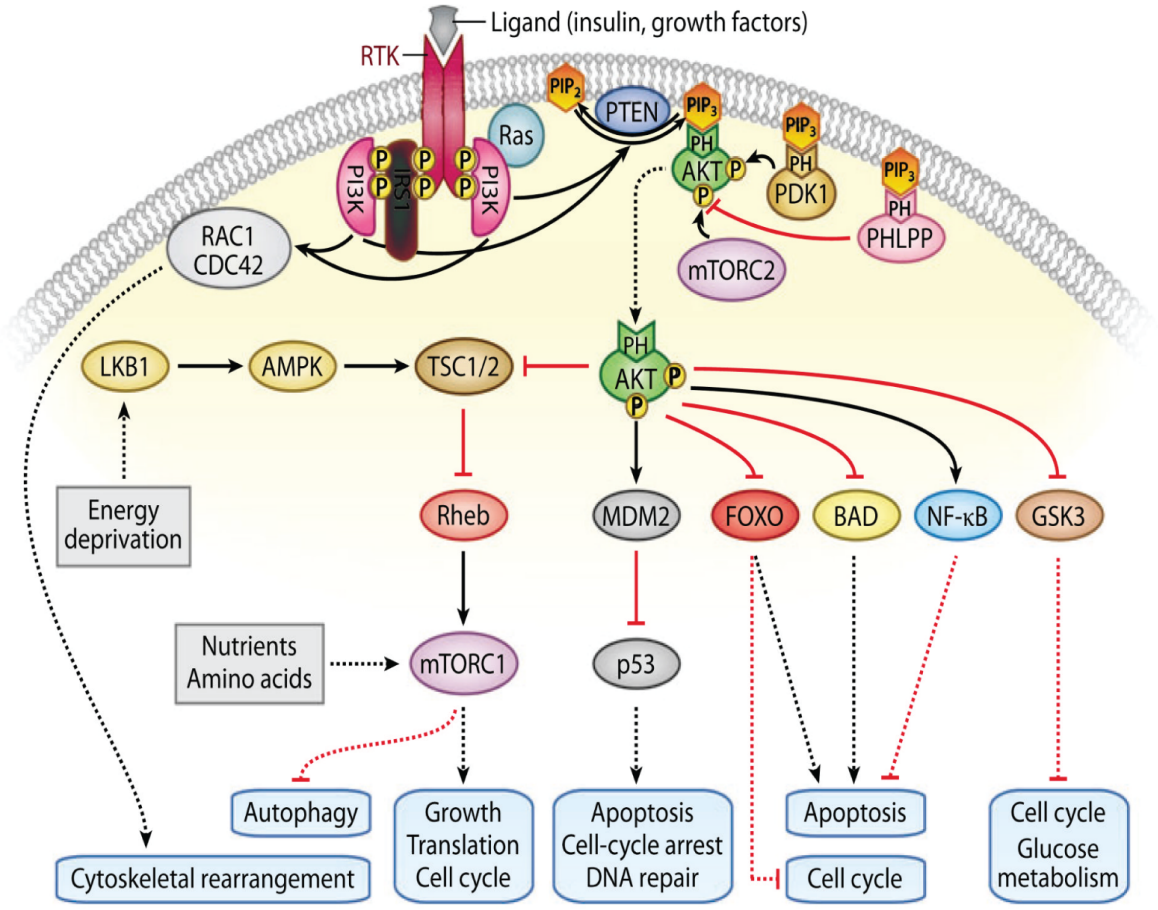

Figure 4 | The PI3K-AKT signalling pathway
Activated receptor tyrosine kinases (RTKs) recruit and activate PI3K, leading to increased phosphatidylinositol-3,4,5-trisphosphate (PIP3) levels. PIP3 recruits many proteins to the membrane by binding to their pleckstrin homology (PH) domains, including the serine/threonine kinases AKT, 3-phosphoinositide-dependent kinase (PDK1), and the phosphatase PH domain and leucine rich repeat protein phosphatase (PHLPP). Activated AKT may phosphorylate a range of substrates, thereby activating or inhibiting these targets and resulting in cellular growth, survival, and proliferation through various mechanisms. Abbreviations: GSK, glycogen synthase kinase; NF-κB, nuclear factor-κB; PIP2, phosphatidylinositol-4,5 bisphosphate; RAC1, Ras-related C3 botulinum toxin substrate 1(Chalchoub N. and Backer S.J., 2009).

AKT controls a plethora of cellular responses, and the three AKT isoforms, AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ) are ubiquitously expressed in all cell types and tissues (Toker A. and Yoeli-Lerner M., 2006). AKT is recruited to the membrane via PIP3 binding of its PH domain and its full activation requires phosphorylation either by PDK1 (3-phosphoinositide-dependent kinase) at threonine 308 (Mora A. et al., 2004) and at serine 473 by the rapamycin-insensitive mTOR complex (mTORC2) (Sarbassov D.D. et al., 2005) or potentially by other kinases in some contexts (Feng J. et al., 2004). Once phosphorylated and active, AKT relocalizes to several subcellular locations where it phosphorylates proteins involved in various cellular processes. A careful search of the literature finds over 100 reported non-redundant AKT substrates, of which approximately 25% do not contain the minimal recognition motif of AKT as R-X-R-X-X-S/T-B (Alessi D.R. et al., 1996).

The diverse cellular roles of AKT do not fall under a one-substrate-one-function paradigm: each physiological response downstream AKT appears to be mediated by multiple targets. Furthermore, some AKT substrates control more than one cellular function, and these functions may vary in a cell- and signal- context-dependent manner (Manning B.D. and Cantley L.C., 2007).

- **Cell survival**

AKT enhances the survival of cells by blocking the function of proapoptotic proteins and processes. AKT negatively regulates the function or expression of several Bcl-2 homology domain 3 (BH3)-only proteins, which exert their proapoptotic effects by binding to and inactivating prosurvival Bcl-2 family members. For instance, AKT directly phosphorylates and inhibits the BH3-only protein BAD, triggering release of BAD from its target proteins (Datta S.R. et al., 2000). AKT also inhibits the expression of BH3-only proteins through effects on Forkhead transcription factors, (FOXO) or on MDM2, an E3

ubiquitin ligase that triggers p53 degradation (Mayo L.D. and Donner D.B., 2001).

- **Cell proliferation**

PI3K activity is required at different steps of the cell cycle, first at the transition G0-G1, a second peak is at mid G1 and it is necessary for the entry in S-phase and induction of DNA synthesis and for the G2-M transition. AKT is involved at all these steps. Its main role in cell cycle control is the inhibition of Glycogen Synthase Kinase 3β (GSK3β) and the transcriptional factors FOXO. GSK3β is a negative regulator of cell cycle since it targets cyclin E, cyclin D and Myc for degradation. FOXO is the O subgroup of Forkhead transcriptional factors (TFs) family. These TFs induce the expression of molecule essential for quiescence maintenance, such as p27KIP , p130 and cyclin G2 (Martinez-Gac L. et al., 2004; Garcia Z. et al., 2006).

- **Protein synthesis**

AKT activates mTOR (mammalian target of rapamycin) inducing upregulation of protein synthesis, at least in part through modulation of two important components of the protein synthesis machinery, 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) and p70S6 kinase (Wullschleger S. et al., 2006).

- **Cell migration**

Current information suggests that one important role for PI3K in mammalian cells is in regulation of the actin cytoskeleton. Since actin remodelling is associated with cell transformation and with matrix- and growth factor-induced motility and cancer cell invasion, the lipid products of PI3K may contribute to malignant conversion via its effects on these cellular processes (Jones R.J. et al., 2000).

Classes IA and IB PI3Ks have been widely implicated in controlling cell migration and polarity (Cain R.J. and Ridley A.J., 2009). Although class II

PI3Ks have been implicated in the regulation of cell adhesion and actin reorganization during cell migration and wound healing in non-immune cell systems (Domin J. et al., 2005), it is likely that their importance has been underestimate. At present, class III PI3K does not have any defined role in cell migration (Backer J.M., 2008).

Primary regulators of actin remodelling are the Rho family of small GTPases, which include Rho, Rac and Cdc42 (Machesky L.M. and Hall A., 1996); each member controls signal transduction through distinct pathways that link membrane receptors to coordination of the cytoskeleton and adhesion complexes. Numerous opportunities for signal integration and crosstalk exist between Rho GTPases and PI3K-mediated signalling pathways; indeed, both Rac and Rho can be activated downstream of phosphoinositide signalling (Cain R.J and Ridley A.J., 2009) (**Fig. 5**).

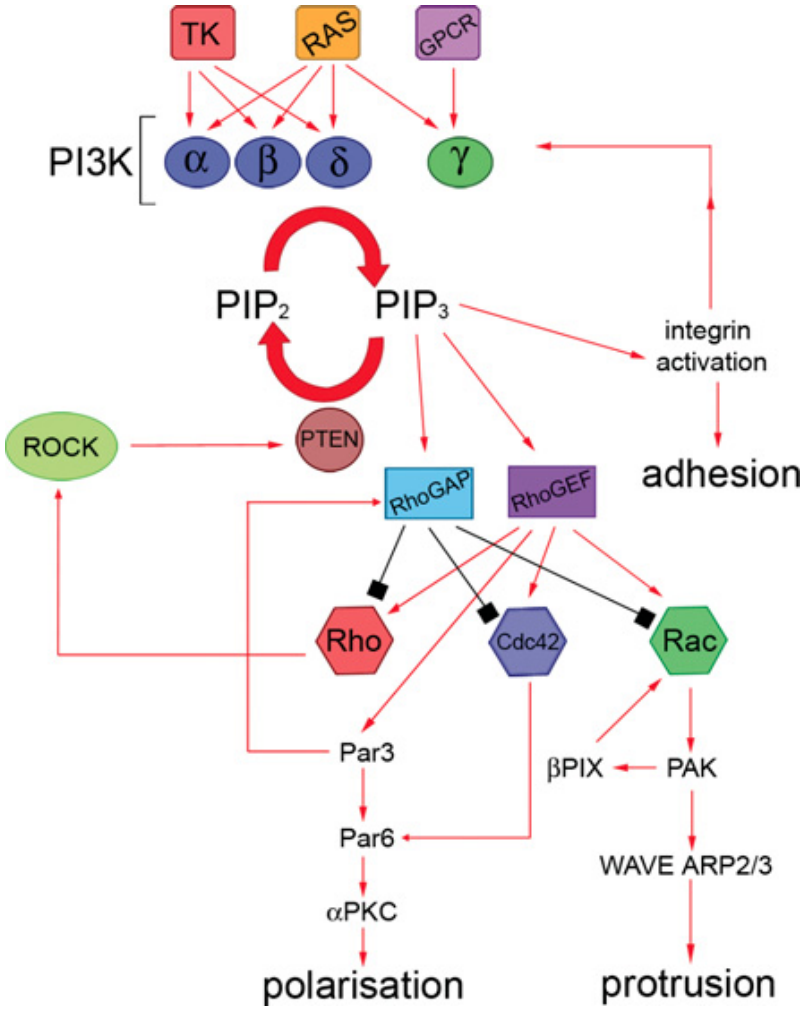

Fig. 5 | PI3K signalling and cell migration
The synergistic and antagonistic relationships between PI3K- and Rho-GTPase-mediated signalling pathways which contribute to cell polarization, adherence and protrusion. Abbreviations: GPCR, G-protein-coupled receptor; PIP2, PtdIns- (4,5)*P*2; PIP3, PtdIns(3,4,5)*P*3; PIX, PAK-interacting exchange factor; ROCK, Rho-associated kinase; TK, tyrosine kinase (Cain R.J. and Ridley A.J., 2009).

Structure and function of $p85^{PI3K}$

Although signalling through all classes of PI3Ks is linked to key growth-regulatory processes, classes IA and IB are the more extensively studied.

Class IA PI3Ks are heterodimeric enzymes made of a catalytic subunit (p110α, p110β, or p110δ) and one of five regulatory subunits (p85α, p55α, p50α, p85β, or p55γ), while the class IB enzyme is a dimer composed of p110γ catalytic subunit and p101 or p84 regulatory subunit (Cantley L.C., 2002; Hawkins P.T. et al., 2006; Vanhaesebroeck B. et al., 2005). The class I catalytic subunit polypeptides p110α, p110β, p110δ, and p110γ are encoded by PIK3CA, PIK3CB, PIK3CD, and PIK3CG, respectively. The regulatory subunits are encoded by five genes: PIK3R1 codes p85α, p55α, and p50α; PIK3R2 codes p85β; PIK3R3 codes p55γ; PIK3R5 codes p101; and PIK3R6 codes p84 (Cantley L.C., 2002; Vanhaesebroeck B. and Waterfield M.D., 1999).

