Exploring Cellular Dynamics: Monitoring PLCβ1 Expression and Visualizing Subcellular Localization in PC12 Cells

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Exploring Cellular Dynamics: Monitoring PLCβ1 Expression and Visualizing Subcellular Localization in PC12 Cells

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Abstract

Phospholipase Cβ1 (PLCβ1) plays a vital role in intracellular signaling by catalyzing the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2) in response to extracellular signals. The activation of PLCβ is controlled by G-protein alpha subunits, particularly Gαq, which initiate downstream signaling cascades that are important for various cellular processes. Additionally, PLCβ1 expression also has other diverse physiological functions, including neuronal differentiation, and has been implicated in cancer and neurological disorders. PC12 cells, derived from adrenal medulla's pheochromocytoma in rats, are used to research neuronal differentiation and neurotoxicity when monitoring the expression of PLCβ1. Differentiation of PC12 cells induces PLCβ1 expression, which we propose influences the localization of Ago2, TRBP, and Egr-1 proteins. This study aimed to investigate the relocalization of Ago2 and TRBP proteins upon PLCβ1 expression modulation. The methods followed for this research involved cell culture, differentiation, transfection for PLCβ1 silencing, fixation, immunostaining, and TIRF-cell imaging. Understanding the interaction between PLCβ1 and the associated proteins in differentiated and transfected PC12 cells opens doors to potential therapeutic targets for neurological disorders and cancer.

Chapter 1 Literature Review

1.1 PLCβ1

Phospholipase Cβ1 catalyzes the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). This reaction is especially important for transmitting numerous extracellular signals within cells, specifically calcium signals in response to hormones and neurotransmitters. While calcium serves as an essential cofactor for this reaction, the activation of this enzyme occurs through one of two G-protein alpha subunits which are alpha-q and alpha-11 (*PLCB1 phospholipase C beta 1 [homo sapiens (human)] - gene - NCBI* 2024). In general, these G-proteins are heterotrimers composed of Gα, Gβ and Gγ subunits. The receptors to which a G-protein trimer interact are primarily determined by the Gα subunit, which interacts with extracellular molecules (such as acetylcholine, serotonin, histamine, bradykinin, endothelin I and angiotensin II). This interaction leads to the binding of those chemical messengers to specific G-protein-coupled receptor (GPCR) thus activating the heterotrimeric G proteins (Rennie et al., 2022).

When a G-protein is activated by a G-protein-coupled receptor (GPCR), the GDP molecule bound to the Gα subunit is exchanged for GTP. This exchange of GDP for GTP leads to a conformational change in the G-protein, allowing it to become activated and initiate downstream signaling cascades. Therefore, when GTP is bound to the Gαq subunit of the G-protein, it becomes activated and enables the activation of PLCβ which initiates intracellular signaling pathways (Weis & Kobilka, 2018). More specifically, the IP_3 product of the PLCβ hydrolysis enters the endoplasmic reticulum where it binds to specific receptors. These IP_3 receptors are responsible for the opening of Ca^{+2} channels, and which release calcium from the intracellular reserves into the cytoplasm. Consequently, these elevated calcium levels interact with numerous enzymes in the organism that affect crucial cellular processes such as neurotransmission, muscle contraction and generating action potential (Jackson et al., 2020).

Besides PLCβ's key role in transmembrane signaling, this enzyme also plays a role in; inducing differentiation to neuronal cells, impacting nuclear activity, gene translation, cancer, and neurological diseases such as schizophrenia (Rennie et al., 2022).

1.2 Ago2 and TRBP

TRBP, also known as TAR RNA binding protein, plays a vital role in various cellular processes, showing its significance within the PC12 cells. TRBP was first identified in aiding the replication of human immunodeficiency virus. This protein has shown to have broader functions, such as the inhibition of protein kinase R (PKR), an important player in innate immune responses and cellular stress reactions. TRBP achieves this inhibition by binding to PACT, an activator of PKR, thereby regulating PKR's activity levels. Additionally, TRBP is an essential component of the minimal RNA-induced silencing complex, where it collaborates with Dicer and Argonaute (Ago) proteins to mediate RNA interference processes . If TRBP's expression is deregulated or its sequence is mutated, it can result in cancer. That is why it is critical for TRBP to have a

balance for its expression levels in normal cellular function, because the depletion or overexpression of TRBP can lead to malignancy ((Daniels & Gatignol, 2012).

