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Overexpression of 14-3-3ζ in cancer cells activates PI3K via binding the p85 regulatory subunit

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Abstract

The ubiquitously expressed 14-3-3 proteins regulate many pathways involved in transformation. Previously, we found that 14-3-3ζ overexpression increased Akt phosphorylation in human mammary epithelial cells. Here, we investigated the clinical relevance and molecular mechanism of 14-3-3ζ overexpression-mediated Akt phosphorylation and the potential impact on breast cancer progression. We found that 14-3-3ζ overexpression was significantly ($P = 0.005$) associated with increased Akt phosphorylation in human breast tumors. Additionally, 14-3-3ζ overexpression combined with strong Akt phosphorylation was significantly ($P=0.01$) associated with increased cancer recurrence in patients. In contrast, knockdown of 14-3-3ζ expression by siRNA in cancer cell lines and tumor xenografts reduced Akt phosphorylation. Furthermore, 14-3-3ζ enhanced Akt phosphorylation through activation of PI3K. Mechanistically, 14-3-3ζ bound to the p85 regulatory subunit of PI3K and increased PI3K translocation to the cell membrane. A single 14-3-3 binding motif encompassing serine 83 on p85 is largely responsible for 14-3-3ζ-mediated p85 binding and PI3K/Akt activation. Mutation of serine 83 to alanine on p85 inhibited 14-3-3ζ binding to the p85 subunit of PI3K, reduced PI3K membrane localization and activation, impeded anchorage independent growth and enhanced stress induced apoptosis. These findings revealed a novel mechanism by which 14-3-3ζ overexpression activates PI3K, a key node in the mitogenic signaling network known to promote malignancies in many cell types.

Keywords

14-3-3ζ; breast cancer; PI3K; Akt

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Introduction

The 14-3-3 proteins constitute a family of highly conserved, dimeric proteins found in all eukaryotic organisms (Aitken, 1995). In humans, seven isoforms have been identified (Aitken, 2006). These 29–31 kDa acidic proteins bind to phosphorylated serine/threonine motifs on target proteins (Aitken, 2006; Muslin *et al.*, 1996). 14-3-3 proteins do not possess any catalytic activity; however, they exert their effect by modulating subcellular localization or catalytic activity of target proteins and by mediating formation of protein complexes (Tzivion and Avruch, 2002). 14-3-3 proteins interact with many proteins that potentially regulate a diverse number of cellular processes (Benzinger *et al.*, 2005; Jin *et al.*, 2004; Pozuelo Rubio *et al.*, 2004). Therefore, 14-3-3 proteins potentially regulate multiple pathways involved in various biological functions and tumor progression.

14-3-3 proteins have been reported to inhibit apoptosis (van Hemert *et al.*, 2001). In response to survival signals, 14-3-3s serve as positive regulators of Akt downstream signaling by binding and sequestering the pro-apoptotic proteins, Bad and FOXO/Forkhead, away from their interaction partners and sites of action (Porter *et al.*, 2006; van Hemert *et al.*, 2001). 14-3-3 proteins have also been shown to interact with other survival promoting proteins such as growth factor receptors and phosphoinositide 3-kinase (PI3K) (Bonnefoy-Berard *et al.*, 1995; Craparo *et al.*, 1997; Lonic *et al.*, 2008; Munday *et al.*, 2000; Oksvold *et al.*, 2004). However, the consequence of these interactions, especially related to cancer progression, are not well understood (Porter *et al.*, 2006; Tzivion *et al.*, 2006).

In response to stimuli, predominantly through receptor tyrosine kinases, PI3Ks regulate many of the same cellular processes as 14-3-3 proteins, including apoptosis (Cantley, 2002). Three classes of PI3Ks are activated in response to different stimuli and have different substrate specificities. Class 1A PI3Ks (hereafter referred to generically as PI3K) are the most studied and the only class clearly implicated in human cancers (Vanhaesebroeck *et al.*, 2005). PI3K is composed of two subunits, a regulatory subunit and a catalytic subunit. The p85 regulatory subunits inhibit the enzymatic activity of the p110 catalytic subunits; yet, they are also necessary for activation and stabilization of p110 (Yu *et al.*, 1998). In general, the p85/p110 heterodimer is activated by membrane recruitment to phosphorylated residues on receptor tyrosine kinases or by adapter proteins via SH2 domains in p85 (Cantley, 2002). PI3K activation promotes accumulation of phospho-lipid substrates in the membrane and activation of downstream signaling pathways, including Akt, that control cell growth and survival. Deregulation and activation of the PI3K/Akt pathway has been identified in various cancers and is associated with increased cancer cell survival. Better understanding of the regulatory mechanisms that promote PI3K activation will bring essential insight into PI3K signaling in normal and cancer cells which may facilitate development of effective targeting strategies.

Although 14-3-3 proteins have been indicated to be involved in cancer, evidence linking 14-3-3 proteins directly to oncogenic functions was minimal for canonical 14-3-3 isoforms (β, γ, ε, η, τ and ζ) (Tzivion *et al.*, 2006; Wilker and Yaffe, 2004). Recently, we discovered that 14-3-3ζ overexpression promoted epithelial-mesenchymal transition in mammary

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epithelial cells and enhanced cancer cell survival, suggesting that 14-3-3ζ promotes malignancy through regulation of common signaling pathways/networks necessary for cancer cell survival and growth (Lu *et al.*, 2009; Neal *et al.*, 2009). Interestingly, we found that high expression levels of 14-3-3ζ were associated with increased Akt phosphorylation/activation in human mammary epithelial cells (Danes *et al.*, 2008). Here, we have investigated the clinical relevance of 14-3-3ζ overexpression-mediated Akt phosphorylation and the molecular mechanism by which 14-3-3ζ mediates Akt activation and cell survival in transformed cells. Our data demonstrated that 14-3-3ζ overexpression leads to PI3K/Akt activation by binding to the p85 regulatory subunit of PI3K and increasing PI3K membrane translocation. A single 14-3-3 binding motif at serine 83 on p85 was largely responsible for 14-3-3ζ-mediated PI3K/Akt membrane recruitment and activation. These findings revealed 14-3-3ζ as a novel regulator promoting PI3K activation that contributed to 14-3-3ζ overexpression-mediated cellular transformation and cancer progression.

Results

14-3-3ζ overexpression is associated with increased Akt phosphorylation in human breast cancers

Our previous data indicated that 14-3-3ζ overexpression led to increased Akt phosphorylation and transformation of the non-transformed MCF-10A human mammary epithelial cells, suggesting 14-3-3ζ may be involved in breast cancer development (Danes *et al.*, 2008; Lu *et al.*, 2009). Therefore, we further investigated whether 14-3-3ζ expression was associated with increased Akt phosphorylation in human breast tumors. We have previously determined by immunohistochemical (IHC) analysis the expression status of 14-3-3ζ in breast tumor sections from 121 patients with invasive breast carcinoma (Neal *et al.*, 2009). Thus, we examined the level of Akt phosphorylation in this cohort with known 14-3-3ζ status (Figure 1a). High levels (score 2+) of Akt phosphorylation were significantly associated with 14-3-3ζ overexpression (score 3+) in these primary breast tumors (Chi square, $P = 0.005$) (Figure 1b). Previously, we have found that 14-3-3ζ overexpression in this cohort is associated with reduced disease-free survival (Neal *et al.*, 2009). Here, we investigated whether 14-3-3ζ overexpression combined with high Akt phosphorylation would predict worse clinical outcome than either marker alone. Indeed, patients with breast tumors characterized as both 14-3-3ζ overexpression and high Akt phosphorylation had significantly reduced disease free survival than patients with breast tumors having either 14-3-3ζ overexpression or high Akt phosphorylation alone (Log rank, $P = 0.01$) (Figure 1c). However, both 14-3-3ζ overexpression and high Akt phosphorylation had no significant impact on patients overall survival compared to either 14-3-3ζ overexpression or high Akt phosphorylation alone (data not shown). Thus, high expression of 14-3-3ζ and strong Akt phosphorylation in breast tumors may stratify a subgroup of patients with increased risk for breast cancer recurrence.