The three different $p85^{PI3K}$ regulatory subunits share a high homology; their architecture consists of an N-terminal SH3 binding domain, two Proline Rich Domains (PRD), a domain homologous to the Rho GTPase-activating protein domain of the BCR gene product (BCR domain), and two SH2 domains (nSH2 and cSH2) separated by an intervening antiparallel coiled-coil (iSH2) (Holt K.H. et al., 1994).

The SH3 and the BCR domains are involved in the dimerization of $p85^{PI3K}$, that could be involved in the stabilization of $p110^{PI3K}$ (Harpur A.G. et al., 1999). The interaction between $p85^{PI3K}$ and $p110^{PI3K}$ is mediated by iSH2 domain, which binds the N-terminal of $p110^{PI3K}$ (Klippel A. et al., 1993).

The $p85^{PI3K}$ regulatory subunit displays a dual effect on PI3K activity, stabilizing the intrinsically unstable $p110^{PI3K}$ subunits, but also keeping them in a low activity state (Berenjeno I.M. and Vanhaesebroeck B., 2009). In the basal state $p85^{PI3K}$ inhibits the catalytic activity of $p110^{PI3K}$ (Yu J. et al., 1998b). Upon growth factor stimulation, the nSH2 and cSH2 domains of $p85^{PI3K}$ bind

to phosphorylated tyrosines (YXXM motif) in activated receptors and adaptors, and this recruitment relieves the p85-mediated inhibition of $p110^{PI3K}$ and bring the catalytic subunits in contact with their lipid substrates at the membranes (Berenjeno I.M. and Vanhaesebroeck B., 2009). The binding of $p110^{PI3K}$ to $p85^{PI3K}$ is necessary to stabilize the catalytic subunit, but it is not sufficient to activate its lipid kinase activity; the activation occurs after the conformational change induced by the phosphopeptide binding to the SH2 domain (Backer J.M. et al., 1992; Carpenter C.L. et al., 1993; Otsu M. et al., 1991).

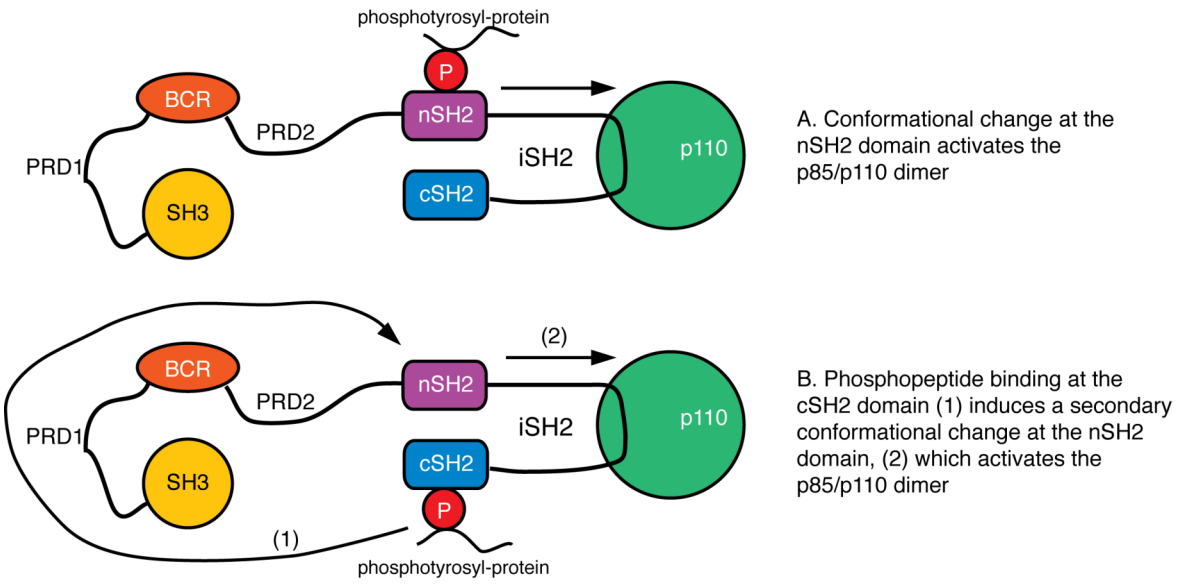

Fig 6 | Activation of PI3K
The binding of phosphoprotein at the nSH2 induces a conformational change at the same domain activating PI3K (A), the binding at the cSH2 induces a conformational change at the nSH2 through the N-terminal of $p85\alpha^{PI3k}$ activating the enzyme (B). (Adapted from Yu J. et al., 1998a)

PKA phosphorylates serine 83 of p85α^{PI3K}

It has been previously shown that PKA efficiently phosphorylates p85α^{PI3K} *in vitro* (Ciullo I. et al., 2001). Sequence analysis of bovine p85α^{PI3K} revealed a PKA consensus site (KKIS), that is highly conserved throughout evolution: KKIS in bovine/human and KRIS in mouse/rat (**Fig. 7**). Moreover, no PKA consensus was found in p85β^{PI3K} (Cosentino C. et al., 2007).

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p85α_bovine  EEIGWLNGYNETTGERGDFPGTYVEYIGRKKISPPTPKPRPPRPLPVAPG
p85α_human   EEIGWLNGYNETTGERGDFPGTYVEYIGRKKISPPTPKPRPPRPLPVAPG
p85α_mouse   EDIGWLNGYNETTGERGDFPGTYVEYIGRKRISPPTPKPRPPRPLPVAPG
p85α_rat     EDIGWLNGYNETTGERGDFPGTYVEYIGRKRISPPTPKPRPPRPLPVAPG
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Fig. 7 | Alignment of p85α^{PI3K} sequences from different species
p85α^{PI3K} presents an highly conserved PKA consensus at the residues 80-83 that is highly conserved in evolution.

PKA phosphorylates p85α^{PI3K} on Serine 83 in vivo, and the corresponding mutants, which prevent or mimic this phosphorylation (p85A or p85D), inhibit or amplify cAMP biological effects on growth and survival. p85^{PI3K} Ser83 is the major, if not the only, PKA phosphorylation site on the protein, since p85A was not phosphorylated neither *in vitro* nor *in vivo*. Furthermore, cAMP in the absence of serum stimulates PI3K, and p85α^{PI3K} mutants selectively interfere with cAMP stimulation of PI3K activity (Cosentino C. et al., 2007).

PI3K in cancer

PI3K has long been associated with cancer. Early evidence for such a connection was the association of phosphatidylinositol kinase activity with two viral oncoproteins, the Src protein of Rous sarcoma virus and the middle T protein of polyoma virus (Whitman M. et al., 1985; Sugimoto Y. et al., 1984). This interaction is mediated by the regulatory subunit p85^{PI3K}, which contains Src homology 2 (SH2) domains that bind to phosphotyrosine on the viral

oncoproteins and bring the catalytic subunit p110α of PI3K into these molecular complexes (Otsu M. et al., 1991; Vogt P.K., et al, 2007). The same modular protein–protein interaction domains also link $p85\alpha^{PI3K}$ and its attached $p110\alpha^{PI3K}$ to activated receptor tyrosine kinases such as epidermal growth factor receptor, platelet-derived growth factor receptor, and mesenchymal-epithelial transition factor, thereby illustrating the participation of this pathway in transducing cancer-relevant cues (Escobedo J.A. et al., 1991; Hu P. et al., 1992). Definitive evidence for the oncogenicity of PI3K was provided by the isolation of a constitutively active $p110\alpha^{PI3K}$ isoform from the genome of the oncogenic avian retrovirus ASV16 (Chang H.W. et al., 1997). This virus was isolated from a spontaneous sarcoma in a chicken and is highly tumorigenic in chickens and transforms chick embryo fibroblasts in culture. The genome of this virus carries as its oncogene a copy of the chicken coding sequence for the PI3K catalytic subunit p110α. The crucial modification that makes the viral $p110\alpha^{PI3K}$ protein oncogenic, in contrast to its innocuous and beneficial cellular counterpart, is the addition of a membrane "address" that is provided by fusion to viral Gag sequences (Aoki M., et al, 2000). This membrane address makes $p110\alpha^{PI3K}$ independent of the $p85^{PI3K}$-mediated recruitment to cellular membranes and results in a constitutively active PI3K (Vogt P.K. et al, 2007).

The PI3K signalling pathway is deregulated in most human cancers by differential gene expression, amplification, or mutation (**Fig. 8**). Several studies have identified common somatic mutations in PIK3CA in a large set of cancers, including colon, rectum, breast, ovary, brain, and liver (Bader A.G. et al., 2005; Samuels I. et al., 2004).

Of particular interest are mutations that occur in the catalytic subunit p110α of class I PI3K, because they confer a strong gain of function upon the enzyme, resulting in enhanced catalytic activity, constitutive signalling, and

oncogenicity in vitro and in vivo (Sun M. et al., 2010). The most striking feature of the mutation profile is the clustering of the mutations in three ‘‘hot-spot’’ regions in the kinase domain and the adjacent helical domain. Changes at three residues, E542K, E545K, and H1047R, comprised ~80% of the mutations in PIK3CA (Gabelli S.B., et al., 2010). All three mutations were found to increase the lipid kinase activity of PI3Kα, with a k_{cat} 2–3 fold higher than that of the wild-type enzyme (Carson J.D. et al., 2008; Gymnopoulos M. et al., 2007) but the molecular mechanisms by which these mutations activate $p110a^{PI3K}$ are just beginning to be elucidated.

One well-defined mechanism of activation of $p110\alpha^{PI3K}$ by mutation in cancer is that of the E545K hotspot mutation in the helical domain of $p110\alpha^{PI3K}$, at the interface with the nSH2 domain. This mutation relieves the basal repression of $p85\alpha^{PI3K}$ on $p110\alpha^{PI3K}$ through disruption of an inhibitory charge-charge interaction between the two proteins. Thus, $p85\alpha^{PI3K}$ no longer inhibits p110α-E545K (Berenjeno I.M and Vanhaesebroeck B., 2009). The H1047R mutation elicits conformational changes that are not at a domain interface, but at the interface between $p110\alpha^{PI3K}$ and the membrane, most likely altering membrane-protein interactions (Mandelker D. et al., 2009).

While oncogenic $p110\alpha^{PI3K}$ mutations are common in cancers, such mutations in the regulatory $p85^{PI3K}$ subunit are not as common (Bader A.G. et al., 2005; Hennessy B.T. et al., 2005). The first oncogenic variant of $p85^{PI3K}$, identified in an X-ray-irradiated mouse lymphoma model, was a truncated form of the protein containing residues 1–571 fused to a fragment of Eph (p65). This variant lacks part of the iSH2 and the cSH2, it can still bind $p110\alpha^{PI3K}$ and can localize the PI3K complex at the plasma membrane, resulting in the constitutive activity of the enzyme and contributing to cellular transformation (Borlado L.R. et al., 2000; Chan T.O. et al., 2002; Jimenez C. et al., 1998). However, this mutation has so far not been found in human cancers (Jaiswal

B.S. et al., 2009). Another oncogenic variant of $p85\alpha^{PI3K}$ is $p76\alpha^{PI3K}$, lacking the cSH2 domain, that was described in a human lymphoma cell line (Jucker M. et al., 2002), but its relevance in oncogenesis is not clear (Horn S. et al., 2008). A low prevalence of $p85\alpha^{PI3K}$ mutation in breast (Wood L.D. et al., 2007), colon, and ovarian (Philp A.J. et al., 2001) tumours has been reported, together with a frequent occurrence of $p85\alpha^{PI3K}$ mutations in glioblastomas (Parsons D.W. et al., 2008; CGARN 2008). However, the ability and role of these mutations in promoting oncogenesis remains to be studied.

Approximately 9% of glioblastomas harbour a mutation in $p85\alpha^{PI3K}$ (Parsons D.W. et al., 2008). The mutations cluster in the inter-SH2 (iSH2) domain of $p85\alpha^{PI3K}$ (CGARN 2008; Jaiswal B.S. et al., 2009) which interacts with the adapter-binding and the C2 domains of $p110\alpha^{PI3K}$ (Huang C.H. et al., 2007 and 2008; Elis W. et al., 2006). The interaction between $p85\alpha^{PI3K}$ and the adapter-binding domain stabilizes $p110\alpha^{PI3K}$, while the interaction between p85α iSH2 and the C2 domain inhibits the enzymatic activity of $p110\alpha^{PI3K}$. The mutations in the iSH2 domain of $p85\alpha^{PI3K}$, such KS459delN and DKRMNS560del, affect primarily the residues that interact with the C2 domain of $p110\alpha^{PI3K}$ and weaken this inhibitory interaction, leading to a gain of function in PI3K activity (Jaiswal B.S. et al., 2009; Sun M. et al., 2010). Studies with isoform-specific inhibitors of $p110\alpha^{PI3K}$ suggest that expression of $p85^{PI3K}$ mutants in fibroblasts leads exclusively to an activation of $p110\alpha^{PI3K}$, and $p110\alpha^{PI3K}$ is the sole mediator of $p85^{PI3K}$ mutant-induced survival signalling and oncogenesis (Sun M. et al., 2010), even if potency in cell transformation is not always correlated with signalling levels measured by the phosphorylation of AKT and other downstream targets (Kang S. et al., 2006; Zhao L. et al., 2008).