Ago2, or argonaute-2, serves as the primary nuclease component within the RNA-induced silencing complex (RISC) and can bind to small interfering RNAs (siRNAs) in their double-stranded form, retaining the parent strand. The complementary strand is degraded to allow hybridization with a target mRNA. When the pairing between the non-parental strand and the mRNA is perfect, as seen with exogenous siRNAs, Ago2 undergoes conformational changes leading to mRNA degradation. On the other hand, imperfect pairing, typical of endogenous microRNAs (miRNAs), prevents those conformational changes that are necessary for Ago2 nuclease activity, resulting in the formation of a stalled complex. Consequently, the formation and stability of these stalled complexes, along with their integration into stress granules, can influence protein populations. This alteration in protein populations may subsequently impact downstream protein-protein interactions, ultimately affecting cellular properties (Qifti et al., 2021).

1.3 PC12 Cells

All experiments of this research were conducted using PC12 cells derived from rat pheochromocytoma. This cell line shows features of mature dopaminergic neurons and characterization regarding neurosecretion, ion channels, and neurotransmitter receptors. These cells are highly versatile for pharmacological studies and are easy to culture, making them ideal for studying proliferation and differentiation processes. Under normal conditions, PC12 cells exhibit adrenal cell-like characteristics, but when treated with nerve growth factor (NGF), they differentiate into sympathetic ganglion neurons both morphologically and functionally (Wiatrak et al., 2020).

1.4 Working Model

The role of PLCβ1 in PC12 cells is significant, particularly in the context of neuronal differentiation and signaling pathways. Previous studies have shown that monitoring the expression of PLCβ1 levels can have impacts on differentiation and neuronal phenotypes, which could be associated with cellular responses that promote neuronal development and function. Understanding the mechanisms involved around PLCβ1 in PC12 cells can provide insights into neurobiology and potential therapeutic targets.

