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# The Breast Cancer Susceptibility Gene Product (γ-Synuclein) Alters Cell Behavior through it Interaction with Phospholipase Cβ

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## Abstract

The breast cancer susceptibility gene protein, also known as γ-synuclein, is highly expressed in human breast cancer in a stage-specific manner, with highest expression in late stage cancer. In model systems, γ-synuclein binds phospholipase Cβ2 which is regulated by Gαq to generate intracellular $Ca^{2+}$ signals. PLCβ2, which is also absent in normal tissue but highly expressed in breast cancer, is additionally regulated by Rac to promote migration pathways. We have found that γ-synuclein binds to the same region of PLCβ2 as Gαq. Using cells that mimic stage 4 breast cancer (MDA MB 231), we show that down-regulation of γ-synuclein reduces the protein level of PLCβ but increases the transcript level over 40 fold. γ-Synuclein down-regulation also promotes the interaction between Gαq and PLCβ resulting in a stronger $Ca^{2+}$ response to Gαq agonists. The ability of γ-synuclein to interfere with Gαq-PLCβ interactions allows more PLCβ to colocalize with Rac impacting Rac-mediated pathways that may give rise to cancerous phenotypes.

### Keywords

γ-synuclein; phospholipase Cβ2; calcium signaling; cell morphology; cell migration

## 1. INTRODUCTION

Synucleins are a family of small, intrinsically disordered proteins, consisting of three members: α, β and γ (for review see [1]). The synucleins share a conserved N-terminal domain but each member possesses a distinct C-terminal region. Synucleins are predominantly expressed in neuronal tissues, where they have been implicated in neurotransmitter homeostasis and release. However, their exact function remains unknown (for review see [1, 2])

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γ-Synuclein is a 127 amino acid protein and possesses a shorter and slightly negative C-terminal domain that distinguishes it from other synucleins [3]. In contrast to the many studies involving α-synuclein which plays a key role in Parkinson's disease, little research has been done on γ-synuclein. However, mouse studies of γ-synuclein have been carried out and these show that knocking-out expression leads to the improvement of working memory, suggesting that γ-synuclein has a role in cognitive function [4].

Aberrant overexpression of γ-synuclein has been observed in various pathological conditions especially in a variety of cancers including prostate, colorectal, pancreatic, ovarian and gall bladder [5, 6]. Surprisingly, γ-synuclein was first discovered in 1996 in breast cancers and was named the Breast Cancer Susceptibility Gene Product, but was later identified as being a member of the neuronal synuclein family [7]. In breast cancer, γ-synuclein is overexpressed in later-stage (Stage III and IV) cancer tissues, but not in healthy or early-stage (Stage I and II) cancer tissue [8]. Even though different types of breast cancers are classified by different markers [9], the presence of γ-synuclein been established as a biomarker for later stages of cancer and has been considered to be a prognosis of poor outcome [10].

Studies have shown that knockdown of γ-synuclein expression in prostate [5] and gall bladder [6] cancer cells greatly reduced the occurrence of cancerous phenotypes such as cell proliferation, migration, invasion and cell cycle arrest. Further studies have shown that downregulation of γ-synuclein expression in MCF7 cells (an early-stage breast cancer cell line) resulted in a drastic reduction in cell migration and proliferation [11], as well as the propensity to form tumors when xenografted into mice [12]. A study performed using the triple-negative breast cancer cell line, MDA MB 231 has revealed that knockdown of γ-synuclein results in an inhibition of cell migration and proliferation [13].

While the exact role of γ-synuclein in the signaling pathways that lead to cancer is currently not known, some studies have shown that γ-synuclein promotes cancerous phenotype by increasing ER-α (Estrogen Receptor) transcription [14], activating MAPK [15], enhancing AKT and ERK signaling [13], and binding and inhibiting BubR1, a mitotic checkpoint protein that prevents the formation of the anaphase promoting complex, thereby allowing the cells to rapidly undergo mitosis [16]. γ-Synuclein is known to regulate signaling pathways by changing its intracellular localization[17] and binding to transcription factors affecting gene expression[18].

Our lab has previously shown that both α- and γ-synuclein interact with the signaling enzyme phospholipase Cβ (PLCβ) to alter $Ca^{2+}$ responses [19, 20]. There are 4 isoforms of PLCβ (PLCβ -4) which are all strongly activated by the Gαq family of heterotrimeric G proteins. Receptors that are coupled to Gαq include those that bind acetylcholine dopamine, angiotensin II, bradykinin as well as endothelin I [21]. Both PLCβ2 and β3 can be activated by Gβγ subunits which may be released in response to activation of other G protein families. PLCβ enzymes catalyze the hydrolysis of the signaling lipid phosphatidylinositol 4,5 bisphosphate to generate two second messengers (diacylglyercol and 1,4,5 inositol trisphosphate) which lead to the activation of protein kinase C and release of $Ca^{2+}$ from intracellular stores, respectively (for review see [22, 23]). Even though they bind strongly to

membranes, PLCβs are soluble and are found both on the plasma membrane and in the cytoplasm. It has been shown that membrane binding of PLCβ2 can be enhanced by Rac which better allows PLCβ to access its $PI(4,5)P_2$ substrate and promote hydrolysis ([24] for review see [25]). It is notable that Rac1 which mediates cytoskeletal changes associated with migration and mobility leading to the idea that PLCβ may also be involved in these processes via its association with Rac. Rac1-RhoA signaling plays a role in cell motility, where Rac-mediated signaling is associated with forward movement and Rho-mediated signaling with the contractile movement. The movements associated with Rac or Rho mediated signaling have also been linked to protease-dependent mesenchymal and protease-independent amoeboid modes of invasion [26].