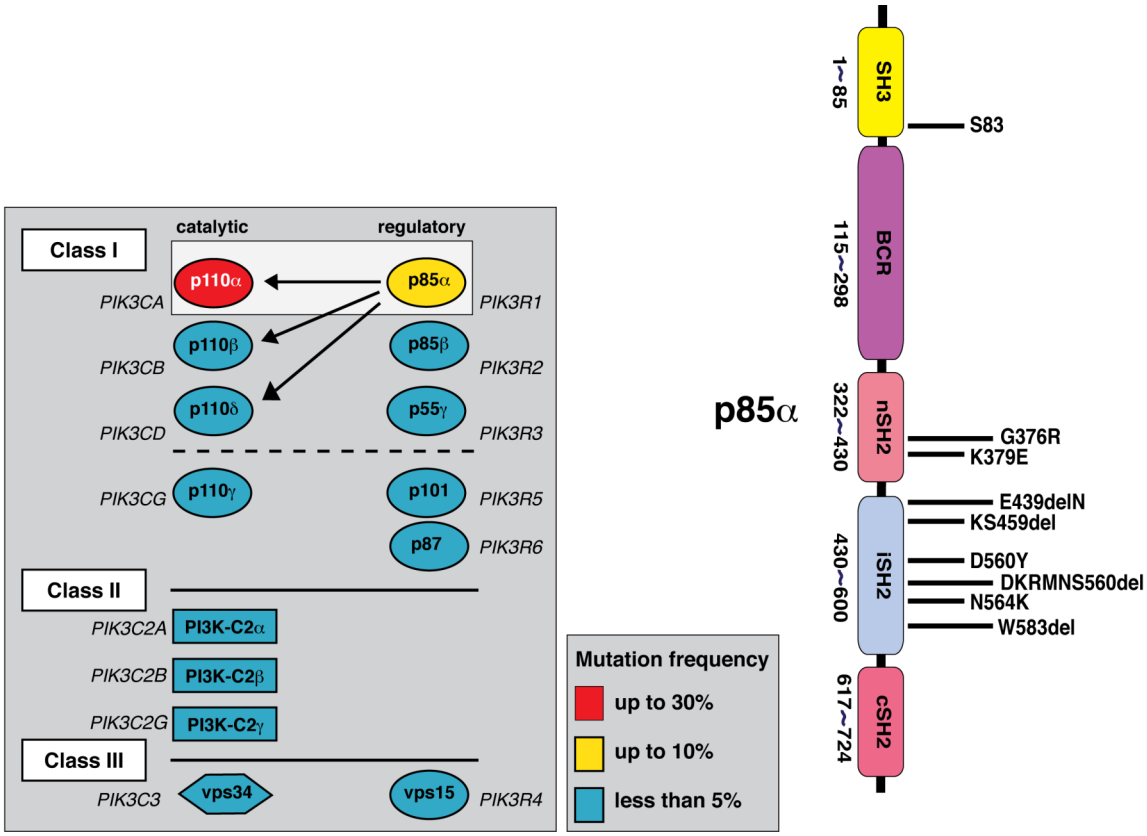

Fig. 8 | Cancer Mutations in PI3K Family Members

The left panel shows the frequency of mutation in cancer in the different PI3K family members. The right panel shows the location of mutations in $p85\alpha^{PI3K}$. (Adapted from Berenjeno I.M and Vanhaesebroeck B., 2009; Sun M. et al., 2010).

Interactions between p85α and Nuclear receptors

Multicellular organisms require a specific intercellular communication to organize the complex body plan properly during embryogenesis and maintain their physiological properties and functions throughout life. Growth factors, neurotransmitters and peptide hormones bind to membrane receptors thereby inducing the activity of intracellular signalling pathways; other small hydrophobic signalling molecules such as steroid hormones, certain vitamins and metabolic intermediates enter target cells and bind to cognate members of a large family of nuclear receptors. Nuclear receptors are of major importance for intercellular signalling in animals because they converge different intra- and extracellular signals on the regulation of genetic programs. Such nuclear receptors are transcription factors that: respond directly through physical association with a large variety of hormonal and metabolic signals; integrate diverse signalling pathways because they correspond themselves to targets of post-translational modifications; regulate the activities of other major signalling cascades. (Bourguet W. et al., 2000).

The nuclear receptor superfamily describes a related but diverse array of transcription factors, which include nuclear hormone receptors (NHRs) and orphan nuclear receptors. NHRs are receptors for which hormonal ligands have been identified, whereas orphan receptors are so named because their ligands are unknown (Kliewer S.A. et al., 1999). All of the nuclear receptors have common structural features (**Fig. 9**), which include a central DNA Binding Domain (DBD) responsible for targeting the receptor to highly specific DNA sequences comprising a Response Element (RE) (Bourguet W. et al., 2000). The Ligand Binding Domain (LBD) is contained in the C-terminal half of the receptor and recognizes specific hormonal and non-hormonal ligands, directing specificity to the biologic response. Nuclear receptors can exist as homo- or heterodimers with each partner binding to specific RE sequences that exist as

half-sites separated by variable length nucleotide spacers between direct or inverted half-site repeats (Olefsky J.M., 2001). Four categories of nuclear receptors are known: Class 1 receptors include the known steroid hormone receptors, which function as homodimers binding to half-site RE inverted repeats. Class 2 receptors exist as heterodimers with RXR receptor partners and function in a ligand-dependent manner. The second two classes include orphan receptors, which function as homodimers binding to direct RE repeats (Class 3) or monomers binding to single site REs (Class 4) (Manglesdorf D.J. et al., 1995).

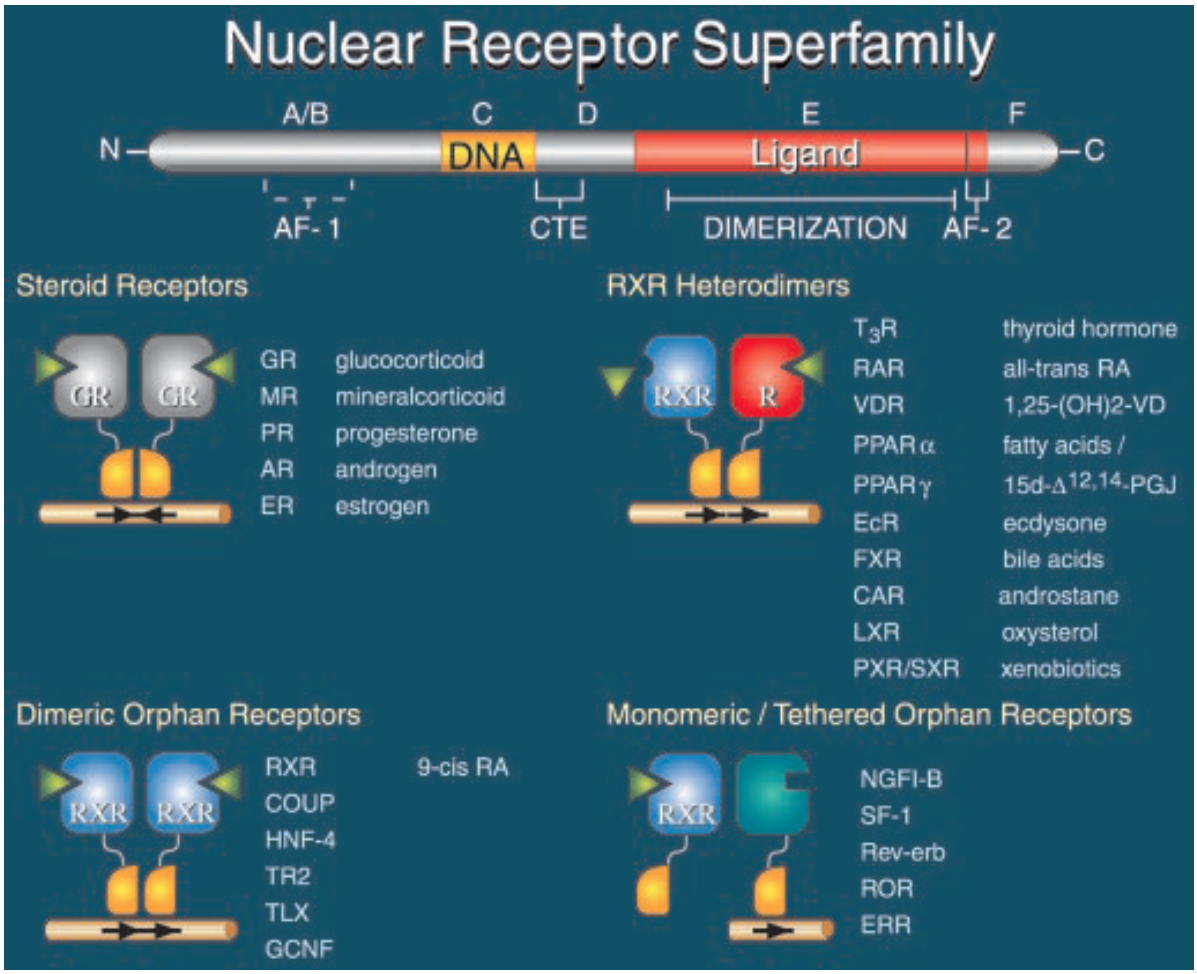

Fig. 9 | Structure/function organization of nuclear receptors
The six domains (A–F) of nuclear receptors comprise regions of conserved function and sequence. All of the nuclear receptors contain a central DNA Binding Domain (region C), which is the most highly conserved domain and includes two zinc finger modules. A Ligand Binding Domain (region E) is contained in the C-terminal half of the receptor. Situated between the DBD and LBD is a variable length hinge domain (region D). Many members of the nuclear receptor family form homo- or heterodimers, and amino acid sequences important for dimerization are contained within the DBD and LBD (Olefsky J.M., 2001).

ER

The estrogen receptor (ER) is perhaps the most well defined nuclear receptor system from the point of view of biologic responses and clinical implications. There are two subtypes of the ER (ERα and -β), which are products of distinct genes; although quite similar in structure, the two ER subtypes display structural differences and can mediate overlapping but different sets of biologic functions (Hall J.M et al., 2001). Besides their classical genomic actions, estrogens can exert nongenomic effects through interaction with and activation of signalling kinases such as IGF-1R, Src, PI3K, MAPK, EGFR and ErbB-2 (Acconcia F. and Kumar R., 2006; Edwards D.P., 2005). Conversely, the cytoplasmic kinases can phosphorylate co-activators possibly modifying ERα activity (Zheng F.F. et al., 2005).

It has been described that estradiol can rapidly activate cytosolic pathways by triggering cytoplasmic complex containing ERα, the tyrosine kinase Src, and the p85 subunit of PI3K, leading to the activation of key downstream signalling kinases such as MAPK and AKT, which orchestrate cell proliferation and survival (Greger J.G. et al., 2007; Castoria G. et al., 2001; Cabodi S. et al., 2004). It has also been demonstrated that methylation of ERα on arginine 260 is a critical upstream signal for the formation of this complex and thereby the activation of downstream transduction cascades (Le Romancer M. et al., 2008). Furthermore, $p85\alpha^{PI3K}$ Ser 83 plays an essential role for estrogens signalling, since its phosphorylation by cAMP–PKA is required for the formation of the complex ER-alpha/p85α (Cosentino C. et al., 2007).

RAR

Nuclear retinoic acid (RA) receptors (RARs) consist of three subtypes, α (NR1B1), β (NR1B2) and γ (NR1B3), encoded by separate genes (Duong V. et Rochette-Egly C., 2010). For each subtype, there are at least 2 isoforms, which are generated by differential promoter usage and alternative splicing and differ

only in their N-terminal regions. RARs are activated by retinoids, which cover natural vitamin A metabolites exemplified by retinoid acid and synthetic active analogs (Rochette-Egly C. and Germain P., 2009).

RARs function as ligand-dependent transcriptional regulators, heterodimerized with retinoid X receptors (RXRs), which also consist of three types, α NR2B1, β (NR2B2) and γ (NR2B3) (Germain P. et al., 2006). There is increasing evidence that the ubiquitin/proteasome machinery degrades the retinoid receptors subsequently to their activation (Zhu J. et al., 1999; Boudjelal M. et al., 2000; Bastien J. and Rochette-Egly C., 2004).