To investigate whether 14-3-3ζ overexpression is responsible for Akt phosphorylation in cancer cells, we inhibited 14-3-3ζ expression with siRNA and examined its effect on Akt phosphorylation. Previously, we showed that 14-3-3ζ siRNA treatment of MDA-MB-435 cancer xenografts reduced tumor growth and increased tumor cell apoptosis (Neal *et al.*,

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2009). Here, we examined 14-3-3ζ siRNA treated MDA-MB-435 xenografts for Akt phosphorylation status by immunoblot of tumor lysates and by IHC staining on tissue sections. Akt phosphorylation was reduced in 14-3-3ζ siRNA-treated tumors compared to control siRNA treated xenografts (Supplementary Figure S1a, b). To assure that the reduced Akt phosphorylation resulted from downregulation of 14-3-3ζ in cancer cells by siRNA independent of the tumor microenvironment, we stably infected MDA-MB-435 cancer cells with 14-3-3ζ siRNA-containing lentiviral constructs and examined Akt phosphorylation following serum stimulation *in vitro*. Akt phosphorylation in serum stimulated MDA-MB-435 cells was reduced after 14-3-3ζ downregulation by siRNA compared with controls (Supplementary Figure S1c). Similarly, serum stimulated Akt phosphorylation was also reduced in MDA-MB-231 cancer cells transiently transfected with 14-3-3ζ siRNA (Supplementary Figure S1d). These data indicated that 14-3-3ζ expression played an important role in Akt activation in cancer cells.

14-3-3ζ enhanced PI3K activity by increasing PI3K membrane localization

We next explored the molecular mechanism of 14-3-3ζ overexpression-mediated Akt activation. Increased Akt phosphorylation by 14-3-3ζ could result from a direct interaction with Akt or through activation of its upstream signaling pathways. Since we did not detect an interaction between 14-3-3ζ and Akt in cancer cells (data not shown), we focused our investigation on the effects of 14-3-3ζ overexpression on PI3K activation, the major upstream signaling event leading to Akt phosphorylation. Indeed, high expression of 14-3-3ζ in MCF10A stable transfectants (MCF10Aζ) (Danes *et al.*, 2008) enhanced PI3K activity compared to control cells (MCF10A.vec) (Figure 2a). Thus, 14-3-3ζ overexpression can lead to increased PI3K activation in these cells.

Membrane recruitment of PI3K can lead to PI3K activation (Cantley, 2002). To investigate whether increased PI3K activation by 14-3-3ζ overexpression was due to increased recruitment of PI3K to the membrane, membrane-bound proteins were separated from cytosolic proteins in cell lysates from MCF10Aζ and MCF10A.vec control cells and examined for p85 and p110 proteins by western blotting. Both p85 and p110 were enriched in the membrane-bound protein pool in the 14-3-3ζ overexpressing MCF10Aζ cells as compared to MCF10A.vec control cells (Figure 2b). Additionally, immunfluorescent staining showed increased p85 and p110 membrane localization in MCF10Aζ cells compared to MCF10A.vec control cells (Figure 2c and Supplementary Figure S2). These data indicated that 14-3-3ζ overexpression led to increased PI3K activation by increasing PI3K membrane localization.

14-3-3ζ binds to and forms a complex with PI3K

14-3-3 proteins frequently modulate protein subcellular localization by binding to target proteins. Therefore, we examined whether 14-3-3ζ promotes PI3K membrane localization and activation by binding to PI3K in cells. Indeed, immunoprecipitation of HA-14-3-3ζ from MCF10Aζ cells readily pulled down the p85 subunit of PI3K (Figure 2d). Similarly, association of endogenous 14-3-3ζ and p85 could be detected in MCF7 cells by immunoprecipitation of 14-3-3ζ or p85, indicating interaction *in vivo* at physiological protein levels (Figure 2e). Notably, 14-3-3ζ co-existed in a complex comprising both p85

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and p110 subunits of PI3K (Figure 2d and Supplementary Figure S3a). These data indicated that 14-3-3ζ formed a complex with PI3K and recruited PI3K to the cell membrane where PI3K became activated.

14-3-3ζ association with PI3K is mediated by serine 83 phosphorylation on p85

14-3-3 association with target proteins usually occurs through a 14-3-3 consensus binding motif centered around a phosphorylated serine residue on the target protein. To map the site of 14-3-3ζ interaction on p85, we examined the p85 protein sequence and identified three potential 14-3-3 binding motifs in the p85α isoform, which were not present in the p85β isoform (Supplementary Figure S3b). One of these sites, surrounding serine 83 (S83), resembles a mode 2 consensus binding site for 14-3-3, and the other two sites, surrounding serine 154 and serine 231, resemble mode 1 consensus sites (Supplementary Figure S3b) (Yaffe *et al.*, 1997). To determine which of these sites are responsible for 14-3-3ζ binding to p85, we generated p85 proteins with mutations in the mode 2 consensus binding site (p85$^{S83\mu}$) or mutations in either or both of the mode 1 consensus binding sites (p85$^{S154\mu}$, p85$^{S231\mu}$, p85$^{S154/231\mu}$) and stably transfected these histidine/Xpress-tagged p85 mutants or wild type p85 (p85^{WT}) into MCF7 cells overexpressing HA-14-3-3ζ (MCF7ζ) (Supplementary Figure S3c and data not shown). Binding of p85$^{S83\mu}$ to 14-3-3ζ was reduced compared to p85^{WT} (Figure 3a). In contrast, p85$^{S154\mu}$, p85$^{S231\mu}$, and p85$^{S154/231\mu}$ bound to 14-3-3ζ at levels similar to p85^{WT} (Supplementary Figure S3d and data not shown). The data indicated that 14-3-3ζ binding to p85 occurs largely through the phospho-serine 83 motif and that phosphorylation of serine 83 on p85 is necessary for 14-3-3ζ binding.

To confirm that the serine 83 site of p85 is phosphorylated in cells *in vivo*, we raised an antibody against a phospho-peptide corresponding to the 14-3-3 binding motif in p85 surrounding serine 83 (p85-P-S83). Immunoprecipitation using p85-P-S83 antibody followed by western blotting with p85-P-S83 antibody demonstrated that the p85-P-S83 antibody recognized serine 83 phosphorylated p85 *in vivo* (Figure 3b, *left*). The serine 83 phosphorylation signal was reduced following treatment with calf intestine alkaline phosphatase (CIAP) both in p85-P-S83 immunoprecipitates and in total cell lysates (Figure 3b, *right*). Additionally, the p85-P-S83 antibody immunoprecipitated p85^{WT} but not p85$^{S83\mu}$ from transiently transfected cells (Figure 3c). The data indicated that p85 is phosphorylated on serine 83 *in vivo* in these cell lines.

We next attempted to identify the kinase responsible for phosphorylation of serine 83 on p85. We implemented an unbiased approach by fractionating MCF7 cell lysates using a glycerol gradient. We collected fourteen fractions and identified molecular weight ranges of the proteins in each fraction (Supplementary Figure S4a). Combined fractions (A, B, C, and D) were used for *in vitro* kinase assays using a GST tagged p85 peptide spanning amino acids 77-101 (GST-p85 1) or GST only as substrates. We found that fraction B contained a kinase(s) (size range 35 –70 kDa) capable of phosphorylating the GST-p85 1 peptide (Supplementary Figure S4b). While we were working on the purification of the kinase in fraction B, it was reported that serine 83 on p85 can be phosphorylated by the 40 kDa protein kinase A (PKA) within the size range of fraction B (Cosentino *et al.*, 2007). Therefore, we tested PKA for phosphorylation of serine 83 on p85 by *in vitro* kinase assays

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using purified PKA and GST fused p85 peptide spanning amino acids 50–109 (GST-p85 2) with either wild type serine 83 (WT) or serine 83 mutated to alanine (SA) as substrate. Indeed, PKA phosphorylated p85^{WT} but not p85^{SA} (Figure 3d). These data demonstrated and confirmed that p85 was specifically phosphorylated on serine 83 by PKA.