Using purified proteins, it was observed that both α- and γ-synuclein bind strongly to the C-terminal region of PLCβ2 [20]. This binding site of γ-synuclein is also the binding site of Gαq, resulting in the competitive inhibition of $G\alpha_q$-mediated PLCβ2 activation. Conversely, the levels of γ-synuclein do not affect the binding of Gβγ or Rac to PLCβ2, since they bind to the N-terminal region which is distant from the Gαq binding site. [20]. It is also notable that the binding site of γ-synuclein on PLCβ2 overlaps the calpain cleavage site and therefore, the presence of γ-synuclein prevents PLCβ2 degradation [27].

Like γ-synuclein, PLCβ2 is abnormally overexpressed in late-stage breast cancer cells and tissues [28, 29]. Knockdown or overexpression of PLCβ2 affects cell migration only in late stage breast cancer cell lines, with no effect on cell proliferation or invasion [29], suggesting that PLCβ2 is part of a signaling pathway that influences transition into the late-stage cancer phenotypes.

From the studies described above, we postulate that γ-synuclein might promote cancer phenotypes through its ability to increase levels of PLCβ2. This idea is supported by observations that in breast cancer cell line MDA MB 231, PLCβ2 and γ-synuclein co-localize with each other and PLCβ2 co-immunoprecipitates with γ-synuclein, suggesting a cellular interaction between the two proteins [20]. Here, we present evidence that γ-synuclein, by its ability to increase PLCβ2 levels, allows for enhanced Rac-mediated signals at the expense of Gαq signals thereby promoting cancerous phenotypes.

## 2. MATERIALS AND METHODS

### 2.1 Cell culture

MDA MB 231 cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 units/mL of penicillin and 50 μg/mL of streptomycin at 37°C and 5% CO2. To knockdown protein expression levels, cells were transfected using Dharmafect reagent with 40pg of γ-synuclein (Sigma), PLCβ2 (Sigma) or non-specific control (Ambien) siRNAs and were incubated for 96 hours before performing an experiment. For migration, invasion and viability assays, cells were transfected with 40pg each of γ-synuclein and PLCβ2 siRNAs or with 40pg each of γ-synuclein and control siRNAs, or with 80 pg of the control siRNA.

### 2.2 Quantitative Real Time Reverse Transcriptase PCR (qRT-PCR)

Total RNA were extracted using RNAeasy kit (Qiagen) according to the manufacturer's instructions. 2 μg of total RNA was used for cDNA synthesis with random hexamers using Reverse Transcriptase kit (Qiagen). Using TaqMan® Gene Expression Assays (ThermoFisher) for SNCG (γ-synuclein) , PLCB2 (PLCβ2) and ACTB (β Actin) as primers and using DNA Engine Opticon® 2 System (BioRad), real-time PCR was carried out with a denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing at 60°C for 15 s and extension at 72°C for 30 s. Upon completion of the cycling steps, the reaction was stored at 4°C. Real-time PCR was carried out Reactions were run in triplicate in three independent experiments. The geometric mean of housekeeping gene ACTB was used as an internal control to normalize the variability in expression levels. Expression data were normalized to the geometric mean of housekeeping gene ACTB to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method [30]

### 2.3 In vitro protein association studies

Binding between purified coumarin-PLCβ2 and purified C3PO in the presence and absence of purified γ-synuclein was carried out using the materials and procedure described in [20]. Briefly, purified proteins were labeled with the thiol-reactive probe7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) at a probe:protein ratio of 4:1 on ice. The reaction was stopped after 60 minutes by adding 10 mM DTT and the protein was purified either by extensive dialysis. Protein solutions were diluted into 20 mM Hepes (pH 7.2), 160 mM NaCl, 1 mM DTT.

Spectral measurements were performed on an ISS spectrofluorometer (Champaign, IL) using 3 mm quartz cuvettes. Peptide and The emission spectrum of CPM-labeled protein was measured from 400 to 550 nm (λex = 380 nm). The background spectra of unlabeled protein or peptide were subtracted from each spectrum along the titration curve, which was also corrected for dilution.

### 2.4 Immunofluorescence

Cells were fixed using 3.7% formaldehyde and permeabilized with 0.2% nonyl phenoxypolyethoxylethanol (NP40) and incubated with 0.2% NP40 in phosphate buffered saline (PBS) for 5 min and then blocked in PBS containing 4% goat serum for 1 h. Cells were then incubated with the primary antibody (anti-PLCβ2, anti-Rac1 or anti-Gαq (Santa Cruz Biochemicals, Inc.) diluted to 1:1000 for 1.5 hours at 37°C, followed by incubation with Alexa-labeled secondary antibody for 0.5 hours at the same temperature. Cells were washed with Tris buffered saline (TBS) buffer after the incubations. Images of the cells were obtained using Olympus Fluoview FV1000 laser scanning confocal microscope, and were analyzed using Olympus (Fluoview) software and Image J (NIH).