RARs regulate the expression of subsets of target genes involved in a variety of biological processes, including cellular differentiation, proliferation and apoptosis (Delacroix L. et al., 2010; Eifert C., et al., 2006), as well as development (Zile MH, 2001), reproduction, immunity, organogenesis and homeostasis (Mark M., et al 2009; Niederreither K., and Dolle P., 2009). In addition to physiological roles, RARs are also involved in several diseases and cancers subsequently to mutations, fusions to other proteins, altered expression levels or aberrant post-translational modifications, resulting into altered functions and disruption of homeostasis genes (Duong V. and Rochette-Egly C., 2010).

Novel non-genomic mechanisms of signal transduction through nuclear hormone receptors have been described. These rapid, non-genomic actions do not rely on gene transcription or protein synthesis, but involve ligand-induced modulation of signal transduction pathways (Wehling M., 1997; Losel R. and Wehling M., 2003; Simoncini T. and Genazzani A.R., 2003). It has been described that RA treatment of neuroblastoma cells results in activation of the PI3K signalling pathway, and this activation is required for RA-induced differentiation (Lopez-Carballo G., et al 2002). This activation of PI3K by RA occurs through a non-genomic action of the classical nuclear RAR (Masià S. et

al., 2007). In contrast to what has been reported for steroid receptors, where interaction of the receptor with the p85 regulatory subunit of PI3K was under the control of ligand, (Simoncini T. et al., 2000; Castoria G. et al., 2001), it has been shown that RARα forms a stable complex with $p85\alpha^{PI3K}$ independently of the presence of RA (Masià S. et al, 2007).

AIM OF THE STUDY

Steroid, growth factor and membrane receptor signalization frequently converge in the PI3K signalling pathway, a key player in cellular homeostasis, whose misregulation can result in tumour development. Extensive research on the molecular mechanisms involved in the action of PI3K signalling, describes the PI3K regulatory subunit p85α as responsible for protein–protein interactions with phosphotyrosine residues of other proteins. The $p85\alpha^{PI3K}$ adaptor protein employs the inter-SH2 (iSH2) region, to bind the $p110^{PI3K}$ catalytic subunit (Klippel A. et al, 1993), and SH3 and SH2 domains, phosphotyrosine residues, and proline-rich motifs to interact with activated tyrosine kinases, adaptor proteins, nuclear receptors and PKA (Hellyer N.J. et al, 1998; McGlade C.J. et al., 1992; Ciullo I. et al., 2001; Cosentino C. et al., 2007). Although the involvement of $p85\alpha^{PI3K}$ SH2 domain in the signal transduction has been extensively studied, the function of the SH3 domain at the NH terminus remains elusive.

In many steroid-dependent cancers, a close crosstalk exists between growth factor and steroid signalling which thus converge in the PI3K/AKT pathway; this signalling cascade is frequently deregulated in breast cancer (Altomare D.A. and Testa J.R., 2005). All-trans Retinoic Acid (RA) is a vitamin A derivative that regulates cell growth, differentiation, and apoptosis (DeLuca L.M., 1991; Sporn M.B and Roberts A.B., 1994). Various synthetic retinoids, including RA, have shown promise for the treatment and prevention of several cancers, including carcinoma of the breast (Veronesi U. et al., 1999; Dragnev K.H. et al., 2000; Guruswamy et al., 2001; del Rincòn S.V. et al., 2003). Numerous groups have found that retinoids potently inhibit the growth of breast cancer cell lines (Zhu W.Y. et al., 1997; Wang Q. et al., 2000), and others have reported the capacity of retinoids to inhibit mammary carcinogenesis in animal models (Anzano M.A. et al., 1994; Moon R.C. and

Constantinou A.I., 1997). Many mechanisms have been proposed to explain this inhibition of breast cancer cell growth exerted by retinoids (Liu Y. et al., 1996; Zhou Q. et al., 1997; Agadir A. et al., 1999). It has been recently demonstrated a rapid, non-genomic actions of Retinoic Acid on PI3K signalling pathway mediated by RARα that does not require new gene transcription or newly synthesized proteins (Masià S. et al, 2007). It has also been described that the PI3K binding to estrogen receptor and PKA RII subunit is finely regulated by the PKA-induced phosphorylation of the Ser83 in the N-terminal/SH3 domain of the $p85\alpha^{PI3K}$ (Cosentino C. et al, 2007; De Gregorio G., et al, 2007). Since $p85\alpha^{PI3K}$ binds several types of receptors and adaptors, the phosphorylation of Ser83 by PKA can induce a conformational change of the PI3K complex, which results in facilitated binding to these partners, thereby modulating PI3K activity (Cosentino C. et al, 2007; De Gregorio G., et al, 2007). This site represents probably a nodal point, where information from several receptors that regulate cell growth, survival, adhesion and motility is channelled to PI3K.

The aim of this work is to analyse the role of the SH3 domain of the PI3K p85α regulatory subunit in Retinoic Acid and cAMP signalling using as model system the breast cancer cell line MCF7, which express functional RAR receptor.

In particular, in the first part of the work we tested whether $p85\alpha^{PI3K}$ Ser83 modulates the binding of the retinoic acid receptor RARα to PI3K after treatment of MCF7 cells with RA and cAMP.

The second part of the work describes the biological consequences of the expression of mutant versions of $p85\alpha^{PI3K}$ in MCF7 cells, focusing on the role of $p85\alpha^{PI3K}$ Ser 83 in cell motility, growth and survival.

MATERIALS AND METHODS

Cell cultures and plasmid transfections

MCF7 (Michigan Cancer Foundation-7) is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman (Soule H.D. et al., 1973). This cell line retained several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes, and therefore represents a useful in vitro model of breast cancer.

MCF7 cells were grown in DMEM with 10% fetal bovine serum supplemented with 2mM L-glutamine and maintained at 37 °C in an humidified atmosphere of 5% CO2. Prior to experiments, cells were grown for 48 h in phenol red-free DMEM and 5% charcoal-stripped serum (Biowest).

Plasmids carrying FLAG-tagged p85α-wild-type or its Ser83 mutated forms were obtained as described (Cosentino C. et al., 2007). pSG5-RARα plasmid was gently provided by Prof. Domingo Barettino, Instituto de Biomedicina de Valencia.

MCF7 cells were grown in 100 mm tissue culture plates to 70/80% confluence and plasmid transfection (8μg) was performed using Lipofectamine 2000™ (Invitrogen) and JetPEI™ (Polyplus transfection) transfection reagents following the manufacturer's instructions.

Wound Healing assays

MCF7 cell were plated in 35 mm dish and cultured to confluence of 90%. Cell layers were then scratched with a p200 pipette tip, and cellular debris has been removed by washing gently. Following wounding, culture medium was replaced with fresh medium and cells were exposed to 30 μM all-trans Retinoic

Acid or 200 μM 8-Br-cAMP (Sigma-Aldrich), or both of them for 16 hours. Images were acquired on a phase contrast microscope Axio Observer (Carl Zeiss, Inc., Oberkochen, Germany) and wound diameter was measured at 0 and 16 hours using the computing software Axio Vision (Carl Zeiss, Inc.) that measures the scratch area colonized by cells during the examined time frame. Each result is the mean of at least three independent experiments.

Clonogenic assays

MCF7 cells (3 x 102) expressing the $p85\alpha^{PI3K}$ mutants were seeded into 6 well plates that were then incubated at 37°C for about 10 days until cells have formed sufficiently large clones (at least 50 cells). Clones were counted after 30' fixing with a mixture of 6% glutaraldehyde and 0.5% crystal violet. Experiments were performed in triplicate.

Proliferation assays

The cell proliferation was assessed by cell counting. Briefly, 3 $x10^4$ cells were seeded into 35 mm plates and allowed overnight at 37°C in a humidified incubator with 5% CO2. Cell numbers were determined using a hemacytometer at time zero (T0), and after 24, 48 and 72 h in serum-free medium. Cells were counted three times by two independent investigators. Inter-observer variation was below 5%.

Thymidine incorporation

MCF7 cells (5 x 10^4) were plated into 24-well plates, cultured untreated or treated with 30 μM all-trans Retinoic Acid or 200 μM 8-Br-cAMP (Sigma-Aldrich), or both of them for 24 and 48 h, and then incubated with 1 mCi/ml/well [^{3}H]thymidine (6.7 Ci/mmol, Amersham) for 4 h. The cells were

washed with phosphate-buffered saline (PBS), pH 7.4, incubated with 10% TCA for 30 min at 4 C, and washed, and the attached cells were extracted with 300 µl/well of 1 M NaOH for 1 h at 37 C. One hundred microliters was collected and counted in scintillation fluid in a Beckman beta counter. The mean and the standard deviation from three different experiments were calculated and reported on a diagram.

MTT Assays

Cell viability and proliferation were assayed using the 3-(4,5- dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (MTT); Sigma, St. Louis, MO). MCF7 cells transfected with $p85\alpha^{PI3K}$ mutants were plated into 24-well plates, (5×10^4) and allowed overnight at 37°C in a humidified incubator with 5% CO2. After indicated 48 h, 100µl MTT (2 mg/ml) was added to the medium and incubated for a further 4 h. The medium containing MTT was then replaced with DMSO and absorbance was measured at 570 nm.

Cell cycle analysis

Cell cycle analysis was performed by fluorocytometric absorbent cell sorter (FACS). 5×10^5 transfected cells (24 h after transfection) were plated in 60 mm dishes and grown in low-serum medium (0.5% fetal bovine serum) for 18 h. Cells were collected, fixed with 70% ethanol and then stained for 30min at RT in 0.1% Triton X-100, 0.2 mg/ml DNase-free RNaseA and 20 µg/ml propidium iodide. Fluorescence was determined by using the FACScan Flow Cytometer (Becton Dickinson). Experiments were performed in triplicate.

Western Blot and Immunoprecipitation

Subconfluent transfected MCF7 cells were stimulated or not for 10 or 30 minutes with 10 or 30 μM of all-Trans Retinoic Acid (Sigma-Aldrich) or 200 μM 8-Br-cAMP (Sigma-Aldrich) or with 10 μM H89 (Sigma-Aldrich) and then lysed for 30 min at 4°C in ice-cold TNE extraction buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl , 1 mM EDTA, 1% TRITON X-100) supplemented with 1 mM phenilmethilsulfonide fluoride, protease cocktail inhibitor containing 1 mg/ml leupeptin, 2.5 mg/ml aprotinin, 1mg/ml benzamide hydrochloride (Sigma-Aldrich) and phosphatase cocktail inhibitor (Sigma-Aldrich). Cell lysate was cleared by centrifugation (15,000g, 5 min, 4°C) and then normalized for protein amount using the BioRad Protein Assay. Equal amount of protein (80 μg) was electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions and then electrotransferred to a PVDF filters (Millipore). Antibodies against RARα, AKT and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against phosphorylated AKT (Ser473), was obtained from Cell Signal Technologies (Beverly, MA). Antibodies against PI3K-p85α and phosphorylated FAK were from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibody against FlagM2 was from (Sigma-Aldrich). Horseradish-peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and Santa Cruz Biotechnology. Chemiluminescent signals were developed with enhanced chemiluminescence (ECL) or ECL Plus (GE Healthcare). For immunoprecipitation, protein (1 mg) from co-transfected MCF7 cells was incubated with 8ug of antibodies against anti-FlagM2 or RARα, or with 5μL of antibody against p85; immunocomplexes were precipitated using anti-rabbit IgG beads (ExactaCruzF, Santa Cruz Biotechnology), following the manufacturer's instructions, and processed for Western blot analysis as described.

RESULTS

1. Role of phosphorylation of p85α^{PI3K} Ser83 in the interaction with Retinoic Acid Receptor alpha

1a. Interactions between RARα and p85α^{PI3K}

MCF7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red for 48 h, and treated with 10μM RA and/or cAMP 200μM, and/or H89 10μM or their vehicles for 30 min.

Cell lysates were immunoprecipitated with anti-RARα antibody, separated on SDS-PAGE and sequentially analysed by Western Blot with anti-p85α and anti-RARα antibodies. The interaction specificity was assessed using IgG immunoprecipitates as negative control. Immunoprecipitation experiments showed a physical association between p85α^{PI3K} and RARα in all conditions examined (**Fig. 10A** upper panel). In particular, RA treatment increased 4 fold the formation of PI3K/RARα complex, while cAMP stimulation increased it 2,5 fold. Interestingly the co-treatment with RA and cAMP amplified synergistically the binding (9 fold). Treatment of MCF7 cells with the PKA inhibitor, H89, did not impair the basal interaction, but abolished synergy with cAMP. The histogram in **Fig. 10B** represents the ratio of p85α/RARα bands in the RARα immunoprecipitates, derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs t test (* $p<0,01$ vs basal; ** $p<0.05$ vs RA+cAMP treated cells).