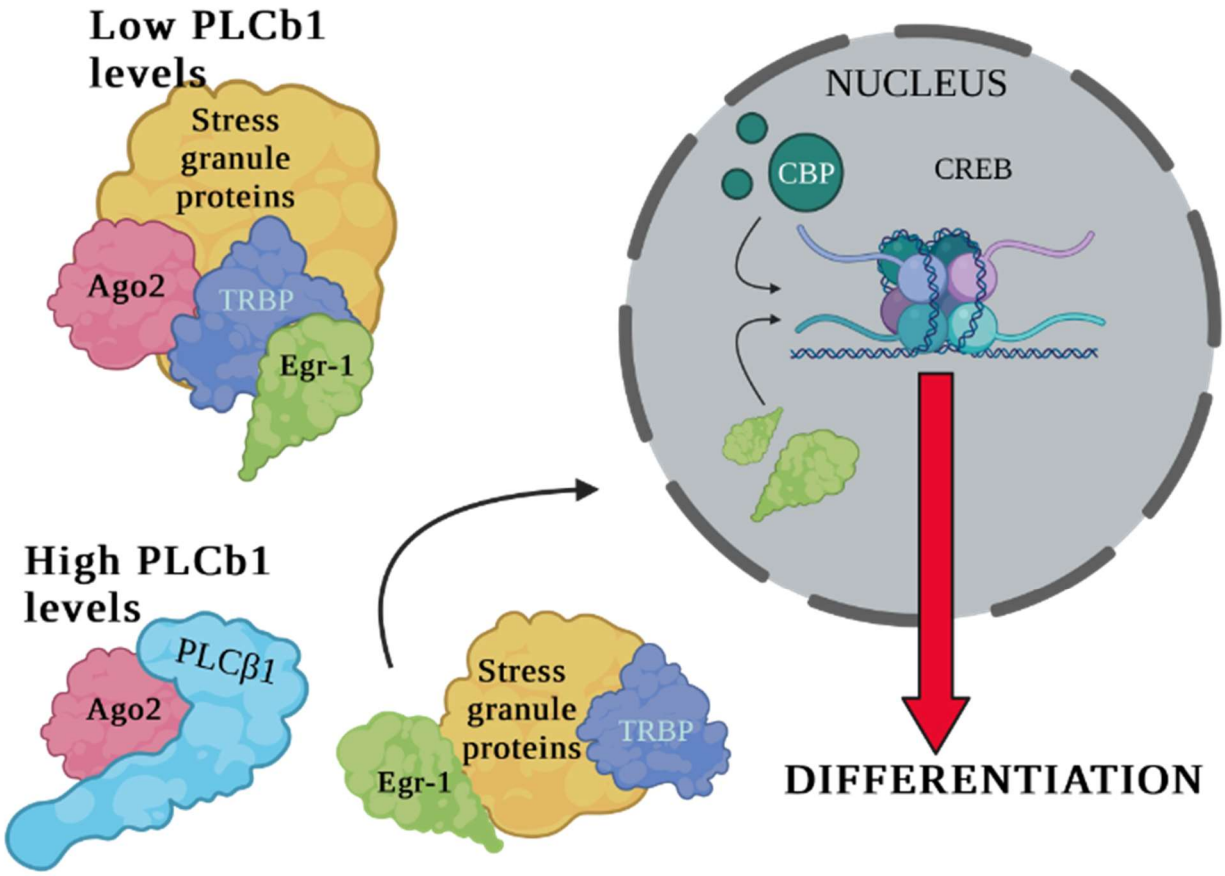

Figure 1: Working model of the localization of Ago2, TRBP and Egr-1 when there are high and low levels of PLCβ1 respectively.

The working model depicted in figure 1 is suggested from previous research done in the Scarlata Lab. It shows the interaction between Ago2, TRBP, Egr-1 and the stress granule proteins which form a complex in the cytosol at low PLCβ1 levels. Alternately, at high PLCβ1 a complex forms between PLCβ1 and Ago2 while Egr-1 moves in the nucleus to induce transcription of different proteins. Previous studies suggest that this relocalization of Egr-1 to the nucleus induces cell differentiation. As part of this research, the interaction between Ago2 and TRBP was investigated to test the validity of the model shown in figure 1 when there are high and low levels of PLCβ1 respectively. Those interactions serve as an important bedrock in understanding neurological disorders and potential therapeutic strategies.

Chapter 2 Materials and Methods

2.1 Cell Culture and Differentiation

The medium used to culture the PC12 cells for the experiments conducted was complete Gibco Dulbecco's Modified Eagle Medium (DMEM) containing 500mL DMEM, 10% horse serum HI, 5% FBS, and 1% pen strep. The PC12 cells were passaged every 2-3 days depending on the desired confluency for each experiment (~60%) and used between passage 8 and 13. A volume of 400µl of cells from a stock dish were split into a new dish containing 9.6ml of DMEM using 2ml of <0.05% trypsin for 2min.

Differentiated PC12 cells that promote a neuronal feature were established using differentiated DMEM and neuronal growth factor (NGF). The differentiated Gibco DMEM contained 500ml Gibco DMEM, 1% horse serum HI, and 1% pen strep. For every 2ml of differentiated DMEM there were 2µl of NGF for each glass bottom dish. All glass bottom dishes contained 50µl of differentiated PC12 cells and 1.5ml of differentiated media. All cells were incubated at 37°C, 5% CO_2 for 48 hours for optimal growth.

2.2 Transfection of PLCβ1

In order to silence the phospholipase Cβ1, a technique known as transfection with lipofectamine and siRNA molecules was utilized. Transfection dilutions consisted of 5µl of lipofectamine and 175µl of Optimem mixed in a single tube while 175µl of optimem were mixed with 4µl of Optimem into a different tube. The two tubes were combined and incubated at room temperature for 15 minutes. Complete media was replaced with 1.75ml antiobiotic-free media for each glass bottom dish and the transfection mix was added. The transfection dilution process repeated if more than one glass bottom dish needed to be transfected. The cells were then placed back in the incubator until confluency reached more than 50%.