### 2.5 Förster resonance energy transfer (FRET)

FRET measurements were performed using an Olympus Fluoview1000 instrument on MDA MB 231 cells co-expressing eCFP-tagged Rac1, eCFP-tagged Gαq and/or eYFP-tagged PLCβ2 proteins as described [31]. eCFP and eYFP were excited using 458 and 515 nm

argon ion laser lines, respectively, and 480–495 and 535–565 nm bandpass filters to collect emission images, respectively. FRET efficiencies were calculated using the Olympus Fluoview software whose algorithm calculates FRET by sensitized emission after correcting for spectral bleed-through using images of control cells expressing only donor or acceptor proteins with the same intensity distributions as the sample.

### 2.6 $Ca^{2+}$ Measurements

Intracellular $Ca^{2+}$ levels in MDA MB 231 cells treated with γ-synuclein siRNA or sham control were harvested and incubated with 1 μM Fura 2-AM in Hanks Balanced Salt Solution (HBSS, Gibco) with 1% BSA. Cells (1 × 107) were incubated with 1 μM Fura 2-AM for 30 min, pelleted, washed twice with HBSS, and incubated for an additional 15 min for de-esterification of Fura 2-AM. Fluorescence measurements were taken as described in earlier publications [32].

### 2.7 Western blotting

MDA MB 231 cells were lysed with 500 ul of buffer containing 150 mM NaCl, 20 mM HEPES, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 ug/ml leupeptin and 10ug/ml aprotinin. The lysates were treated in sample buffer at 95°C for 3 minutes and were loaded onto a 12% PAGE for SDS-PAGE and were transferred into polyvinylidene difluoride membranes. After the transfer, the membranes were blotted with anti- γ-synuclein and anti-PLCβ2 antibodies. Protein levels were calculated using ImageJ and are normalized with the levels of housekeeping genes (β-actin).

### 2.8 Migration and Transwell matrigel invasion assays

For Transwell migration assays, MDA MB 231 cells were trypsinized and suspended in DMEM 96 hours after siRNA transfection. Equal numbers of cells ($2.5 \times 10^4$ −$6.25 \times 10^4$) were loaded on to each of transwell chambers (Corning) that were placed in a 24 well tissue culture plate (Falcon), containing DMEM supplemented with 10% FBS, 50 units/mL of penicillin and 50 μg/mL of streptomycin. The plate is then incubated at 37°C and 5% $CO_2$ for 4 hours. The cells that migrated across the transwell membrane were then fixed with 3.7% Formaldehyde, stained with DAPI and imaged in situ.

For transwell invasion assay, 25ul of Matrigel (BD Biosciences) solution, diluted according to manufacturer's instructions was added to transwell chambers, which were then allowed to dry overnight in a dry incubator. The chambers, now coated with Matrigel, are allowed to rehydrate by incubating with DMEM for 2 hours, after which the same protocol as transwell migration assay, but with an incubation time of 20 hours.

### 2.9 MTT assay

A total of 100,000 cells diluted in 0.5 ml growth media were plated on 12-well flat bottom plates and were then transfected as described earlier. At the indicated times, 120 ul of 5 mg/ml MTT solution in PBS were added to each well for 2.5 h. After removal of the medium, 900 ul of dissolving buffer (0.04M HCl diluted in Isopropanol) were added to each well to dissolve the formazan crystals. The contents of the wells were transferred into plastic cuvettes and absorbance at 570 nm and 650 nm was determined using a spectrophotometer.

### 2.10 Circularity

The shape of the cell was quantified by using the Circularity function of ImageJ (NIH) software, which was calculated using the formula below based on outline of a cell:

$$Circularity = 4\pi \frac{Area}{(Perimeter)^2}$$

## 3. EXPERIMENTAL

### 3.1 Protein and mRNA levels of γ-synuclein and PLCβ2 in MDA MB 231 cells are correlated

Our previous studies of α-synuclein showed that the binding site of α-synuclein on PLCβ2 overlaps with the calpain cleavage site[27]. Since calpain is activated by elevated $Ca^{2+}$, then PLCβ2 degradation by calpain is thought to be involved as a feedback mechanism to help extinguish G protein-mediated $Ca^{2+}$ signals. To determine whether this is the case for γ-synuclein, we down-regulated the protein with siRNA in MDA MB 231 cells and compared the protein levels of PLCβ2 with samples using control siRNA. Estimates from western blot analysis indicated that this treatment reduces γ-synuclein expression by 65%. In **Fig. 1A,** we show that reducing the level of γ-synuclein by ~65% also reduces the level of PLCβ2 by about 40%.

To determine whether the reduced production of PLCβ2 with γ-synuclein expression is on the transcriptional level, we measured the change in mRNA(PLCβ2) as γ-synuclein is down-regulated. Unexpectedly, we find that down-regulation of γ-synuclein increases the level of mRNA(PLCβ2) by a factor of 70 (**Fig. 1B-C**). This finding is consistent with γ-synuclein being involved in transcriptional regulation [15].