Western Blot performed on total lysates with anti RARα antibody showed that RA treatment induced a down-regulation of RARα protein, which is probably

due to proteasomal degradation (Bastien J. and Rochette-Egly C., 2004). p85α and β-actin expressions in inputs were shown (**Fig. 10A** lower panel).

These findings collectively established that endogenous RARα and $p85\alpha^{PI3K}$ stably interact in MCF7 cells and that stimulation by cAMP–PKA is required for the enhancement of this binding.

1b. RARα interacts with p85αPI3K independently from PKA-modification of Ser83

In order to study if the formation of PI3K/RARα complex depends from phosphorylation of $p85\alpha^{PI3K}$ Ser83, quiescent MCF7 cells were transiently transfected with a construct containing bovine p85WT tagged with FLAG (pSG5-FLAG-p85WT), or with p85A (pSG5-FLAG-p85A) or with p85D (pSG5-FLAG-p85 D).

Cell lysates were immunoprecipitated with anti-FLAG antibody or with non-immune IgG. As shown in **Fig. 11A**, overexpression of the mutated forms of $p85\alpha^{PI3K}$ did not significantly impair the interaction between RARα and $p85\alpha^{PI3K}$.

The histogram in **Fig. 11B** represents the ratio of FLAG/RARα bands in the FLAG-$p85\alpha^{PI3K}$ immunoprecipitates, derived from three independent experiments.

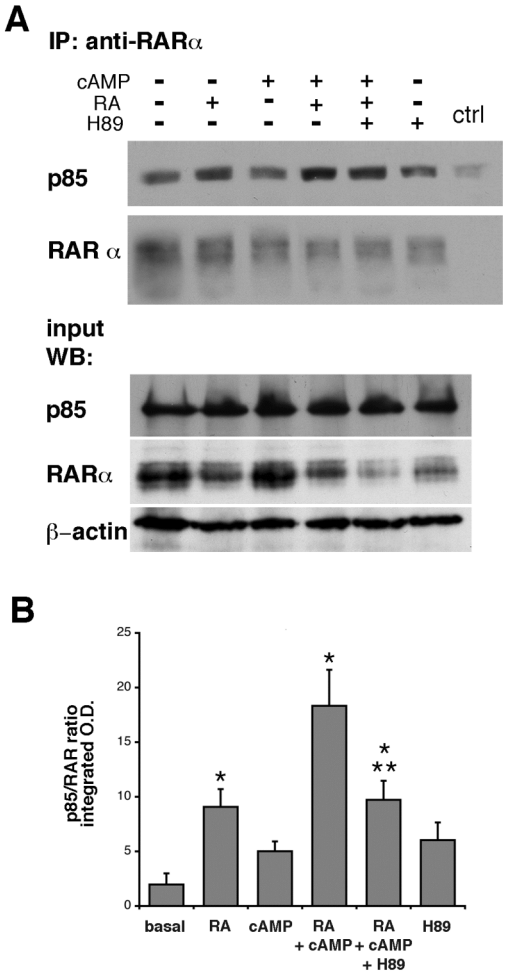

Fig. 10 | Interactions between RARα and p85α[PI3K]

A. Total cell extracts were prepared from MCF7 quiescent cells treated with 10 μM RA and/or cAMP 200μM, and/or H89 10μM or their vehicles for 30 min. About 1mg protein from control or RA-treated cell extracts were incubated with 5 μL of p85α antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated using antirabbit IgG beads (SantaCruz), following the manufacturer's instructions, and suspended in sample buffer containing freshly added inhibitors. After Western blot the filter was sequentially developed with anti-p85α and anti-RARα antibodies, using horseradish peroxidase-conjugated Rabbit ExactaCruzF (SantaCruz) as secondary antibodies.

B. The histogram represents the ratio of p85/RARα bands in the RARα immunoprecipitates, derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs *t* test: * $p<0.01$ vs basal; ** $p<0.05$ vs RA+cAMP treated cells.

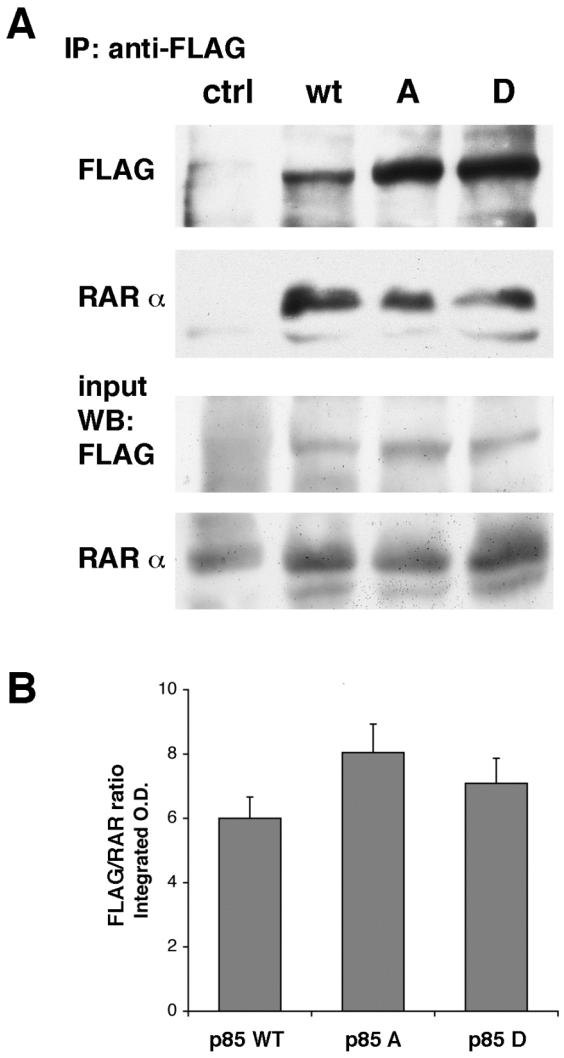

Fig. 11 | RARα interacts with p85α^{PI3K} independently from PKA-modification of Ser83

A. MCF7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red and transiently transfected with p85α^{PI3K}-flag wild type, p85A or p85 D; 48 h after transfection, cell lysates were immunoprecipitated with non-immune IgG or 8μg of anti-Flag antibody for 15 h. Sample aliquots were immunoblotted with anti-RARα and anti-Flag antibodies using horseradish peroxidase-conjugated Rabbit ExactaCruzF (SantaCruz) as secondary antibodies to detect the PI3K/RARα complex. B. The histogram represents the ratio of FLAG/RARα bands in the FLAG-p85α^{PI3K} immunoprecipitates, derived from three independent experiments.

1c. cAMP/RA synergistic enhancement of RARα/p85α^{PI3K} binding is dependent from PKA-modification of Ser83

In order to test the role of p85α^{PI3K} Ser83 in modulating the RARα/p85α binding after RA treatment, MCF7 cells were transiently transfected with p85α wt or mutant encoding vectors and treated for 30 minutes with RA and/or cAMP 200μM, and/or H89 10μM or their vehicles. Cell lysates were immunoprecipitated with anti-RARα antibody, separated on SDS-PAGE and analyzed by Western Blot with anti-p85α and anti-RARα antibodies.

As demonstrated in **Fig. 12**, the ability to form the p85α/RARα complex was maintained in all the examined conditions, but overexpression of p85α^{PI3K} mutants significantly abolished the synergistic effect of cAMP/RA treatment. Moreover, the reduction of p85α/RARα binding observed in the p85 wt overexpressing cells after H89 treatment, was completely lost in both p85 mutants transfected cells. The histogram in **Fig. 12B** represents the ratio of p85α/RARα bands in the RARα immunoprecipitates, derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs t test (* $p<0.01$ vs basal).

Taken together, these data demonstrated that p85α^{PI3K} Ser83 is crucial for the synergistic enhancement of RARα/p85α binding induced by cAMP/RA.

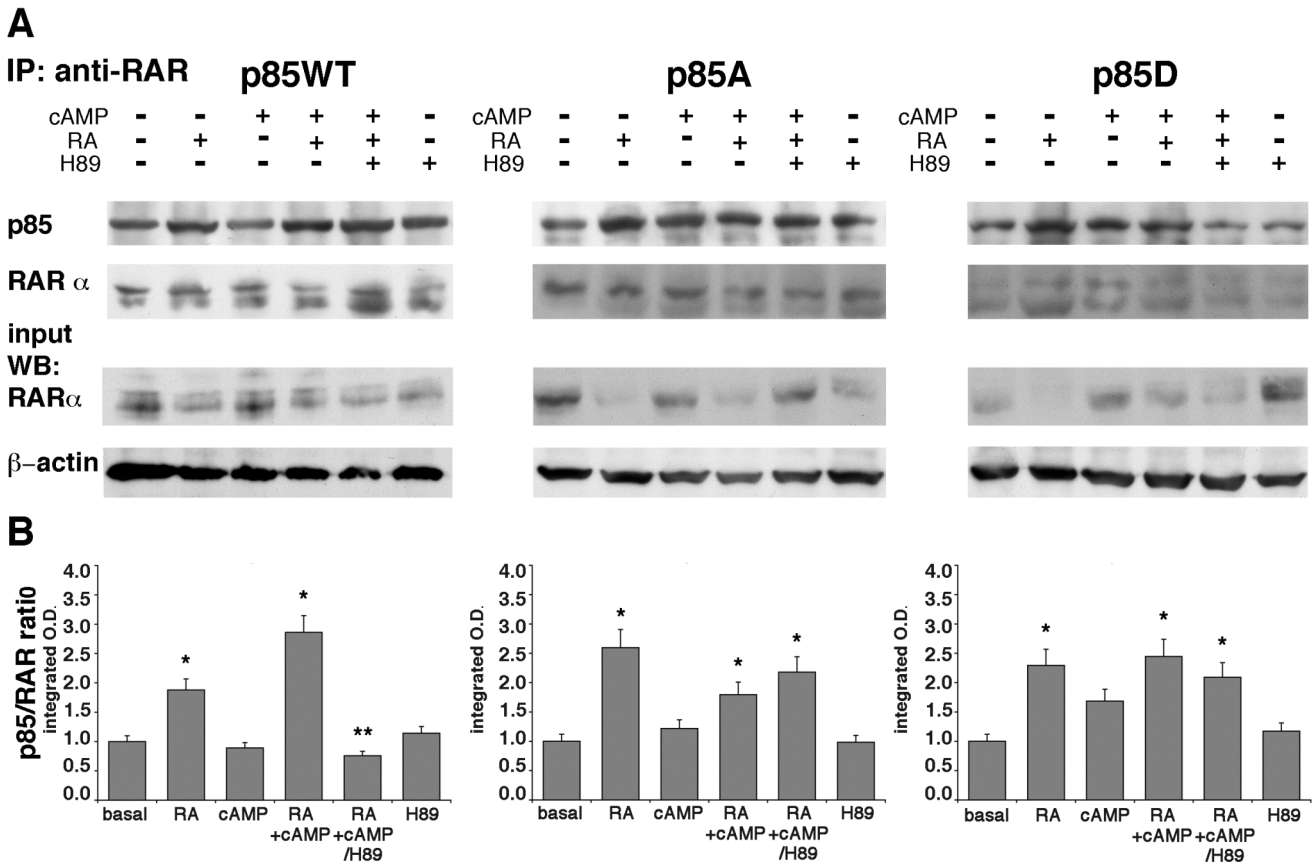

Fig. 12 | p85 Ser83 is important for the cAMP-PKA-modulation of the RARalpha/p85 complex

A. MCF7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red and transiently transfected with p85α^{PI3K}-flag wild type, p85A or p85 D; 48h after transfection cells were treated with 10 μM RA and/or cAMP 200μM, and/or H89 10uM or their vehicles for 30 min. About 1mg protein from control or RA-treated cell extracts were incubated with 8 μg of RARα antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated using anti-rabbit IgG beads (SantaCruz), following the manufacturer's instructions, and suspended in sample buffer containing freshly added inhibitors. After Western blot the filter was sequentially developed with anti-p85α and anti-RARα, using horseradish peroxidase-conjugated Rabbit ExactaCruzF (SantaCruz) as secondary antibodies.

B. The histogram represents the ratio of p85α/RARα bands in the RARα immunoprecipitates, derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs *t* test: * $p<0.01$ vs basal.

2. Role of p85α^{PI3K} Ser83 in the non-genomic actions of retinoic acid on PI3K signalling pathway

It has been recently demonstrated that administration of RA resulted in activation of the PI3K signalling pathway (Lopez-Carballo G. et al, 2002) through a rapid non-genomic action mediated by the retinoic acid receptor (Masià S. et al, 2007).