Mutation of the 14-3-3 binding motif on p85 decreased PI3K membrane localization and activity

Our data have indicated that 14-3-3ζ overexpression increased PI3K membrane localization and activity (Figure 2b, c and Supplementary Figure S2). We next investigated whether 14-3-3ζ binding to p85 through the phospho-serine 83 motif is important for the recruitment of PI3K to the membrane in 14-3-3ζ overexpressing cells. To this end, we collected cell lysates from MCF7ζ cells expressing either p85^{WT} or p85$^{S83\mu}$, separated membrane-bound proteins from cytosolic proteins, and examined p85 and p110 subcellular localization by western blot. Compared to MCF7ζ.p85^{WT} cells, MCF7ζ.p85$^{S83\mu}$ cells had reduced p85 and p110 membrane localization (Figure 4a). Furthermore, immunofluorescent staining in MCF7ζ.p85^{WT} and MCF7ζ.p85$^{S83\mu}$ cells also demonstrated that p85$^{S83\mu}$ with mutation of the 14-3-3 binding motif had a reduced membrane localization compared to p85^{WT} (Supplementary Figure S5). These data indicated 14-3-3ζ binding to p85 on phospho-serine 83 contributed to the increased PI3K membrane localization in 14-3-3ζ overexpressing cancer cells.

We next investigated whether 14-3-3ζ binding to the phospho-serine 83 motif of p85 is required for 14-3-3ζ-mediated PI3K activation. PI3K kinase assays showed that increasing p85^{WT} expression in 14-3-3ζ high-expressing MCF7ζ breast cancer cells led to activation of PI3K and increased Akt phosphorylation, whereas p85$^{S83\mu}$ has lost these functions (Figure 4b). Although p85^{WT} and p85$^{S83\mu}$ expression did not significantly affect basal PI3K activity in 14-3-3ζ low-expressing MCF10A.vec cells, expression of p85^{WT} in 14-3-3ζ high-expressing MCF10Aζ cells induced PI3K activation, indicating p85^{WT} is involved in 14-3-3ζ high-expression-mediated PI3K activation (Figure 4c). However, expression of the 14-3-3ζ-binding defective p85$^{S83\mu}$ mutant in 14-3-3ζ high-expressing MCF10Aζ cells failed to induce PI3K activation compared to p85^{WT}, suggesting that the phospho-serine 83 motif is needed for 14-3-3ζ-mediated PI3K activation (Figure 4c). Furthermore, mouse embryonic fibroblast (MEF) cells from the p85 knockout mouse reconstituted with p85^{WT} had increased serum-induced Akt phosphorylation compared to p85 knockout MEF cells reconstituted with vector control, while p85 knockout MEFs reconstituted with p85$^{S83\mu}$ did not have the same effect (Figure 4d). To further determine whether single mutation of serine 83 (p85^{S83A}) was sufficient to disrupt 14-3-3ζ mediated PI3K/Akt activation, we transiently transfected the p85^{WT}, p85$^{S83\mu}$, and p85^{S83A} constructs into MCF7ζ cells and compared their impact on Akt phosphorylation/activation. Indeed, the p85^{S83A} single mutant reduced Akt activation as effectively as p85$^{S83\mu}$ (Supplementary Figure S6). Thus, 14-3-3ζ binding to p85 via the phospho-serine 83 motif is important for 14-3-3ζ mediated membrane translocation and activation of PI3K/Akt pathway.

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Inhibition of 14-3-3ζ binding to p85 reduced transformation-related properties in cancer cells

Deregulation of the PI3K/Akt signaling pathway contributes to the pathogenesis of multiple human cancers. Since 14-3-3ζ binding to the serine 83 motif on p85 contributed to PI3K activation and increased Akt phosphorylation, we next investigated whether 14-3-3ζ binding to serine 83 on p85 contributes to transformation-related properties of cancer cells (Figure 5). Indeed, $p85^{S83\mu}$ expression in MCF7ζ cells led to a reduced cell proliferation and an increased apoptosis under low serum conditions compared to the control cells expressing $p85^{WT}$ (Figure 5a, b). In addition, $p85^{S83\mu}$ expression reduced anchorage independent growth of MCF7ζ cells compared to the $p85^{WT}$ expressing cells (Figure 5c). These data suggested that 14-3-3ζ binding to serine 83 on p85 contributes to 14-3-3ζ-mediated transformation through PI3K/Akt activation.

We previously reported that 14-3-3ζ overexpression in MCF10A cells reduced p53 protein levels via Akt-MDM2 mediated degradation and induced epithelial to mesenchymal transition (EMT) via TGFβ pathway (Danes *et al.*, 2008; Lu *et al.*, 2009). We further investigated whether either phenotype was modulated by 14-3-3ζ activation of PI3K. First, we investigated the p53 levels in the MCF7 (with wt p53) panel of transfectants (as shown in Figure 5d). Indeed, Akt phosphorylation was increased in MCF7.ζ cells compared to MCF7.vec cells and the increased Akt phosphorylatioin was associated with reduced p53 protein levels in MCF7.ζ cells. Conversely, expression of $p85^{S83\mu}$ in MCF7.ζ cells reduced Akt phosphorylation and partially rescued p53 protein levels (Figure 5d). Similarly, $p85^{S83\mu}$ expression in MCF10Aζ cells also inhibited Akt phosphorylation and partially rescued p53 protein levels (Supplementary Figure S7a). Furthermore, treatment of MCF10Aζ cells with the PI3K inhibitor LY290042 also rescued p53 protein levels (Supplementary Figure S7c) (Danes *et al.*, 2008). These data indicated that 14-3-3ζ mediated p53 degradation in MCF7 and MCF10A cells is primarily regulated by PI3K/Akt activation. Second, to test whether 14-3-3ζ-mediated PI3K/Akt activation contributes to the induction of EMT, we stably expressed in MCF10Aζ cells $p85^{WT}$ or $p85^{S83\mu}$ using a lentiviral expression system. Expression of $p85^{S83\mu}$ in MCF10Aζ cells inhibited 14-3-3ζ mediated Akt activation but did not reverse the EMT phenotype (Supplementary Figure S7a, b). To further investigate if PI3K-Akt pathway activation contributes to 14-3-3ζ-dependent EMT phenotype in MCF10A cells, we also treated MCF10Aζ cells with the PI3K inhibitor LY294002. LY294002 effectively inhibited 14-3-3ζ-mediated Akt activation but did not reverse the EMT phenotype (Supplementary Figure S7c, d). These data indicated that EMT in MCF10A.ζ cells is independent of the PI3K-Akt pathway but is primarily dependent on the TGFβ-smad-SIP1 signaling pathway as we previously reported (Lu *et al.*, 2009).

Discussion

Our data highlighted a novel mechanism underlying the potential oncogenic function of 14-3-3ζ, whereby, 14-3-3ζ binding to p85 on the phospho-serine 83 motif in the N-terminal domain of p85 contributes to PI3K membrane localization, PI3K activation, and cancer cell proliferation and survival. 14-3-3 proteins impact multiple pathways implicated in tumorigenesis; however, it is not clear which 14-3-3ζ interacting proteins or regulated

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pathways may contribute to cellular transformation. The current study using the $p85^{S83\mu}$ mutant demonstrated that 14-3-3ζ binding to the phospho-serine 83 motif on p85 plays an important role in activation of the PI3K/Akt pathway. We demonstrated that mutation of the 14-3-3-binding motif on p85 effectively reduced PI3K activation and impaired many general properties of cellular transformation. Although other mechanisms may contribute to 14-3-3ζ's tumor-promoting function, our data demonstrated that 14-3-3ζ activation of PI3K could serve as a target for multiple cancer types.