2.3 Fixation

PC12 cells were fixed using paraformaldehyde (PFA) to preserve their morphology and structure in order to be analyzed under a microscope. A solution of PFA 37% was diluted to 10-fold with PBS for a final concentration of 3.7%. Prior to fixation, the media is aspirated from the cells under the hood, and the cells are then rinsed with PBS to remove any debris. The glass bottom dishes containing the cells are then transferred to a lab bench where 3ml of the prepared fixative solution is added to each one. The fixation time of the PC12 cells was 20 minutes. Once 20 minutes went by, the fixative solution was removed and the cells were rinsed three times with 1ml non-sterile PBS for 5 minutes each time. An additional 1ml of non-sterile PBS was added in each glass bottom dish and then placed in the cold room.

2.4 Immunostaining

Following fixation, the cells were then ready for staining with fluorescent dyes and antibodies. The desired antibodies used to stain the cells were targeting Ago2 and TRBP. The glass bottom dishes were brought to the lab bench and rinsed with PBS 3 times for 10 minutes each time. This step helped remove any residual fixative and prepared the dishes for antibody staining. Then, 2ml of blocking solution per dish was added to the cells to block nonspecific binding sites for 20 minutes. The blocking solution contained 15ml PBS, 0.1875ml 10% goat serum, 0.15g BSA and 0.05625g glycine for a total blocking of 6 glass bottom dishes (2ml per dish). First, the primary antibody, anti-TRBP or anti-Ago2, is diluted in a 1:1000 ratio in PBS + 1% BSA and 500µl were used for each dish for 1 hour. The primary antibody was then washed with 1ml PBS 3 times for 10 minutes each time to remove any residual non-binded antibody. Following, a secondary antibody that specifically recognizes the primary antibody is diluted in a 1:2000 ratio with PBS + 1% BSA and 500µl were used to stain each glass bottom dish for 1 hour. The secondary antibodies used were conjugated with fluorophores which allowed the visualization under the microscope of the target proteins. The specific wavelengths chosen fluoresced either green at 588nm or red at 647nm. Lastly, all glass bottom dishes were washed 3 times with PBS for 10 minutes each time.

2.5 TIRF- Cell Imaging

The glass bottom dishes containing the stained PC12 cells were visualized under a Total Internal Reflection Fluorescence (TIRF) microscope which is equipped with appropriate filter sets for detecting fluorophores used for antibody labeling. Images of the stained cells were captured using a 60x magnification for analysis. Once images were captured, they were transferred and analyzed through a software named ImageJ. This software saved the images with appropriate brightness setting and allowed them to be ready for further interpretation.

Chapter 3 Results and Discussion

Three proteins shown in figure 1 are argonaute-2 (Ago2), TAR RNA-binding protein (TRBP) and early growth response protein 1 (Egr-1). The rationale for exploring the subcellular localization of TRBP and Ago2 stems from previous research conducted in the Scarlata lab. Previous studies have focused on understanding PLCβ1 role in the relocalization of Egr-1. Therefore, this research focuses on TRBP and Ago2 to further unravel the molecular mechanisms between PLCβ1 expression and the localization of RNAi components.