### 3.2 PLCβ2-Gαq interactions in MDA MB 231 cells are affected by γ-synuclein knockdown

In previous in-vitro studies using purified proteins, we found that the binding of γ-synuclein to PLCβ2 reduces enzymatic activity [20] . We also found that γ-synuclein binds to the same site as Gαq on PLCβ2 suggesting that γ-synuclein inhibits Gαq-mediated activation. Because MDA MB 231 cells over-express both γ-synuclein and PLCβ2, it is possible that Gαq-mediated activation of PLCβ2 is diminished due to competition by γ-synuclein.

Based on our previous work, we postulate that lowering the levels of γ-synuclein would promote association between Gαq and PLCβ2. We first tested this idea by reducing the protein levels of γ-synuclein in MDA MB 231 cells using siRNA and monitoring the change in colocalization between PLCβ2 and Gαq by immunofluorescence. We find a significant increase in colocalization between PLCβ2 and Gαq when γ-synuclein levels are reduced **(Fig. 2A)** which is consistent with the idea that γ-synuclein competes with Gαq for PLCβ binding.

To confirm that loss of γ-synuclein promotes physical association between PLCβ and Gαq, we measured the FRET between eCGP-Gαq and eYFP-PLCβ2 expressed in MDA MB 231 cells. We find a reduction in the amount of γ-synuclein causes a small but significant increase in FRET ($p<0.001$) (**Fig. 2B**). The values of FRET, when compared to the value

obtained for the positive control (eCFP-X-eYFP) and negative controls (free eCFP and free eYFP, or eCFP-TRAX and YFP-membrane marker), show an increase in association from 19.1±0.2% to 32.2±0.6% when γ-synuclein is reduced approximately in half.

If reducing γ-synuclein promotes PLCβ2-Gαq association, then it is likely $Ca^{2+}$ signals generated through Gαq will be enhanced. We tested this idea by measuring the release of $Ca^{2+}$ with carbachol addition to suspensions of MDA MB 231 cells loaded with the fluorescent $Ca^{2+}$ indicator Fura-2. We find that cells in which γ-synuclein has been down-regulated by 65% using siRNA show a significant increase in $Ca^{2+}$ release despite the correlative reduction in PLCβ levels (**Fig. 2C**) suggesting that high levels of γ-synuclein expression may quench $Ca^{2+}$ signals mediated through Gαq.

### 3.3 γ-Synuclein inhibits the association between PLCβ2 and C3PO

In a yeast two hybrid study, we found that the C-terminal region of PLCβ1 associates to the nuclease Translin-Associated Factor X (TRAX) [33]. TRAX binds to the oligonucleotide binding protein translin to form the octamer C3PO [34]. C3PO has been shown to play an integral role in RNA-induced silencing by removing the passenger strand of the double-stranded silencing siRNA [35]. The association between PLCβ and C3PO reduces the ability of C3PO to process specific genes [36]. Because the binding site for Gαq and C3PO on PLCβ2 overlap, we determined whether γ-synuclein could interfere with PLCβ2-C3PO association as well. To answer this question, we labeled purified PLCβ2 with a fluorescent probe and monitored the change in fluorescence as C3PO binds in the absence and presence of γ-synuclein (**Fig. 3A**). This observation was corroborated by enhanced colocalization between TRAX and PLCβ2 upon knockdown of γ-synuclein in MDA MB 231 cells (**Fig. 3B**). This result suggests that γ-synuclein may indirectly affect miR population by interfering with PLCβ-C3PO association.

### 3.4 The presence of γ-synuclein affects PLCβ2- Rac interactions and Rac-mediated events

Phosphoinositides are required by RhoGEFS to regulate Rac (e.g [37]). Rac has been shown to bind to the N-terminal region of PLCβ2 which is structurally distant than the region where γ-synuclein and Gαq bind [38]. Rac promotes the activation of PLCβ2 by recruiting and stabilizing its association to the plasma membrane (see [39]). Because the PLCβ2 /γ-synuclein complex does not bind to Gαq, the presence of γ-synuclein would make more PLCβ available to impact Rac-mediated pathways, such as cell invasion and cell morphology [40-43]. Alternately, down-regulating γ-synuclein would make less PLCβ2 available to Rac due to reduction of the cellular amount of enzyme and by promoting Gαq association.

We first measured changes in the colocalization between PLCβ2 and Rac in control and γ-synuclein knockdown MDA MB 231 cells and found a significant reduction with reduced γ-synuclein expression (**Fig. 4A**). We note that western blotting shows that the protein level of Rac is not affected by γ-synuclein knock-down. We supported the co-immunofluorescence studies by FRET in which cells were transfected with eYFP-PLCβ2 and eCFP-Rac1. We find that the level of FRET slightly decreases with γ-synuclein knock-down. We note that

this small change in FRET may reflect the over-expression of the proteins which mask larger changes at endogenous levels (**Fig. 4B**).