Our data demonstrates an interaction between 14-3-3ζ and p85 in breast cancer cells. In addition, 14-3-3ζ was also found to interact with the p85 subunit of PI3K in platelets and 14-3-3τ was found to interact with the p110 subunit of PI3K in T cells; however, neither study defined an interaction site for 14-3-3 on either PI3K subunit (Bonnefoy-Berard *et al.*, 1995; Munday *et al.*, 2000). The sequence surrounding serine 231 is not highly conserved among species, and mutation of serine 231 and serine 154 in our study did not impact 14-3-3ζ binding to p85. In contrast, the serine 83 motif is highly conserved and mutation of this motif abrogated 14-3-3ζ binding, indicating that the serine 83 motif on p85 is likely the major motif mediating 14-3-3ζ binding. However, we do not rule out that other sites may also contribute to overall 14-3-3 binding. We have also demonstrated that 14-3-3ζ overexpression could activate PI3K in breast cancer cells. In contrast, 14-3-3τ overexpression reduced PI3K activity in activated T cells (Bonnefoy-Berard *et al.*, 1995). Interestingly, i*n vitro* experiments utilizing platelet cytosol determined the effect of recombinant 14-3-3ζ on PI3K activity was biphasic. At low concentrations, 14-3-3ζ inhibited PI3K, but at higher concentrations, 14-3-3ζ enhanced PI3K activity (Munday *et al.*, 2000). Signaling pathways are highly dynamic and have well established dose dependent responses. It is reasonable to speculate that activation of PI3K by 14-3-3ζ in cancer cells is a consequence of the 14-3-3ζ overexpression above a threshold level. Additional cofactors and stimuli in different cell types may also affect the interaction of 14-3-3ζ and p85, thereby changing PI3K activity and downstream biological processes.

Because the interaction between 14-3-3ζ and p85 is dependent on phosphorylation of serine 83 on p85, it is important to study the regulation of serine 83 phosphorylation. As we were investigating along this line, protein kinase A (PKA) was identified to phosphorylate serine 83 on p85 in response to thyroid hormone stimulation in non-transformed thyroid cells (Cosentino *et al.*, 2007; De Gregorio *et al.*, 2007), which supports our finding that serine 83 on p85 was phosphorylated *in vivo*. Here, we confirmed that PKA could phosphorylate serine 83 on p85 and that serine 83 phosphorylation contributes to 14-3-3ζ-mediated transformation of cancer cells. These data suggest differential regulation of p85 serine 83 phosphorylation, and thus differential regulation of PI3K signaling, between non-transformed cells and 14-3-3ζ overexpressing cancer cells. Our data also raise questions as to whether 14-3-3ζ binding to serine 83 of p85 controls other cellular process and disease states dependent on PI3K. The PI3K pathway not only impacts cancer, but is also involved in immunity, obesity, diabetes, energy balance, angiogenesis, hematopoiesis, and stem-cell self-renewal, as well as the survival and function of neurons, heart, bone, muscle, and liver tissue (Katso *et al.*, 2001; Kim *et al.*, 2005; Vanhaesebroeck *et al.*, 2005). Further

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investigation is needed to determine whether the ubiquitously expressed 14-3-3ζ modulates these important biological functions by targeting and activating PI3K.

Development of clinically applicable, specific inhibitors of the PI3K/Akt pathway is a current focus in the area of targeted therapy. In our patient cohort, high levels of both 14-3-3ζ expression and Akt phosphorylation in tumors was associated with breast cancer recurrence (Fig. 1), indicating that the14-3-3ζ/PI3K interaction is important in breast cancer progression and interfering with this interaction may provide clinical benefit. Directly targeting 14-3-3ζ may lead to toxic side effects due to a potential role of 14-3-3 in normal cellular homeostasis. However, disrupting the interaction of 14-3-3ζ and p85 by targeting the 14-3-3/p85 interface may be a drugable target since our data demonstrated that mutation of serine 83 on p85 reduced transformation-related properties of cancer cells. Understanding the dynamic regulation of 14-3-3ζ interaction with p85 may allow development of effective therapies targeting this pathway.

14-3-3 binding to target proteins may act as a "molecular anvil" to induce a conformational change in the target protein, which may lead to enhanced interactions with other proteins, post-translational modifications, or altered subcellular localization (Yaffe, 2002). We showed that 14-3-3ζ binding to phospho-serine 83 on the p85 subunit of PI3K increased PI3K membrane localization in cancer cells. The N-terminal domain of p85 is known to have a negative regulatory function on PI3K activity that is relieved by receptor tyrosine kinase interaction (Yu *et al.*, 1998). Similarly, 14-3-3ζ binding to serine 83 in the N-terminal domain may relieve the inhibitory function of p85, facilitate the interaction of PI3K with other adapter molecules, or enhance membrane localization and activity. This suggests 14-3-3ζ overexpression and serine 83 phosphorylation on p85 could represent alternative mechanisms to PI3K gene mutations or PTEN-loss to activate PI3K pathway in cancer. Interestingly, although MCF-7 cells harbor an activating mutation in exon 9 of p110 that makes PI3K activation independent of p85, our data showed that mutation of serine 83 on p85 could still reduce PI3K membrane localization and activation in MCF-7 cells. This suggested that serine 83 phosphorylation on p85 may define a novel regulatory mechanism of PI3K activation. This model implies that targeting 14-3-3ζ or pathways regulated by 14-3-3ζ may be effective anti-cancer strategies in patients whose tumors overexpress 14-3-3ζ.

Materials and Methods

Patient Information and Immunohistochemical staining

Clinical data and patient sample information has been previously described (Neal *et al.*, 2009). Briefly, after deparaffinization and rehydration, sections were subjected to heat-induced epitope retrieval in 0.01 mM citrate buffer (pH6.0). Endogenous peroxidase activity was blocked for 30 min in 3% hydrogen peroxide. Nonspecific binding was blocked by treatment with blocking reagent (DAKO, Carpinteria, CA, USA) for 30 minutes at room temperature. The slides were then incubated with 14-3-3ζ polyclonal antibody (C-16; Santa Cruz, Santa Cruz, CA, USA) or phospho-Akt Ser473 (Cell Signaling, Danvers, MA, USA) at 4°C overnight. Immunodetection was performed using streptavidin-biotin detection with the LSAB2 system (DAKO). 3-3'-diaminobenzidine was used for color development and

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hematoxylin was used for counterstaining. 14-3-3ζ and phospho-Akt Ser473 staining was scored as positive when more than 10% of tumors cells showed staining for the marker. Levels of staining (0, 1+, 2+, 3+) were scored based on staining intensity within the tumor sample.