In order to understand whether this rapid non-genomic action involved cAMP-PKA signalling, Western Blot analysis was performed to assess the state of phosphorylation of AKT in the MCF7 cells upon stimulation with RA 30μM and/or cAMP 200μM for 5, 10, 20 and 30 minutes.

The data presented in **Fig. 13** demonstrated that both RA and cAMP treatment were able to induce AKT phosphorylation on Ser473 (* $p<0,01$ vs basal), but not significant synergy was observed after co-treatment. The histogram in the lower panel represented the ratio of P-AKT/AKT derived from three independent experiments.

In order to study the role of p85α^{PI3K} Ser83 in the regulation of the non-genomic effects induced by Retinoic Acid, quiescent MCF7 cell were transfected with p85α wt or mutant encoding vectors and treated for 10 minutes with or without RA 30μm. As shown in **Fig. 14** the phosphorylation of P-AKT was present in all conditions examined. The histogram represents the ratio of P-AKT/AKT derived from three independent experiments.

These results demonstrated that p85α^{PI3K} Ser83 is not involved in the rapid-non genomic activation of P-AKT induced by RA.

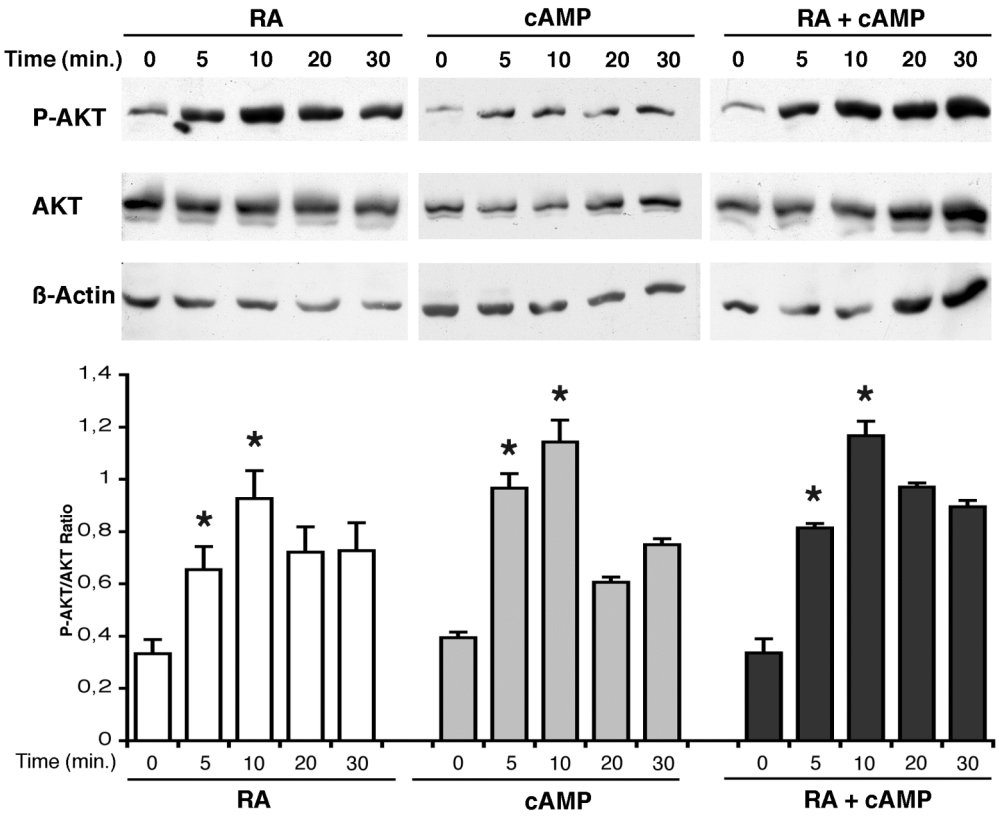

Fig.13 | Activation of PI3K-AKT pathway in response to RA and cAMP
MCF7 cells were treated with RA (30μM) and/or cAMP (200μM) for the times indicated in the figure, and 80μg of total cell extracts were analyzed by western blot. The phosphorylation state of Akt was analyzed by using specific antibodies against AKT phosphorylated in Ser473 (upper panel) and the filter was reprobed with antibodies against total AKT (middle panel) and β-actin (lower panel). The histogram represents the ratio of P-AKT/AKT derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs *t* test: * p<0,01 vs basal.

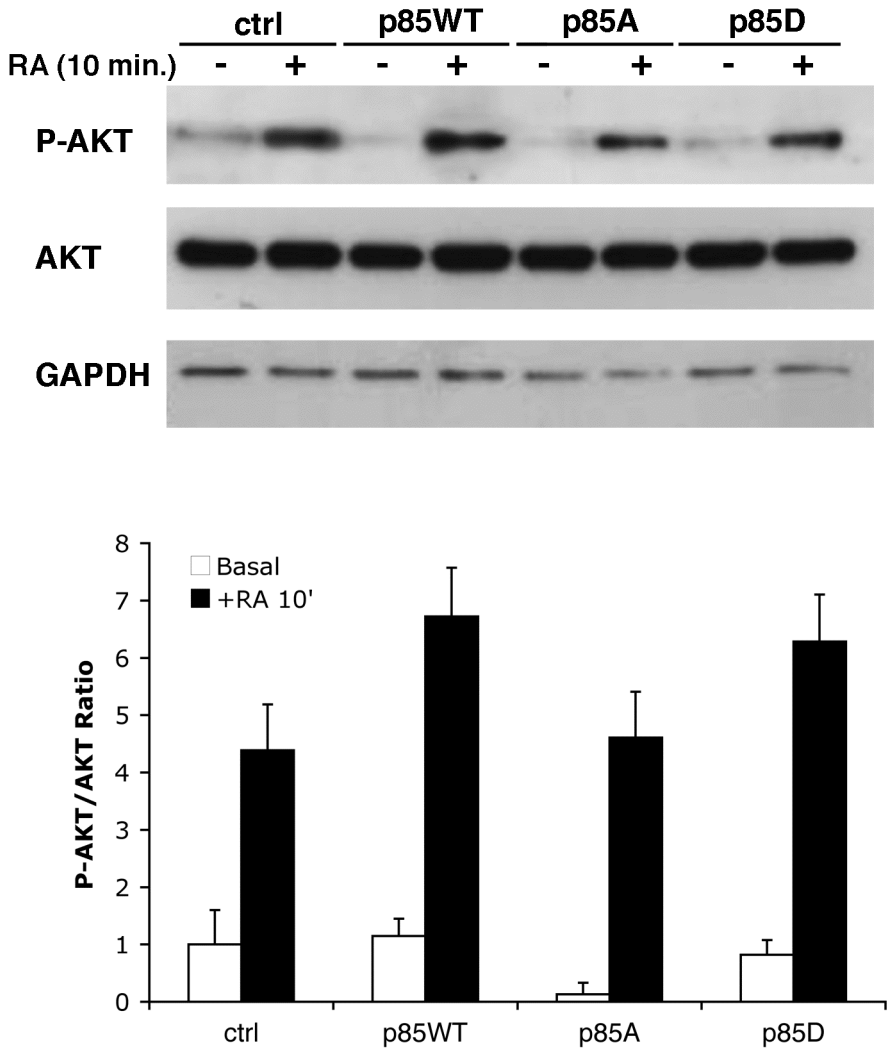

Fig. 14 | Effect of the substitution of p85α^{PI3K} Ser83 on AKT phosphorylation

Quiescent MCF7 cells were transfected with p85α^{PI3K} wild type or p85A or p85D. After 48 h, the cells were treated with 30 μM RA for 10 min. Cell lysates were analysed by Western blot with anti P-AKT Ser473 (upper panel), AKT (middle panel) or GAPDH (lower panel) antibodies. The histogram represents the ratio of P-AKT/AKT derived from three independent experiments.

3. Biological effects following the phosphorylation of p85α^{PI3K} Ser83 in MCF7 cells

To determine the overall survival of MCF7 tumour cells overexpressing the p85α^{PI3K} mutants, clonogenic assays were performed. MCF7 cells (3 x 10^2) transiently transfected with the p85α^{PI3K} mutants were seeded into 6 well plates that were then incubated at 37°C for about 10 days until cells have formed sufficiently large colonies. Clones made by at least 50 cells were counted after fixing with a mixture of 6% glutaraldehyde and 0.5% crystal violet. As shown in **Fig. 15**, overexpression of p85 A induced a statistically significant reduction of the ability of a single cell to grow into a colony (* p< 0.05 p85 A vs empty vector or p85 wt or p85 D).

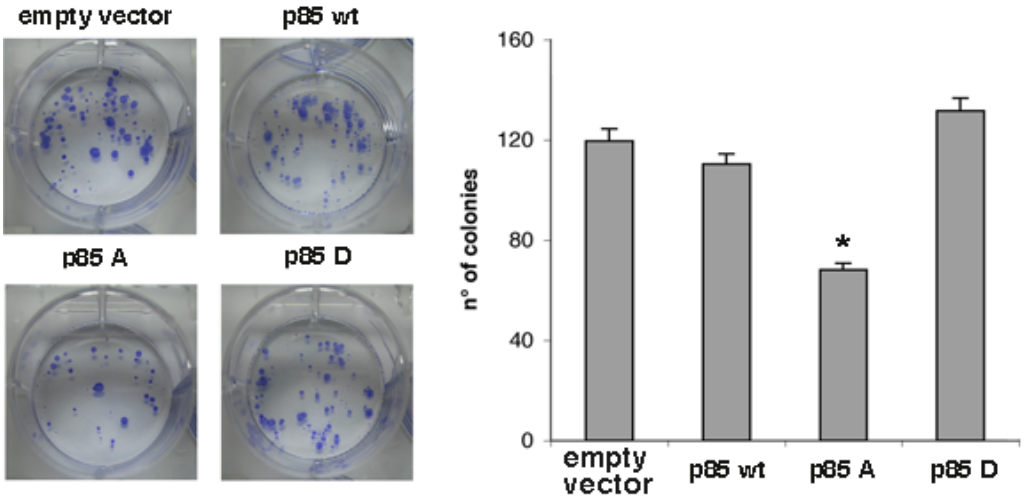

Fig. 15 | p85 A expression influences MCF7 survival
MCF7 cells expressing the p85α mutants were seeded into 6 well plates that were then incubated at 37°C for about 10 days; clones made by at least 50 cells were counted after 30' fixing with a mixture of 6% glutaraldehyde and 0.5% crystal violet. The histogram represents the average number of colonies of four separate experiments. (* indicates a high statistical significance (Student's t-test, P<0.001) compared to empty vector or p85 wt or p5 D).

To investigate the role of p85α^{PI3K} mutants in the control of cell proliferation, growth curves, colorimetric MTT assay and cell cycle analysis by fluorocytometric absorbent cell sorter (FACS) have been performed on MCF7 cells transiently transfected with p85 wt, A or D or with the empty vector. Cells were counted at time 0, at 24 h, at 48 h and at 72 h. **Fig. 16A** demonstrated that overexpression of p85 A caused a lower proliferation rate with respect to p85 wt or p85 D. The trypan blue test performed in all proliferation assays (data not shown) demonstrated that the reduction of proliferation observed in these cells was not due to reduction of cellular viability. Similar results were obtained by colorimetric MTT assay (**Fig. 16B**) and by FACS analysis (**Fig. 16C**).

All together these results demonstrated that p85α^{PI3K} Ser 83 plays an important role in the control of MCF7 cells proliferation.