### 3.5 γ-Synuclein / PLCβ2 interactions may be linked to cancerous phenotypes

MDA MB 231 cells are known to be able to follow both amoeboid and mesenchymal pathways of invasion. The elongated morphology is often associated with a mesenchymal pathway, whereas circular morphology is often associated with an amoeboid pathway. We determined the ability of γ-synuclein to affect the morphology of MDA MB 231 cells by comparing cells treated with siRNA(γ-synuclein) or control siRNA. We find that knocking down γ-synuclein expression results in cells with a more elongated and less circular morphology (**Fig. 5A**). Because γ-synuclein increases the protein level of PLCβ2, then more of its N-terminus is available for Rac1 binding resulting in a decrease in inhibition of RhoA. RhoA signaling is associated with a more circular amoeboid morphology [44], while Rac is associated with more elongated mesenchymal morphology (for review see [25]).

Migration pathways can be mediated by Rac [45]. Previous studies [13, 29] found that the levels of both γ-synuclein and PLCβ2 independently affect the migration of MDA MB 231 cells. Based on this work, we performed Transwell migration assay using cells treated with two siRNAs (γ-synuclein and control siRNAs, γ-synuclein and PLCβ2 siRNAs, PLCβ2 and control siRNAs, or double the amount of control siRNA). We observe that knocking down the levels of both PLCβ2 and γ-synuclein results in decreased cell migration, corroborating previous results [11]. However, treatment with both PLCβ2 and γ-synuclein siRNA revealed that knockdown of PLCβ2 attenuates the decrease in cell migration caused by the knockdown of γ-synuclein in breast cancer cells, without completely eliminating the effect. This result suggests that γ-synuclein/ PLCβ2/Rac1 complexes is one of the interactions that utilized by γ-synuclein to induce cell migration (**Fig. 5B**). Treatment with γ-synuclein siRNA on MDA MB 231 cells decreases cell invasion as quantified by Transwell matrigel assay which was also attenuated by simultaneous treatment with PLCβ2 siRNA **(Fig. 5C)**.

To understand whether these migration effects could be due to changes in cell viability (see [13]), we determined whether simultaneous knockdown of PLCβ2 attenuated reduced viability due to γ-synuclein. We find that this is not the case **(Fig. 5D)** supporting the idea that PLCβ2 / Rac pathways are promoted by γ-synuclein.

## 4. DISCUSSION

In this study, we have shown that changes in the interaction between PLCβ2 and γ-synuclein may promote cancerous phenotypes though several mechanisms. Breast cancer is typified by changes in cell morphology and migration, which are brought about by changes in the levels of specific genes and their expressed proteins. Changes in gene expression were first examined by Ji and coworkers [7] who identified a gene that is absent in normal tissue but highly overexpressed in breast cancer tissue. Surprisingly, this gene was later identified to be the neurological protein, γ-synuclein. γ-Synuclein has since been found in other types of cancer where it may be involved in transcriptional regulation of cellular proteins. This regulation was seen here by the impact of γ-synuclein on the message level of PLCβ2.

Most likely γ-synuclein levels significantly affect the mRNA levels of other regulatory proteins but in ways that are difficult to predict. For example, in breast cancer cells, activator protein AP-1, a complex composed of c-Jun homodimers or c-Jun/c-Fos heterodimers, was found to promote the expression of γ-synuclein [46]. Conversely γ-synuclein was found to bind to and mediate AP-1 activity in neuronal and retinoblastoma cells [18, 47], and to activate c-JunN-terminal kinase 1 (JNK1) in breast cancer [15]. Meanwhile PLCs, including PLCβ2 and transcription factors c-Jun and c-Fos reciprocally regulate their respective expression levels in cardiomyoctes [48].

Previous studies have shown that like γ-synuclein, PLCβ2 is absent in normal breast tissue but is found at high levels in breast cancer [28]. It is noteworthy that changes in PLCβ expression appear in many types of cancer [49] showing the complexity of lipid signaling pathways [50]. Here, we show the positive correlation between protein levels of γ-synuclein and PLCβ2. A similar correlation was previously found in analogous studies of α-synuclein and PLCβ1 where we have shown that α-synuclein protects PLCβ2 from degradation by the calcium-activated protein enzyme, calpain [27]. While we do not know the extent of regulation of PLCβ2 levels by calpain under normal cellular conditions, it is probable that γ-synuclein physically protects PLCβs from other forms of degradation as well. It is possible that the interactions between γ-synuclein, PLCβ2 and transcription factors might cause a feedback effect in response to the higher PLCβ2 degradation in γ-synuclein knockdown MDA MB 231 cells compared to control, causing an increase in PLCβ2 mRNA in response to γ-synuclein knockdown.

Cancer cells are less responsive to external regulation. Our studies showing that reduction of γ-synuclein by 40% significantly enhances $Ca^{2+}$ signals mediated through Gαq stimulation is consistent with a loss in regulation by external sources in cancerous states when γ-synuclein is over-produced. Additionally, we find that down regulation of γ-synuclein increases both the co-localization of PLCβ and Gαq, and their physical association as seen by FRET, which also contributes to a stronger $Ca^{2+}$ response.

PLCβ enzymes are distinguished from other PLCs by their long ~400aa C-terminal tails which contain the Gαq binding site (see [23] ). We have previously found that PLCβs can bind to the promoter of RNA interference and this binding impacts siRNA down-regulation. Because the binding region between PLCβ and C3PO overlaps with Gαq , it is not surprising that γ-synuclein interferes with PLCβ2 – C3PO association. Although much more work would be needed to determine whether specific targets or pathways are altered by γ-synuclein through this mechanism, existing literature and our results suggest that the association between γ-synuclein and PLCβ may influence transcriptional and post-transcriptional gene regulation.