Constructs, Cell Lines and Antibodies

MCF7 cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) with plasmids expressing hemagglutinin-tagged 14-3-3ζ (pcDNA3-HA-14-3-3ζ) or empty vector as control (pcDNA3) and selected with neomycin. MCF7ζ cells were transfected with plasmids (pcDNA4/HisC) (Invitrogen) expressing polyhistidine/Xpress tagged wild type p85 ($p85^{WT}$), p85 with a double mutation (S83A, P85T) in the mode 2 consensus binding site ($p85^{S83\mu}$), p85 with a single mutation (S83A) in the mode 2 consensus binding site ($p85^{S83A}$) or mutations (S154A, S231A) in both mode 1 consensus binding sites ($p85^{S154/231\mu}$). MCF7ζ.p85 stable transfectants were selected with Zeocin (Invitrogen). MCF10A.vec and MCF10Aζ cells were described previously (Danes *et al.*, 2008). MCF10Aζ cells were infected with pLove (Addgene, Cambridge, MA, USA) lentiviral constructs expressing untagged $p85^{WT}$ and $p85^{S83\mu}$ (pLove-$p85^{WT}$ and pLove-$p85^{S83\mu}$). MCF-7, MCF10A, and HEK 293T cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and the MDA-MB-435 cell line was obtained from Dr. Janet Price, UT M. D. Anderson Cancer Center. The $p85\alpha^{-/-}$, $p85\beta^{-/-}$ knockout MEFs were obtained from Dr. Lewis Cantley, Harvard Medical School (Brachmann *et al.*, 2005) The His/Xpress-p85 mutants were generated using Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's protocol and confirmed by DNA sequencing. Antibodies used for immunoprecipitation and immunoblot were: PY20, ADH, 14-3-3ζ and His (Santa Cruz); β actin (Sigma, St. Louis, MO, USA); p85 (Millipore, Billerica, MA, USA); p110, Akt and phospho-Akt Ser473 (Cell Signaling); Xpress (Invitrogen); HA (Covance, Princeton, NJ, USA) and ErbB2 (Lab Vision, Fremont, CA, USA). Antibodies used for immunofluoresence assays were: p110 (Millipore); HA (Roche, Indianapolis, IN, USA) and p85 (Millipore). The anti-p85-phospho-Ser83 antibodies were affinity purified from the serum of rabbits injected with the IGRKKIpSPPTPK peptide by Zymed Laboratories (San Francisco, CA, USA)

RNA Interference of 14-3-3ζ

Transient transfection of oligo duplex siRNA has been previously described (Neal *et al.*, 2009). Stable transfection of 14-3-3ζ shRNA in MDA-MB-435 was done using the Mission 14-3-3 shRNA lentiviral system according to the manufacturer's protocol (Sigma).

Phosphatidylinositol 3-Kinase Assay

The PI3-kinase assay was performed as previously described with minor modifications(Nagata *et al.*, 2004). Cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10 mM Na4P2O7, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO, 10 μg/ml aprotinin, and 10 μg/ml leupeptin on ice for 30 min. Immunoprecipitation was performed on clarified cell lysates (15,000 rpm for 15 min at 4°C) using PY20, Xpress or

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p85 antibodies for 1.5 hours followed by protein G-sepharose beads (Roche) for 1.5 hours. Immune complexes were then washed several times with a final wash in kinase buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA). Beads were resuspended in 50 μl of kinase buffer containing 0.2 mg/ml of phosphatidylinositol (sonicated 15 min at 4°C) (Sigma), 20 μCi of [-P]ATP and 20 mM MgCl2 for 10 minutes at room temperature. Reactions were terminated by adding 150 μl of chloroform/methanol/11.6 M HCl (50:100:1), and phosphatidylinositol was extracted with 100 μl of chloroform. The organic phase was washed with methanol/1 M HCl (1:1) and lyophilized. Phosphatidylinositol, resuspended in 15 μl of chloroform, was spotted on a silica gel 60 thin layer chromatography plate and resolved in chloroform/methanol/28% ammonium hydroxide/water (86:76:10:14) for 45 min. Phosphorylated products were visualized by autoradiography and quantified by the Fujix Bio-Imaging Analyzer Bas 1000.

Membrane/Cytoplasm Separation

Cytosolic proteins were extracted with 0.05% Saponin (Sigma) buffer followed by membrane protein extraction in triton X-100 containing lysis buffer as previously described (Nagata *et al.*, 2004).

Imunofluoresence assays

Assays were performed as previously described (Danes *et al.*, 2008). Confocal microscopy was done using an Olympus FV300 laser scanning confocal microscope.

Immunoprecipitation and Phosphatase Treatment

Cells were harvested in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X) supplemented with protease cocktail inhibitor, 20 mM NaF, 1 mM Na_3VO_4 and 20 mM β-glycerophosphate (Sigma). Immunoprecipitation was performed by preclearing total cell lysate with control IgG antibody and protein G agarose (Roche) followed by incubation of immunoprecipitation antibody overnight. Protein G agarose beads were then added and incubated for 2 hours followed by four washes with lysis buffer. The immunocomplexes were subjected to Western analysis. When performing CIAP treatment, 1mg of cell lysate was immunoprecipitated with anti-p85-P-S83 antibody and then treated with 40U CIAP (New England Biolabs, Ipswich, MA, USA) for 1 hour at 30°C.

Glycerol Gradient Sedimentation and In vitro Kinase Assays

Glycerol gradient separation was performed as previously described with minor modifications (Barboric *et al.*, 2005). Briefly, MCF7 cell lysates were fractionated on a 3% to 15% gradient. After fractionation, lysates were diluted in kinase buffer and incubated with GST bound p85 proteins at 35oC for 30 minutes. Reactions were washed, separated on SDS-PAGE gels and subjected to autoradiography. Protein kinase A reactions were performed for 30 min at 30°C using the recombinant catalytic subunit of PKA (Millipore) in a reaction buffer containing 10 mM MOPS, pH 7.0, 30 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM ATP and 10 μCi [γ^{32}P-ATP] in the presence of GST- p85 fusion proteins.

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Growth Rate, Soft Agar Colony Formation and TUNEL Assay

Assays were performed as previously described with minor modifications (Tan *et al.*, 1997). Apoptosis was analyzed using the APO-BRDU (Phoenix Flow Systems, San Diego, CA, USA) staining kit following the manufacturer's protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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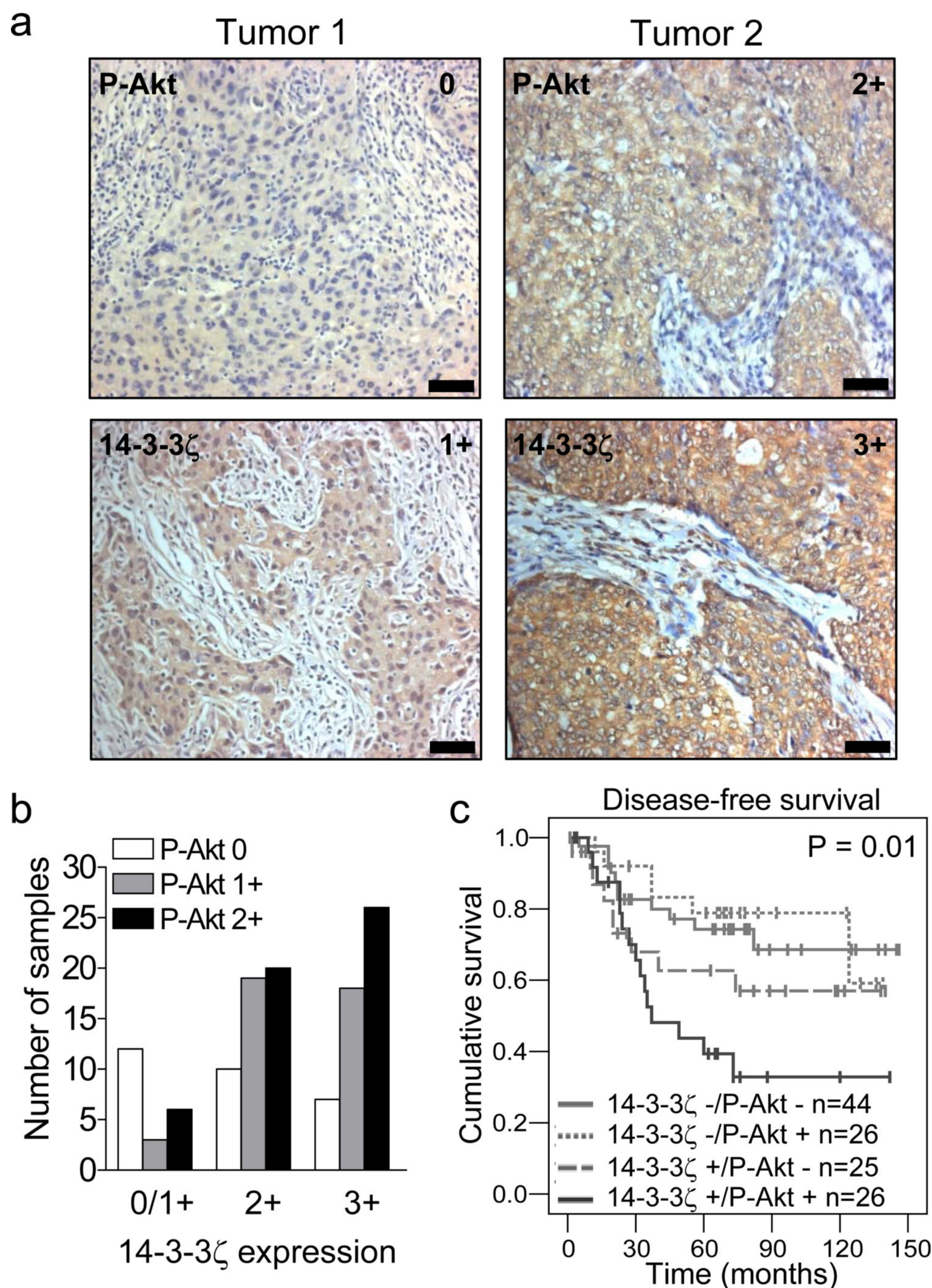