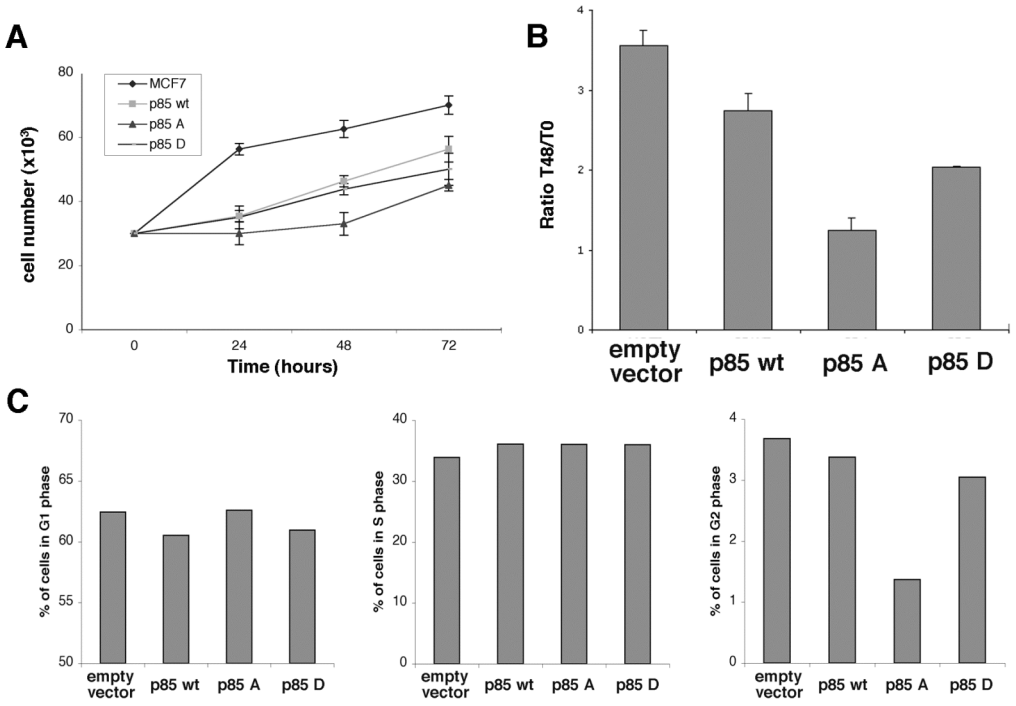

Fig. 16 | p85α^{PI3K} Ser83 is important for MCF7 cell proliferation
A. Cells transiently transfected with empty vector or with p85α mutants were counted three times at time 0, at 24 h, at 48 h and at 72 h after transfection. B. Using the colorimetric MTT assay, cell proliferation was evaluated 48 h after transfection C. Transfected MCF7 cells were serum starved for 12h and then analyzed by FACS to determine the percentage of cells in G1, G2 and S-phase. The data are the mean of three independent experiments.

4. Effect of All Trans Retinoic Acid and cAMP on cell growth and survival in MCF7 cells overexpressing p85α^{PI3K} mutants

cAMP regulates cell proliferation in different manner depending on cell type (Pastan I.H. et al., 1975). In fact, cAMP induces proliferation in such cells as thyroid cell FRTL5 (Lee Y.H. et al., 1998; Ariga M. et al., 2000), while it inhibits proliferation in most cell types, such as NIH 3T3 (Magnaldo I. et al., 1989). It has also been described a differential regulation of vascular smooth muscle and endothelial cell proliferation in vitro and in vivo by cAMP/PKA-activated p85α^{PI3K} (Torella D. et al., 2009).

Retinoic acids, including vitamin A and its analogues, regulate growth and differentiation; they suppress tumour formation in animals and have shown to be effective chemotherapeutic agents in many cancers (Dutta A. et al., 2009). The antitumour effect of retinoids is most often attributed to the induction of differentiation, but these compounds were also shown to arrest the growth of tumour cells by inducing apoptosis or accelerated senescence. (Taraboletti G. et al., 1997). Numerous groups have found that retinoids potently inhibit the growth of breast cancer cell lines (Zhu W.I. et al., 1997; Wang Q. et al., 2000).

The responsiveness to RA and cAMP growth inhibition has been evaluated in MCF7 cells by proliferation assays. The cell growth rate was first analysed by [^{3}H]thymidine incorporation in MCF7 cells overexpressing p85α^{PI3K} mutants that were untreated or treated with RA 30 μM, cAMP 200 μM or both of them for 48 h (**Fig. 17A**).

A consistent inhibition of cell replication was evident in the cells that had undergone RA treatment, with an inhibition of 38%, 43%, 45% and 44%, respectively for MCF7 cells transfected with empty vector, p85 wt, p85 A and p85 D. cAMP treatment was responsible of a stronger reduction of proliferation of the transfected cells (61%, 57%, 65% and 67% of inhibition respectively for the empty vector, p85 wt, p85 A and p85 D). As already

remarked for the p85α/RARα binding, the co-treatment with RA and cAMP also reflect a synergistic effect on inhibiting the growth rate of all the transfected cells (75%, 76%, 80% and 86% respectively). Inhibition of cell growth was evidenced also by growth curves (**Fig. 17B**)..

Differences between treatments were tested for statistical significance using Student's matched pairs t test (*P<0.05; **P<0.001). The differences in the percent of inhibition displayed by the mutants were not significant

Taken together these results indicate that RA and cAMP inhibition of MCF7 cells proliferation in vitro is independent from PKA modification of $p85\alpha^{PI3K}$.

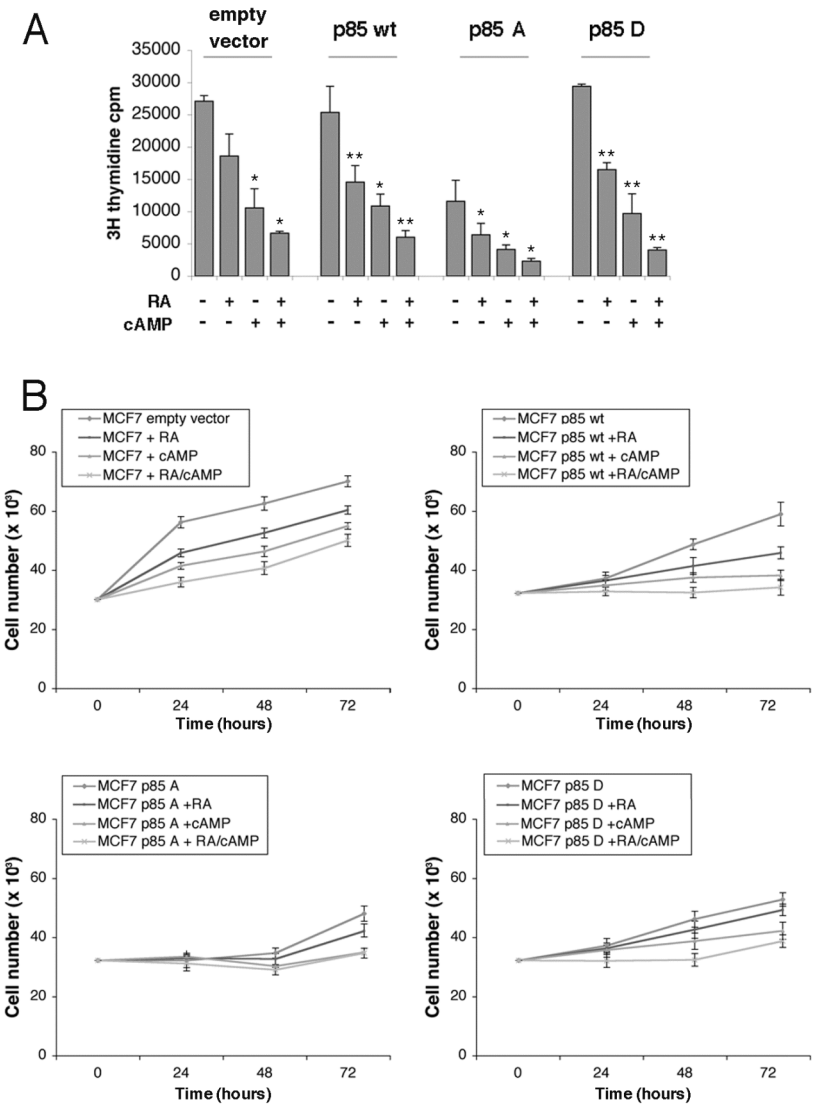

FIG 17 | RA and cAMP inhibit MCF7 cells proliferation in vitro independently from PKA modification of p85α^{PI3K}.

A. Effect of 48 h of RA 30µM, cAMP 200µM or RA/cAMP treatment on [^{3}H]thymidine incorporation of MCF7 cells transiently transfected with the p85α^{PI3K} mutants. B. Cells overexpressing p85 wt, A or D, or the empty vector, grown in the presence or the absence of RA 30µM, cAMP 200µM or RA/cAMP were counted three times by two independent investigators. Inter-observed variation was below 5%. Values represent the mean of triplicate determination ±S.D. of three experiments. (* and ** indicate a statistical significance (Student's t-test), $P<0.05$ and $P<0.001$ respectively).

5. Role of phosphorylation of p85α^{PI3K} Ser83 in the control of migratory behaviour of MCF7 cells

In order to evaluate the role of phosphorylation of p85α^{PI3K} Ser83 in MCF7 cells migration, we performed a wound healing assay. To this end, confluent monolayers of MCF7 cells transiently transfected with empty vector or with p85 wt, p85 A or p85 D wounded with a pipet tip were treated in the presence or absence of RA 30 μM, and wound width was evaluated after 16h of treatment.

As shown in **Fig. 18**, expression of p85A had an effect *per se* on MCF7 migration displaying a statistically significant reduction of migration (89% vs empty vector, 84% vs p85 wt and 88% vs p85 D). p85α^{PI3K} Ser83 was also essential for the reduction of motility induced by RA, since p85 D mutant was insensitive to RA impairment of MCF7 motility.

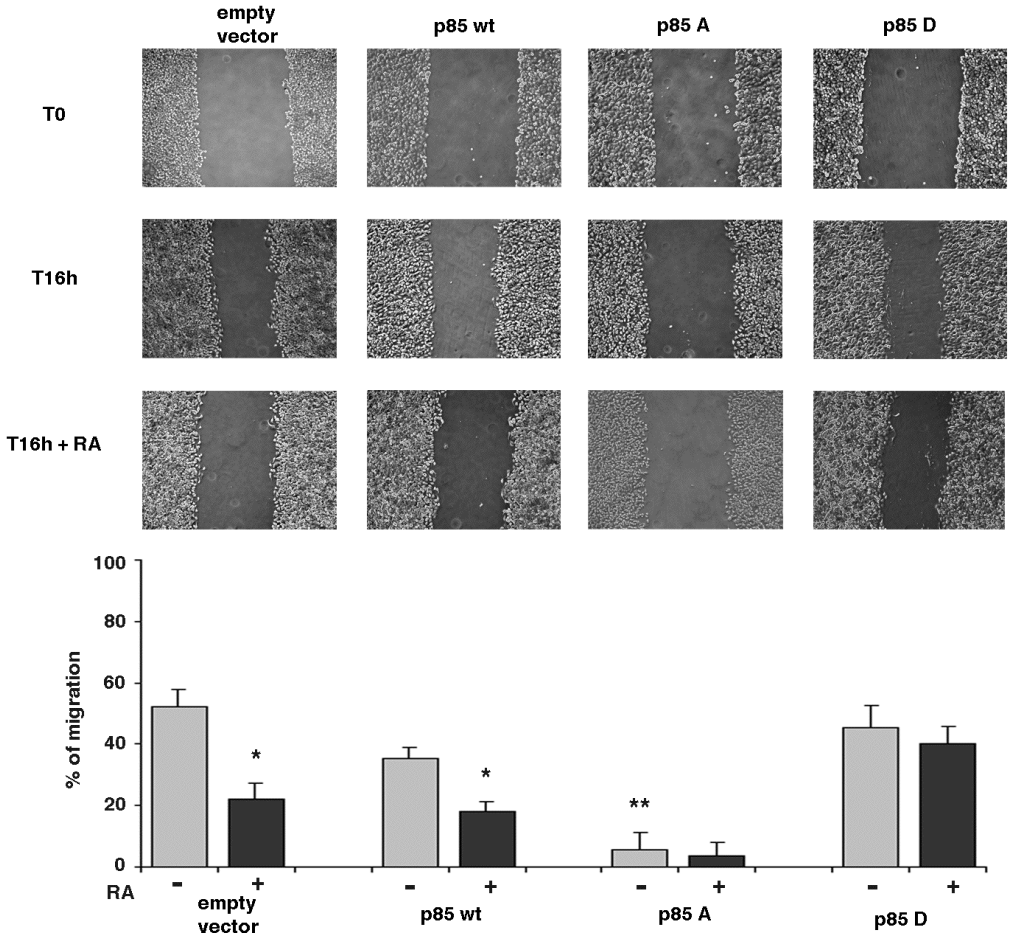

FIG 18. | Migratory behaviour of MCF7 transfected cells
Wound-healing assay was performed in confluent monolayers of MCF7 cells overexpressing p85 mutants untreated or treated with RA 30 µM for 16 h, and the extension of the area colonized by the cells was estimated using Axio Vision, Zeiss software. Histogram represents quantification of the RA treatment effect on cell motility (% of migration) (** indicates a high statistical significance (Student's t-test, $P<0.001$) compared to empty vector or p85 wt or p5 D; * $p< 0.05$ vs basal). Images are representative of three separate experiments.

It has been demonstrated that p85α^{PI3K} interacts with FAK (Focal adhesion kinase) a cytoplasmic tyrosine kinase involved in integrin-mediated signal transduction pathways (Chen H.C. et al., 1996); FAK influences both focal adhesion assembly and disassembly, and is required for the dynamic regulation of integrin focal adhesions during cell migration (Jones R.J. et al., 2000).