Rac1 is involved with processes related to cytoskeletal rearrangements and has been shown to recruit PLCβ2 from the cytosol to the plasma membrane promoting PLCβ activation [24, 51]. Similarly, PLCb2 has been shown to promote migration pathways in breast cancer cells. It is interesting that PLCβ2 is the only PLC isoform that correlates with breast cancer and is specifically activated by Rac1. Because Rac binds to the N-terminal domain of PLCβ2, it should be unaffected by γ-synuclein levels. However, our studies show that when γ-

synuclein is down-regulated, association between PLCβ and Rac is reduced. We interpret this reduction as being due to increased interaction between PLCβ and Gαq due to the loss of γ-synuclein and due to a decrease in the protein levels of PLCβ. It is known that Rac1 inhibits Rho activity, and that the balance between Rac and Rho signaling influences cell migration and invasion [26, 41, 52]. Therefore it is possible that γ-synuclein promotes Rho signaling by sequestering Rac by binding to PLCβ2. This theory is partly corroborated by Pan and co-workers' findings that γ-synuclein was found to activate ERK pathway by enhancing Rho GTPase activation which may play a major role in cell migration [14]. The overall functional effects of the regulation of Rac-Rho signaling by γ-synuclein and PLCβ, can be seen by the changes in the Rac-mediated processes of cell migration and morphology.

## 5. CONCLUSIONS

In conclusion, we have shown that γ-synuclein can have multiple effects on cell function through its association with PLCβ, such as reduced calcium responses due to G protein activation, changes in modulation of RNA interference, and changes in Rac-mediated cytoskeletal arrangement that modulate cell shape and migration. While it is certain that γ-synuclein works through alternate mechanism to promote cancerous phenotypes, these studies show that PLC-γ-synuclein interactions may play an important role.

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## HIGHLIGHTS

- γ-Synuclein and PLCβ2 are linked to cancer and γ-synuclein increases PLCβ2 levels.
- γ-Synuclein competes with Gαq for PLCβ2 binding impacting Gαq-mediated $Ca^{2+}$ responses.
- Reducing γ-synuclein promotes PLCβ2-C3PO association which may affect RNA interference mediated by RNA-induced silencing complex (RISC).
- γ-Synuclein impacts PLCβ2- Rac interactions changing cell morphology and migration.

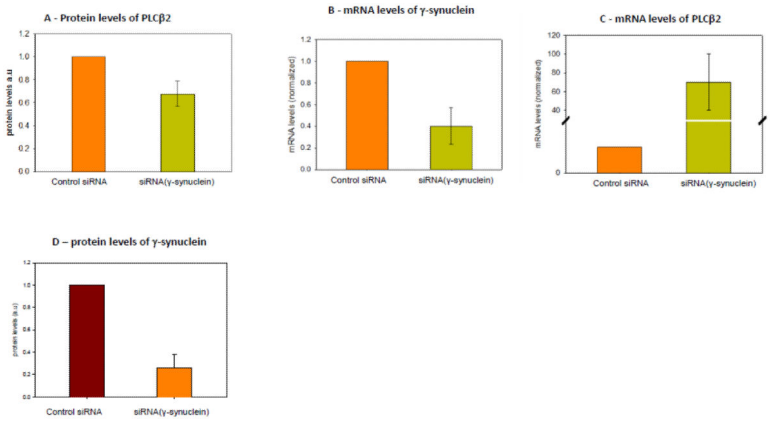


**Figure 1. Correlation between PLCβ2 and γ-synuclein expression**

**A-** Compilation of band intensities from western blots (n=7) showing that the protein level of PLCβ2 in MDA MD 231 cells is reduced when γ-synuclein is knocked-down using siRNA relative to control siRNA. **B-** mRNA levels of γ-synuclein and (**C**) PLCβ2 showing that reducing the message level of γ-syncuclein results in a sharp increase in the mRNA level of PLCβ2 when compared to control. mRNA levels normalized to β-actin mRNA and to the control siRNA) (n=9). **D-** Reference plot showing the reduction of g-synuclein protein levels with siRNA treatment (n=12).