Figure 1. Overexpression of 14-3-3ζ is associated with increased Akt phosphorylation

(**a**) Representative immunohistochemical (IHC) staining of phospho-Akt Ser473 (P-Akt) (IHC score: 0, negative; 1+, moderate positive; 2+, strong positive) and 14-3-3ζ (0/1+, weak positive; 2+, moderate positive; 3+, strong positive) in matched breast cancer specimens. Bar represents 50 μm.

(**b**) Graphical representation of 14-3-3ζ expression correlated with levels of phospho-Akt Ser473 (P-Akt) by immunohistochemical staining in breast cancer specimens (n=121) (Chi square, P = 0.005).

(**c**) Disease-free survival rates of patients examining combined 14-3-3ζ expression and phospho-Akt Ser473 expression. 14-3-3ζ scores 0, 1+, and 2+ were considered negative (–) and 3+ was considered positive (+). P-Akt scores 0 and 1+ were considered negative (–) and 2+ was considered positive (+). P value was determined by log rank analysis.

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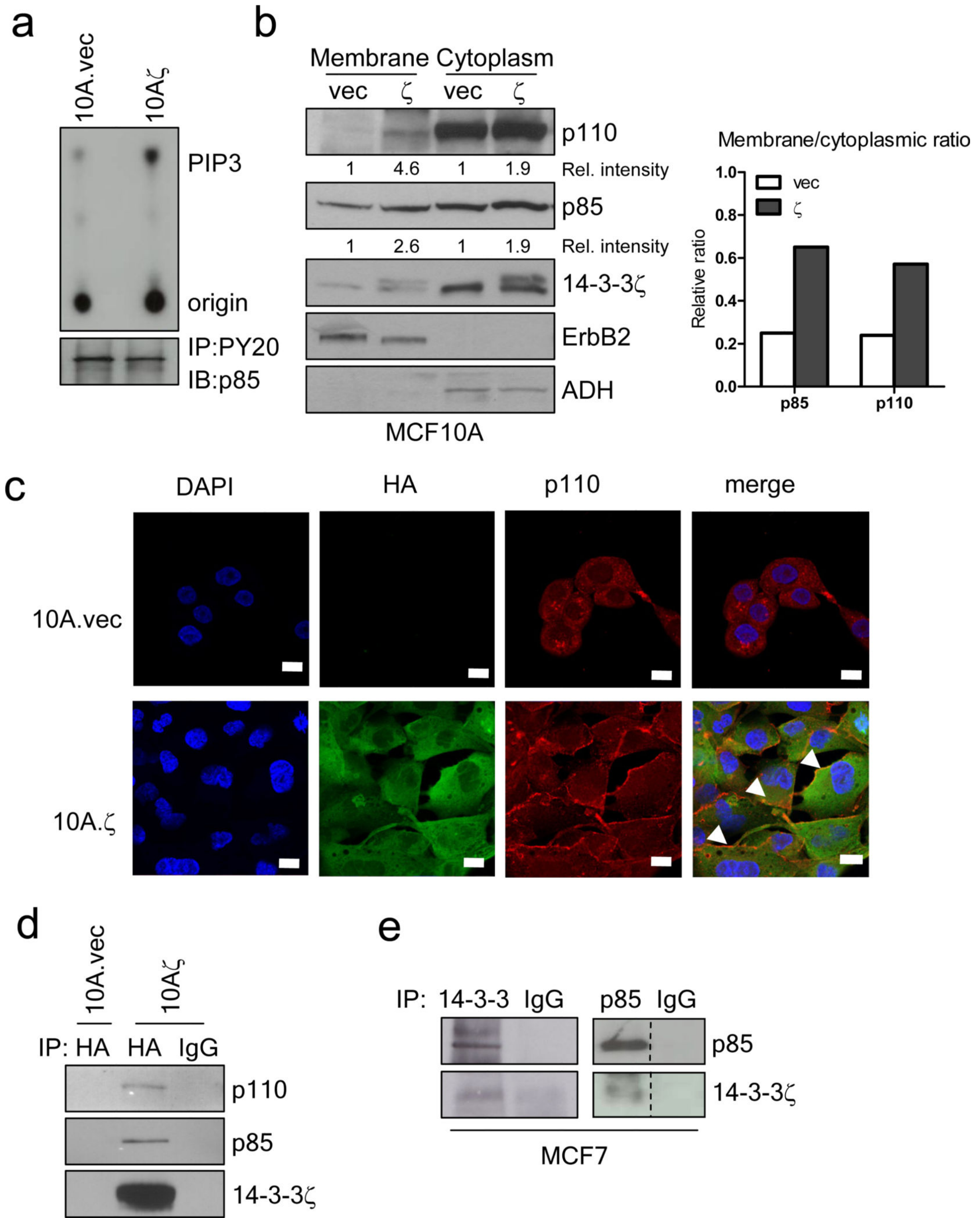

Figure 2. 14-3-3ζ binds to p85 and enhances PI3K membrane localization and activation

(**a**) MCF10A cells stably transfected with HA-14-3-3ζ (10Aζ) or vector as control (10A.vec) were immunoprecipitated (IP) with PY20 antibody and subjected to PI3K assays. Immunoblot (IB) of total p85 showed similar protein recovery following immunoprecipitation.

(**b**) Immunoblot of membrane and cytoplasmic proteins from MCF10A transfectants. ErbB2 and alcohol dehydrogenase (ADH) were used as membrane and cytoplasmic markers, respectively. Relative intensity was quantified by densitometry using ErbB2 and ADH as

loading controls and standardized to vector control. The membrane to cytoplasmic ratio was determined by dividing the corrected value obtained by densitometry of the membrane fraction by the corrected value obtained by densitometry of the cytoplasmic fraction.

(**c**) Immunofluorescent staining of MCF10A cells stably transfected with vector (10A.vec) or HA-tagged 14-3-3ζ (10A.ζ). Cells were stained with anti-HA (14-3-3ζ) and p110 specific antibodies. DAPI staining represents nuclei. Arrows indicate areas of membrane localization. The scale bars represent 20μm.

(**d**) MCF10A HA tagged 14-3-3ζ transfectants and their respective vector control cells were immunoprecipitated (IP) with anti-HA or IgG followed by immunoblot with p110, p85, and 14-3-3ζ antibodies.

(**e**) MCF7 cells were immunoprecipitated (IP) with 14-3-3 or p85 antibodies to detect endogenous association of 14-3-3ζ and p85.