To understand the deepest mechanisms grounding the differences in motility displayed by p85α^{PI3K} mutants, we set out to determine the involvement of P-FAK. Quiescent MCF7 cells were transiently transfected with p85α^{PI3K} wt or p85A or p85D and treated with 30 μM RA for 10 min. Cell lysates were immunoprecipitated with anti-p85α antibody and then sequentially analysed by Western Blot with anti-P-FAK (Y397), anti-RARα and anti-p85α antibodies. As shown in **Fig. 19**, in basal condition p85α^{PI3K} wild type was found associated with P-FAK; this association was significantly enhanced in cells expressing p85A and reduced in p85D expressing cells.

In the presence of RA, the formation of P-FAK/PI3K complex was strongly stimulated in all the conditions examined.

These results indicate that phosphorylation of p85α^{PI3K} Ser83 plays an important role in the formation of the PI3K/P-FAK complex, and in the modulation of this binding mediated by RA.

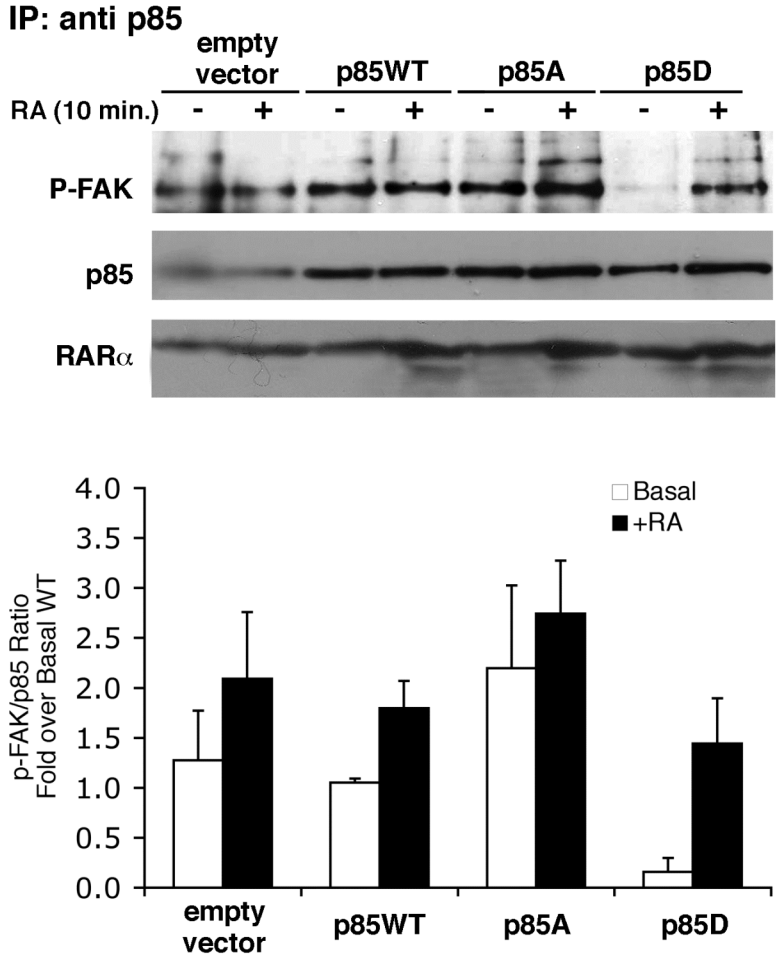

Fig. 19 | Effect of the substitution of p85α^{PI3K} Ser83 on the formation of P-FAK/PI3K complex

MCF7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red and transiently transfected with p85α^{PI3K}-flag wild type, p85A or p85 D; 48h after transfection cells were treated with or without 30 μM RA for 10 min. About 1mg protein from control or RA-treated cell extracts were incubated with 2 ug of p85α antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated using antirabbit IgG beads (SantaCruz), following the manufacturer's instructions, and suspended in sample buffer containing freshly added inhibitors. After Western blot the filter was sequentially developed with anti-p85-PI3K and anti-P-FAK (Y397), using horseradish peroxidase-conjugated Rabbit ExactaCruzF (SantaCruz) as secondary antibodies.

B. The histogram represents the ratio of P-FAK/p85 bands in the p85 immunoprecipitates, derived from three independent experiments. Differences between treatments were tested for statistical significance using Student's matched pairs *t* test: * p<0,01 vs basal.

DISCUSSION

The data presented here indicate that p85α^{PI3K} Ser 83 and the near SH3 domain represent a nodal point modulating information from several signalling cascades converging on PI3K, such as RA and cAMP pathways.

Several studies demonstrated that members of the superfamily of nuclear receptors, such as estrogen receptors (Simoncini T. et al.; Castoria G. et al., 2001; Le Romancer M. et al., 2008) and thyroid receptors (Cao X. et al., 2005), interact with p85α^{PI3K} and activate PI3K/AKT pathway. A stable physical interaction between RARα and p85α has been demonstrated in SH-SY5Y neuroblastoma cells (Masià S. et al., 2007) and in MCF7 cells (Ohashi E., et al., 2009).

It has been already described that the serine residue (Ser83) in the N-terminal/SH3 domain of the p85α^{PI3K} represents the physical link between Ras, estrogen receptor and cAMP–PKA, since its phosphorylation stimulates both the activity of PI3K kinase and its binding to estrogen receptor and PKA RIIβ subunit (Cosentino C. et al., 2007; De Gregorio G., et al., 2007). Virtually, all receptors binding p85α^{PI3K} can cooperate with cAMP–PKA signals via phosphorylation of p85α^{PI3K} Ser83, thus explaining the pleiotropic nature of the effects exerted by cAMP–PKA on several, apparently unrelated, signalling cascades (Cosentino C. et al, 2007).

This work demonstrates that RARα interacts with p85α^{PI3K} independently from PKA-phosphorylation of p85α^{PI3K} Ser83, but this residue plays an important role in the fine regulation of the binding induced by cAMP-PKA. The ability of the cAMP/PKA signalling to synergize with RA has been already shown in different cellular models (Gaillard E. et al., 2006; Parrella E., et al, 2004; Srivastava R.K., et al, 2000). The results presented here demonstrate that the co-treatment of MCF7 cells with RA and cAMP amplified synergistically the

binding between RARα and p85α^{PI3K}, and that the presence of Ser83 in p85α^{PI3K} is crucial for the maintenance of this synergy.

Recent studies highlighted the importance of p85α^{PI3K} Ser83 in the control of cell growth in different cellular models, such as FRTL-5 thyroid cells (De Gregorio G., et al., 2007), NIH3T3 cells (Cosentino C., et al., 2007) and vascular smooth muscle cells (Torella D., et al., 2009), but it is worth noting that the same phosphorylation leads to opposing phenotypes depending on the cell type. In fact the phosphorylation of p85α^{PI3K} Ser83 inhibits cell proliferation in fibroblasts and VSMC, while it is essential for the correct cell cycle progression in thyroid cells, and does not affect cell proliferation of endothelial cells (De Gregorio G. et al., 2007; Cosentino C. et al., 2007; Torella D. et al., 2009). These differences can be explained by the fact that PKA phosphorylating p85α^{PI3K} Ser83 stabilizes the complex Ras-PI3K, triggering to the activation of AKT pathway, that leads to cell survival, growth and proliferation depending on the cell type (Cosentino C. et al., 2007).

Starting from this premise, the results obtained show that phosphorylation of p85α^{PI3K} Ser83 is essential for MCF7 cells survival and proliferation, as demonstrated by the low growth rate observed in MCF7 cells overexpressing the p85A mutant.

Cell proliferation assays demonstrate that RA and cAMP inhibit MCF7 cells proliferation in vitro, as already described in different models (Zhu W.Y. et al., 1997; Wang Q. et al., 2000; Dutta A. et al., 2009; Magnaldo I. et al., 1989); co-treatment cAMP/RA determines a synergistic reduction of proliferation of all the transfected cells. Therefore, despite its role in regulating the physical interaction between p85α^{PI3K} and RARα, Ser83 does not seem to be essential to mediate the inhibitory effects of RA and/or cAMP. Both treatments indeed are able to reduce cell proliferation in MCF7 cells overexpressing p85A,

suggesting the existence of a different pathway, independent from PKA direct phosphorylation of $p85\alpha^{PI3K}$ Ser83.

PI3K isoforms have multiple roles in cell migration in many cell types and systems, but the relative contribution of PI3Ks to different steps of migration depends on the cell state and the combinations of stimuli to which it is exposed (Cain R.J. and Ridley A.J., 2009). Direct PI3K activation is sufficient to disrupt epithelial polarization and induce cell motility and invasion (Keely P.J. et al., 1997). Recent results indicate that PI3Ks act redundantly with multiple other pathways to co-ordinate polarity, and it may be important to optimize polarization and subsequent migration only under certain conditions (Cain R.J. and Ridley A.J., 2009).

To date, the role of $p85\alpha^{PI3K}$ Ser83 in the migratory behaviour of cells has not yet been investigated. This work highlights that the overexpression of p85A induces a highly significant suppression of MCF7 cells motility with respect to p85 wt or p85D ($p<0.01$), indicating that the phosphorylation of this residue is a critical regulator of this process.

Some authors demonstrated that RA and other biologically active retinoids inhibit cellular migration in Airway Smooth Muscle Cells and in MCF7 cells (Day R.M. et al., 2006; Dutta A., et al., 2009).

Wound healing assays demonstrate that the presence of $p85\alpha^{PI3K}$ Ser83 is essential for the reduction of motility induced by RA, since p85D mutant is insensitive to RA impairment of MCF7 motility.

Numerous studies indicate FAK as an important player in signalling cascades associated with cancer progression and metastasis (van Nimwegen M.J. et al., 2007). Increased FAK expression has been correlated with increased cell motility, invasiveness and proliferation (Owens L.V. et al., 1995; Slack J.K., et al., 2001; Wang D. et al., 2000). Moreover, FAK phosphorylation has been demonstrated in different tumour cell types (Aronsohn M.S. et al., 2003;

Kallergi G., et al., 2007b; Recher C. et al., 2004), and several studies demonstrated that p85α^{PI3K} interacts with FAK (Chen et al 1996; Le Romancer M., et al, 2008).

Immunoprecipation experiments performed with anti-p85 antibody and then followed by Western Blot analysis with anti-P-FAK (Y397), anti-RARα and anti p85α antibodies, clearly demonstrate the existence of a ternary complex p85α^{PI3K}/RAR/P-FAK. In basal condition p85α^{PI3K} wt binds P-FAK; this association is significantly enhanced in cells overexpressing p85A and reduced in p85D overexpressing cells. These differences in the ability to bind P-FAK displayed by the p85α^{PI3K} mutants may be responsible of the different migratory behaviour observed in wound healing experiments. In fact, it has been widely demonstrated that the activation of FAK/PI3K/Rac1 signalling inhibits MCF7 cell motility, possibly reflecting differential activation of FAK via phosphorylation of additional sites (Kallergi G. et al., 2007a). To date, six tyrosine phosphoacceptor sites have been identified in FAK, (tyrosines 397, 407, 576, 577, 861 and 925), most of which seem to play positive regulatory roles. (Calalb M.B. et al., 1995; Schlaepfer D.D. et al., 1996). However, phosphorylation of FAK Tyr397 has been found correlated to an inhibited cell migration in a human breast cancer cell line (Wang F. et al., 1999), and in squamous cancer cell lines (Lorch J.H. et al., 2007).

In the presence of RA, the formation of P-FAK/PI3K complex was strongly stimulated in all the conditions examined; this enhancement of the P-FAK/p85 binding may explain the inhibition of migration observed in MCF7 cells after RA treatment. Further investigations are required to understand the discrepancy observed in MCF7 cells overexpressing p85D mutant, where RA treatment on one hand induces the P-FAK/p85α^{PI3K} complex formation, but on the other hand is not able to inhibit migration.

CONCLUSIONS

In conclusion, this study gives evidence of the existence of a complex RARα/PI3K/P-FAK in MCF7 cells, that is fine regulated by phosphorylation of $p85\alpha^{PI3K}$ Ser83 and administration of RA. Changes in binding affinity of this complex are responsible of cell migration impairment.

The data presented above have broad implications since they point to $p85\alpha^{PI3K}$ Ser83 as the physical link between different pathways (cAMP-PKA, RA and FAK), and as an important regulator of MCF7 cells proliferation and migration.

Further work will be necessary to determine the mechanisms by which the complex RARα/PI3K/P-FAK inhibits MCF7 cells motility, in order to evaluate the clinical implication of these findings.

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