3.1 Low PLCβ1 PC12 Cells

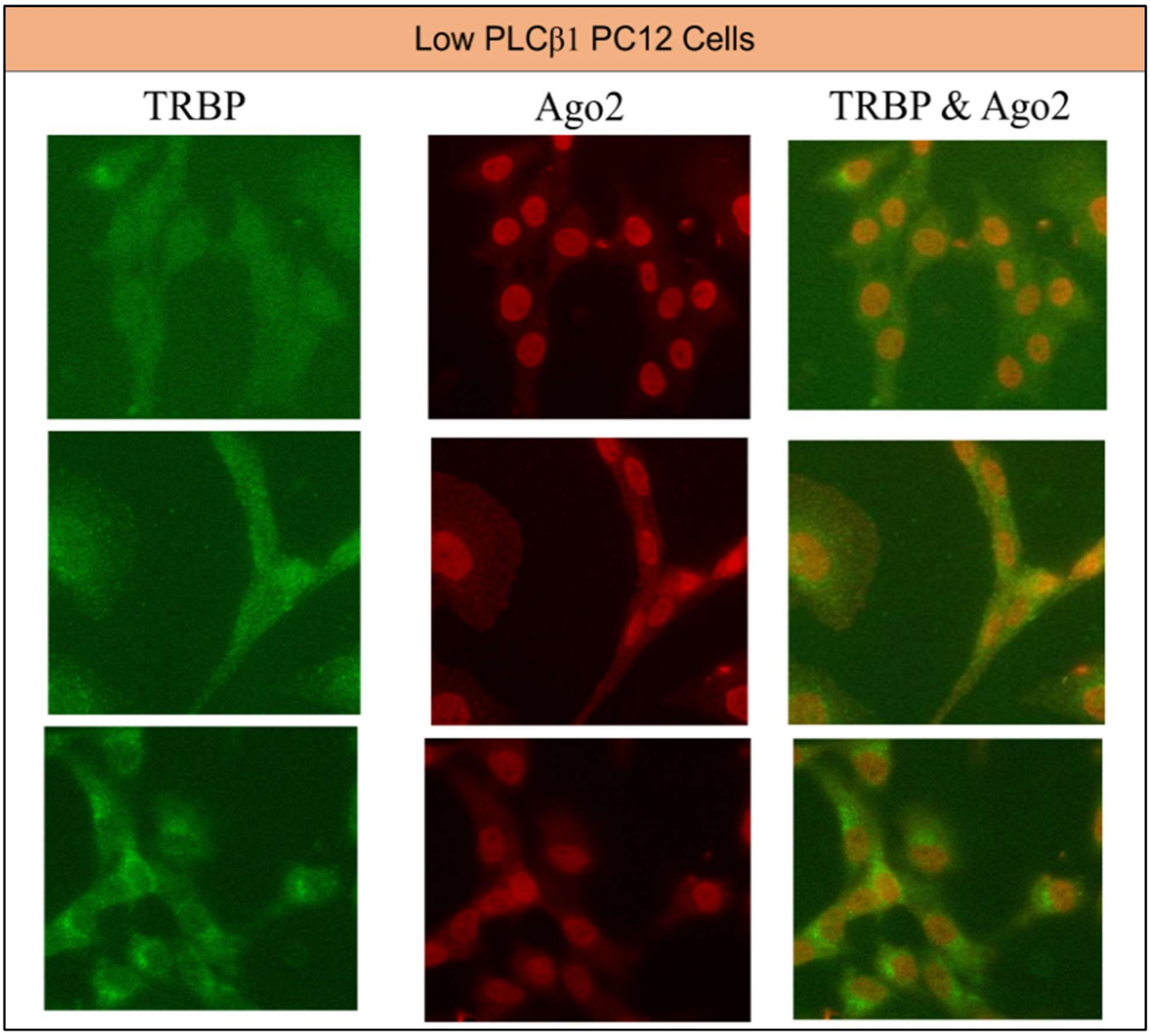

Figure 2: PC12 cell images under low PLCβ1 conditions (transfected cells) captured using a TIRF microscope. The images on the far-left column show TRBP stained cells with fluorophores that fluoresce color. TRBP is shown to be distributed throughout the cell. The second column suggests an Ago2 localization that is primarily in the nucleus exciting red color. While the third column on the right shows a combination of TRBP and Ago2 molecules.