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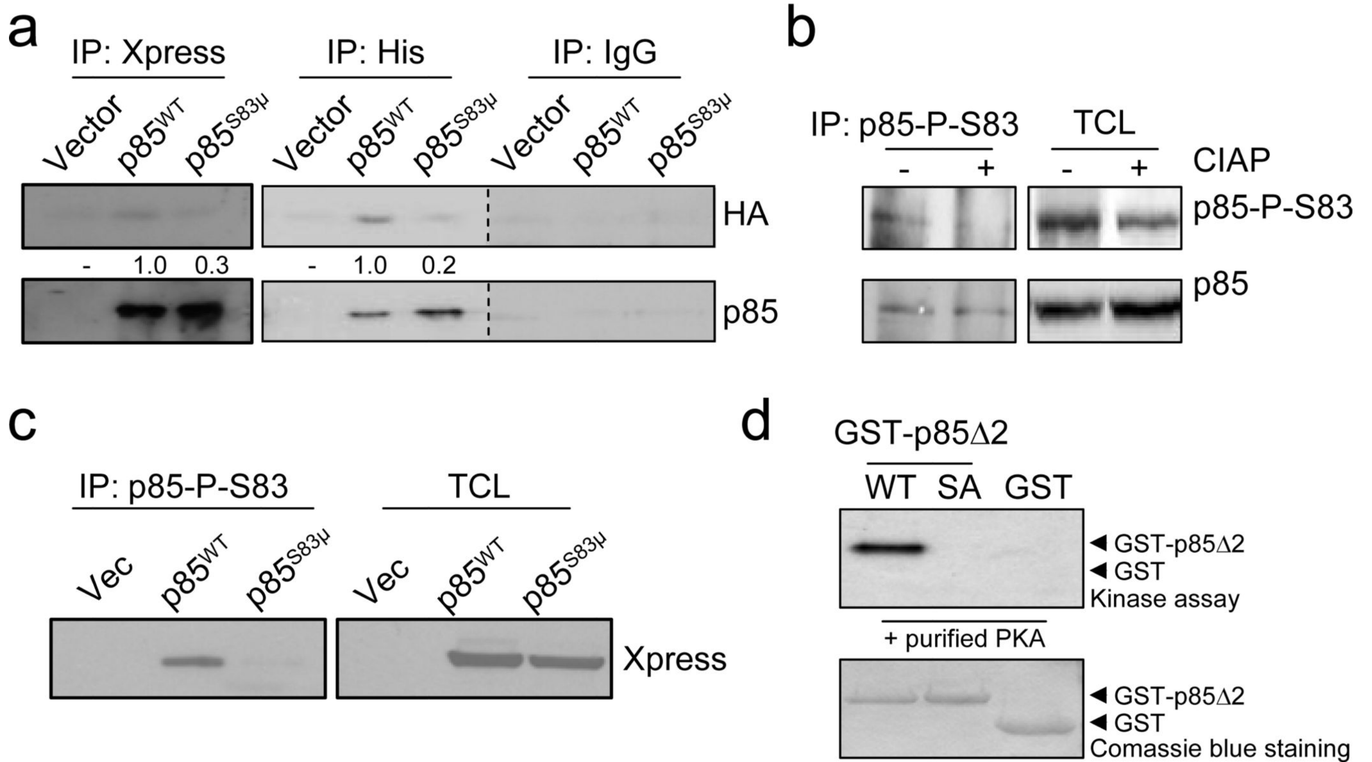

Figure 3. Phosphorylation of serine 83 on p85 defines a 14-3-3 binding motif

(**a**) MCF7 14-3-3ζ transfectants (MCF7ζ) were stably transfected with empty vector (vector), histidine/Xpress-tagged (His/Xpress) wild type p85 ($p85^{WT}$), or tagged p85 mutated in the 14-3-3 binding site encompassing S83 ($p85^{S83\mu}$). Exogenous p85 was immunoprecipitated (IP) with Xpress or His antibodies followed by immunoblot with HA to detect exogenous 14-3-3ζ. Immunoprecipitation efficiency of Xpress or His-tagged p85 was determined by immunoblot with p85 antibody. Relative intensity was quantified by densitometry and standardized to $p85^{WT}$.

(**b**) p85 phospho-serine 83 antibody (p85-P-S83) immunoprecipitates (IP) from MCF7ζ cells or total cell lysates from MCF7ζ (TCL) were untreated (–) or treated (+) with calf intestine alkaline phosphatase (CIAP) followed by immunoblot with p85-P-S83 and p85 antibodies.

(**c**) 293T cells were transiently transfected with $p85^{WT}$ or $p85^{S83\mu}$. Lysates were immunoprecipitated with p85-P-S83 antibody followed by Xpress immunoblot to confirm p85 expression and loss of serine 83 phosphorylation on $p85^{S83\mu}$.

(**d**) top: *in vitro* kinase assays using purified protein kinase A (PKA) and either GST fused full length p85 (GST-p85) or GST fused p85 peptide spanning amino acids 50-109 (GST-p85 2) as substrate. Serine 83 was either wild type serine 83 (WT) or serine 83 mutated to alanine (SA). *Bottom*: Comassie blue staining of purified GST-p85, GST-p85 2 or GST showing equal amounts used in the kinase assay.

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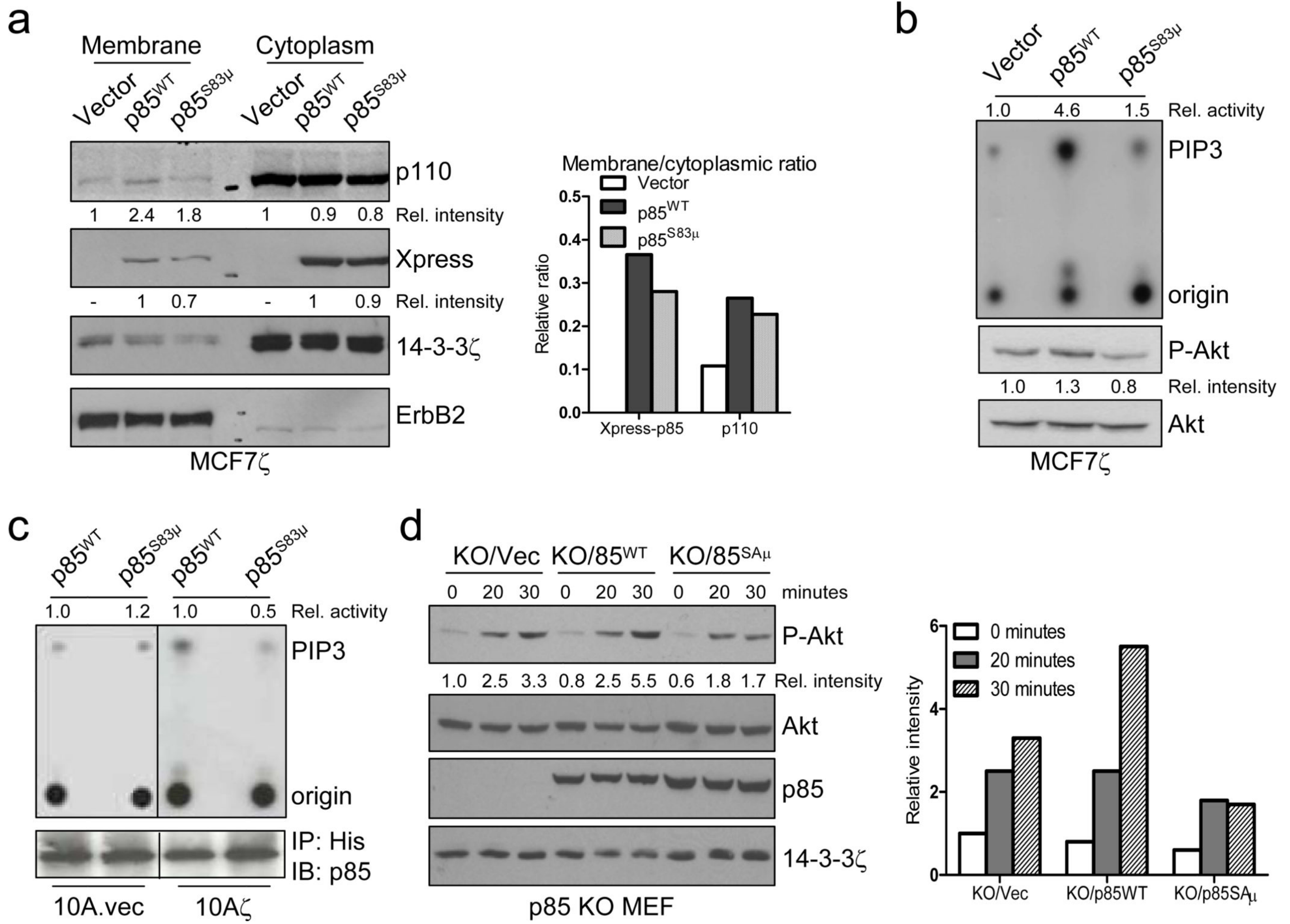