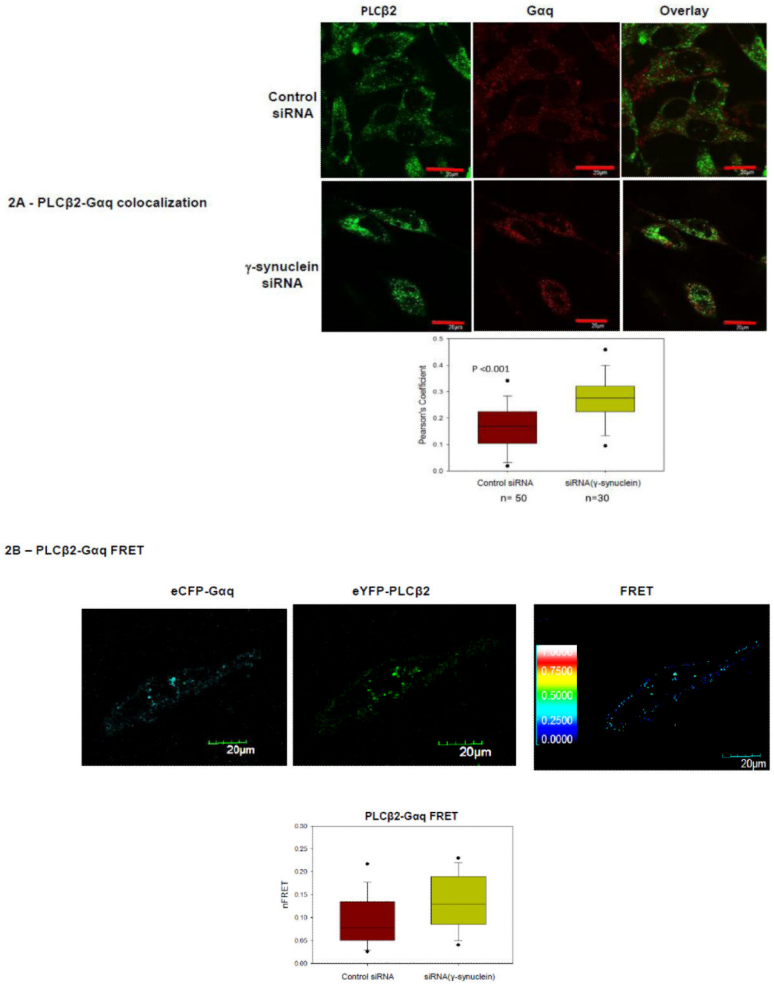


**Figure 2. PLCβ2-Gαq interactions in MDA MB 231 cells are affected by γ-synuclein knock-down**

**A-** Co-immunofluorescence studies showing the colocalization of PLCβ2 (green,) and Gαq (red) in MDA MB 231 cells treated with control (n=50) or γ-synuclein (n=30) siRNA where the compiled colocalization values are shown in the box plot. Pearson's coefficient between

the two groups is statistically significant ($p<0.001$, Student's t-test. Positive control= 0.65 +/− 0.12, negative control= 0.03 +/− 0.08). **B-** Sample raw FRET image of a MDA MB 231cell expressing eCFP- Gαq and eYFP- PLCβ2 with corresponding images at CFP and YFP wavelengths where the normalized FRET efficiencies for cells treated with γ-synuclein (n=30) and control (n=50) are given in the box plot were the values are found to be significantly different ($p<0.001$, Rank Sum Test. (Positive control = 0.44+/− 0.06, Negative control = 0.03 +/− 0.02). **C-** Functional results showing that reduction of γ-synuclein causes a more robust $Ca^{2+}$ response. $Ca^{2+}$ release was measured in MDA MB 231 cell suspensions upon the addition of 100 μM carbachol using Fura-2, where cells were treated with control or γ-synuclein siRNA, n = 6. The mean ± SEM is shown.

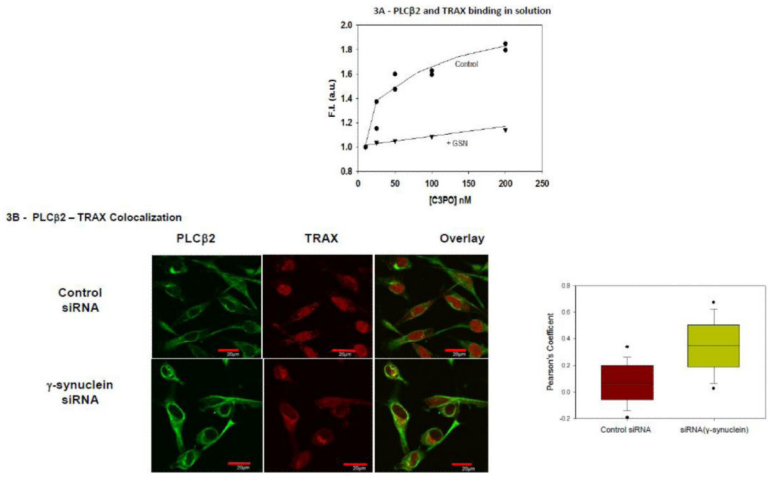


**Figure 3. PLCβ2-TRAX interactions in MDA MB231 cells are affected by γ-synuclein knockdown**

**A-** Normalized change in fluorescence intensity of 2 nM CPM- PLCβ2 in solution as purified TRAX is added in the presence or absence of 100nM γ-synuclein. **B-** Co-immunofluorescence studies showing the colocalization of PLCβ2 (green) and TRAX (red) in MDA MB 231 cells treated with control (n=41) or γ-synuclein (n=75) siRNA. Pearson's coefficient between the two groups is statistically significant ($p<0.001$, Student's t-test. Positive control= 0.65 +/− 0.12, negative control= 0.03 +/− 0.08).