Figure 4. Serine 83 phosphorylation on p85 is necessary for PI3K activation and membrane translocation in 14-3-3ζ overexpressing cells

(**a**) Immunoblot of membrane and cytoplasmic proteins from MCF7ζ.p85 stable transfectants. Exogenous p85 localization was determined by Xpress antibody. ErbB2 and 14-3-3ζ were used as membrane and cytoplasmic markers, respectively. Relative intensity was quantified by densitometry using ErbB2 and 14-3-3ζ as loading controls and standardized to vector control for p110 or $p85^{WT}$ for Xpress. The membrane to cytoplasmic ratio was determined by dividing the corrected value obtained by densitometry of the membrane fraction by the corrected value obtained by densitometry of the cytoplasmic fraction.

(**b**) Exogenous p85 was immunoprecipitated with His antibody from the indicated MCF7ζ transfectants followed by PI3K assays (top). Akt phosphorylation was determined from whole cell lysates by immunoblot with phospho-Akt Ser473 (P-Akt) (bottom). Relative activity and intensity were quantified by densitometry and standardized to vector control.

(**c**) MCF10A.vec (10A.vec) and MCF10Aζ (10Aζ) cells were transiently transfected with $p85^{WT}$ or $p85^{S83\mu}$. Exogenous His/Xpress-p85 was immunoprecipitated (IP) with His antibody followed by PI3K assays (upper panel). Immunoprecipitation efficiency of His-

tagged p85 was determined by immunoblot (IB) with p85 antibody (lower panel). Relative activity was quantified by densitometry and standardized to p85^{WT} for each cell line.
(**d**) Mouse embryonic fibroblasts (MEF) with knockout p85 (KO) were re-constituted with histidine/Xpress-tagged (His/Xpress) p85 wild type (85^{WT}), p85 14-3-3 binding mutant (85$^{S83\mu}$) or empty vector (Vec). Cells were serum starved then stimulated with FBS for the indicated times. Akt activation was assessed by immunoblot for phospho-Akt Ser473 (P-Akt). Relative intensity was quantified by densitometry and standardized to KO/Vec for each cell line.

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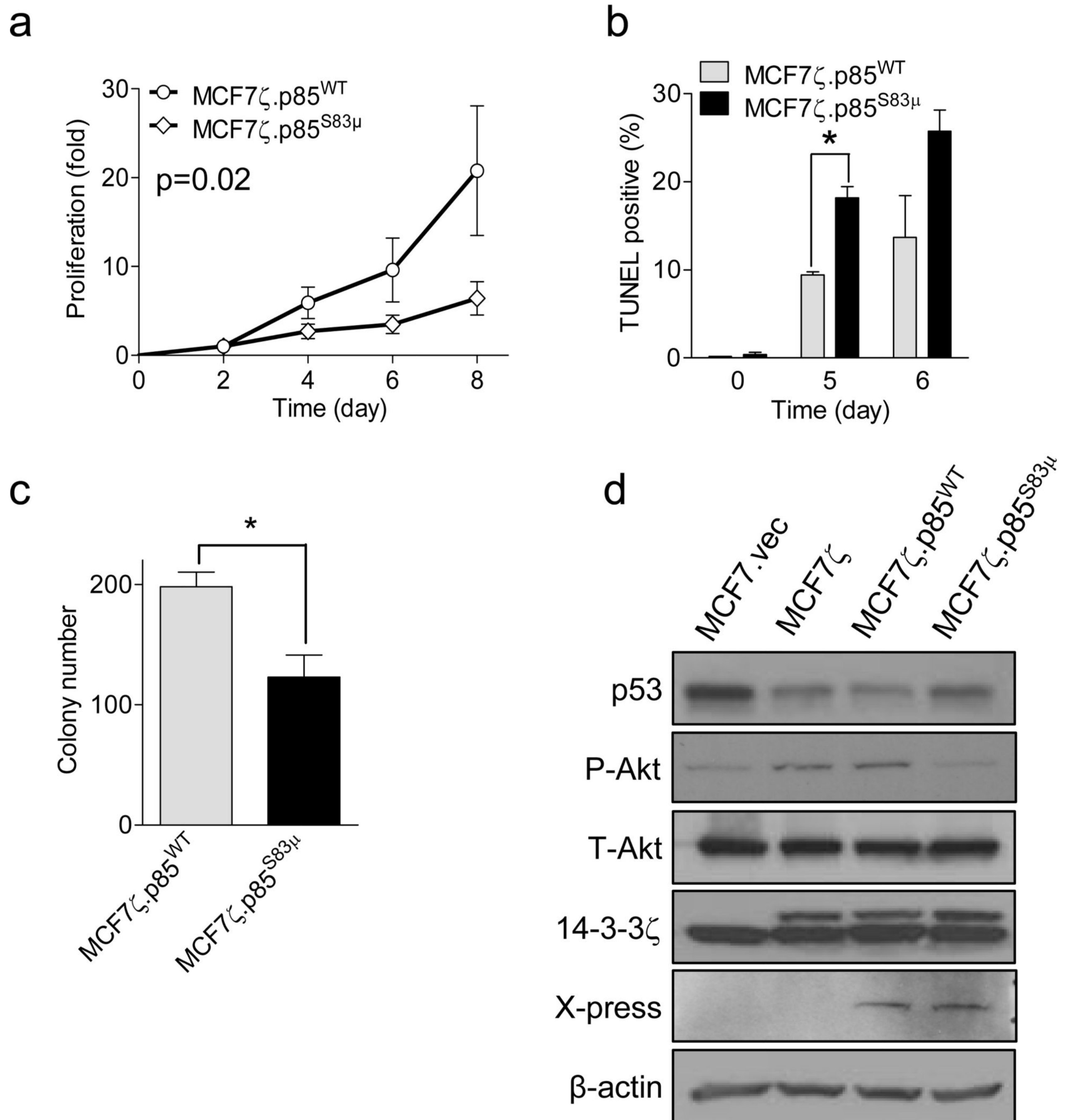

Figure 5. p85 serine 83 phosphorylation and 14-3-3ζ binding modulates transformation in 14-3-3ζ overexpressing cells

(**a**) Proliferation assay of MCF7ζ.p85^{WT} or p85^{S83μ} stable transfectants in 0.5% serum (linear regression, p=0.02)

(**b**) MCF7ζ.p85^{WT} or p85^{S83μ} stable transfectants were serum starved for the indicated times. Apoptotic cells were identified by TUNEL staining (t test, *, p=0.003).

(**c**) Soft agar colony formation of MCF7ζ.p85^{WT}, or p85^{S83μ} transfectants (t test, *, p=0.03). Error bars in (A–C) represent SEM.

(**d**) MCF7 stable transfectants were grown in normal serum conditions. Cell lysates were immunblotted with the indicated antibodies. Akt activation was assessed by immunoblot for phospho-Akt Ser473 (P-Akt). Exogenous p85 levels were detected with Xpress antibody.

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