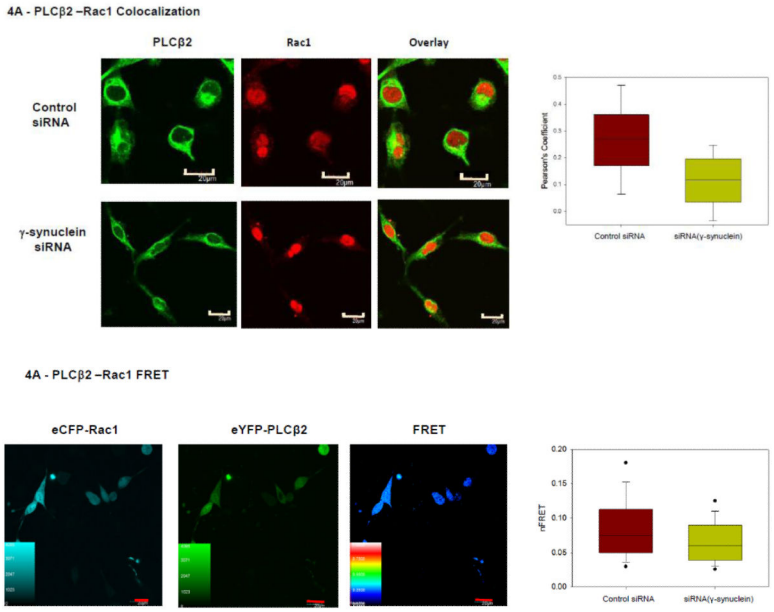


**Figure 4. PLCβ2 and Rac1 interactions in MDA MB 231 cells are affected by γ-synuclein knock-down**

**A-** Co-immunofluroescence studies showing the colocalization of PLCβ2 (green) and Rac1 (red) in MDA MB 231 cells treated with control (n=29) or γ-synuclein (n=30) siRNA. As shown in the accompanying box plots, Pearson's coefficient between the two groups is statistically significant ($p<0.001$, Student's t-test. Positive control= 0.65 +/− 0.12, negative control= 0.03 +/− 0.08). **B-** Sample FRET images of an MDA MB 231 cell expressing eCFP- Rac1 and eYFP- PLCβ2 where the accompanying box plot shows changes in the normalized FRET efficiencies of cells treated with γ-synuclein (n=83) or control (n=80) siRNA ($p<0.001$, Rank Sum Test., Positive control = 0.44+/− 0.06, Negative control = 0.03 +/− 0.02).

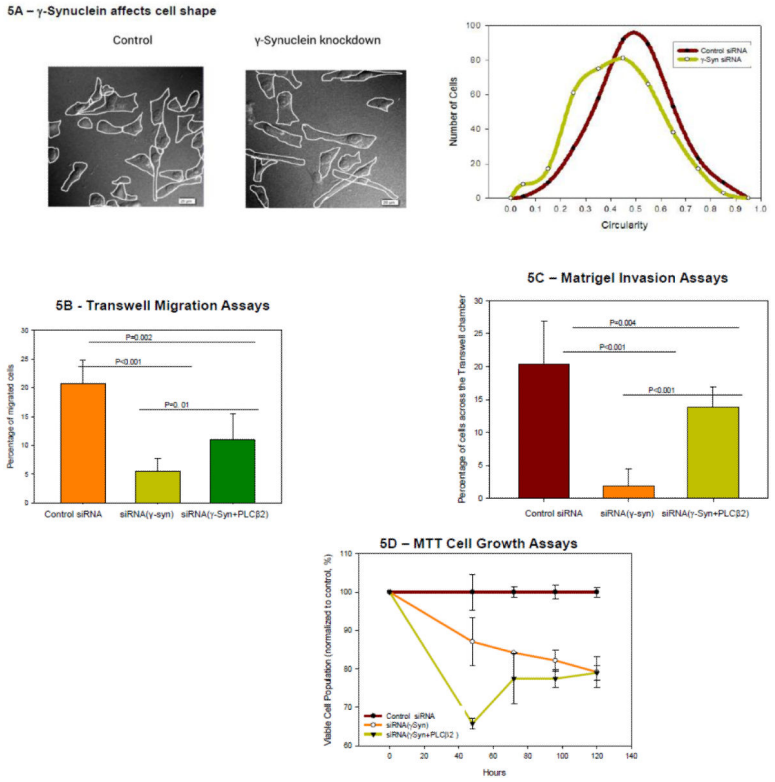


**Figure 5. γ-Synuclein and PLCβ2 affect MDA MD 231 cell phenotypes**

**A-** Sample DIC images of MDA MB 231 cells mock treated or treated with γ-synuclein siRNA and compiled results showing a significant difference in the circularity of cells between the two groups (both n=360, $p<0.001$, Student's t-test). **B-** Transwell migration assay showing the average number of cells treated with control siRNA, γ-synuclein and control siRNA, or γ-synuclein and PLCβ2 siRNA, that migrated per field, determined after fixing the transwell membrane and staining with DAPI after 4 hours of incubation (n=5, ANOVA). **C-** Transwell Matrigel invasion assay showing the average number of cells treated with control siRNA or γ-synuclein siRNA, that migrated per field, which was determined after fixing the transwell membrane and staining with DAPI after 4 hours of incubation (n=5, $p<0.001$ Student's t-test). **D**- MTT assays were used to evaluate cell growth after treatment with control siRNA, γ-synuclein and control siRNA, or γ-synuclein and PLCβ2 siRNA. Data were expressed as a percentage of the absorbance of control cells at 570nm. The mean ± SEM is shown (n=6).