3.2 High PLCβ1 PC12 Cells

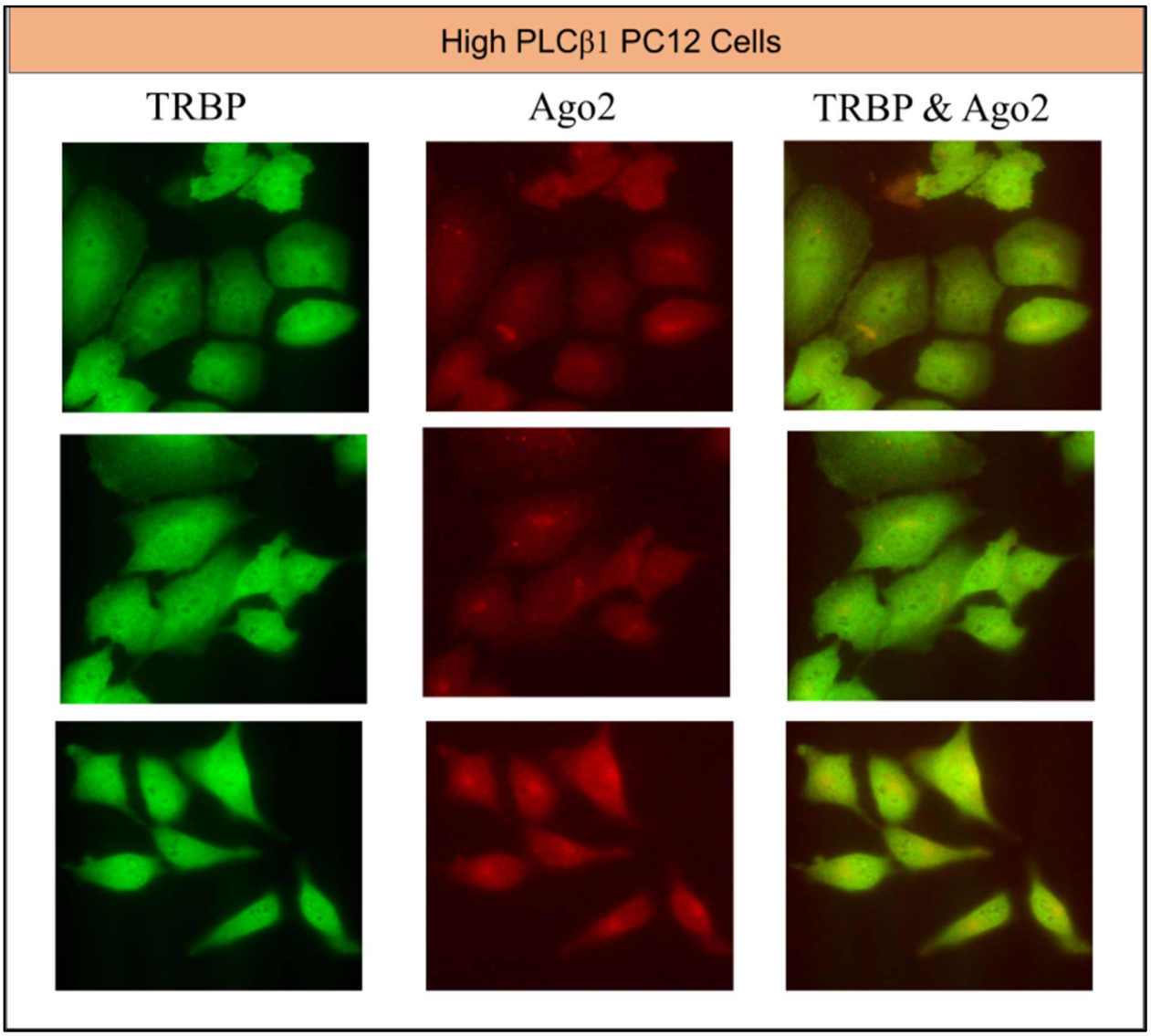

Figure 3: PC12 cell images under high PLCβ1 conditions (differentiated cells) captured using a TIRF microscope. The images on the far left column show TRBP stained cells with fluorophores

that fluoresce green. TRBP is shown to be distributed throughout the cell with a slight transition towards a more nuclear activity. The second column suggests an Ago2 localization that is distributed throughout the cell exciting red color. While the third column on the right shows a combination of TRBP and Ago2 molecules.

3.3 Discussion

In this study, I utilized TIRF microscopy to investigate the subcellular localization of TRBP and Ago2 under both low and high levels of PLCβ1 expression. Both figures 2 and 3 illustrate the results of multiple trials of cell imaging under various conditions. Previous research in the Scarlata lab has shown that PC12 cells gain a neuronal form upon differentiation. Furthermore, differentiating the cells leads to high PLCβ1 expression, shown in figure 3, as NGF promotes the activation of various signaling pathways within the cell, including the PLCβ1 pathway. Figure 2 shows PC12 cells after transfection of silencing RNA, which is the process conducted to reduce PLCβ1 expression using siRNA and lipofectamine, thus leading to low PLCβ1 levels.

Figure 2 presents images of PC12 cells at low PLCβ1 conditions. The first column of the figure shows TRBP staining with green fluorophores which reveals a diffuse distribution throughout the cell, which indicates its presence in both the cytoplasm and the nucleus. This observation is consistent with previous studies highlighting TRBP's function in various cellular processes beyond just in RNA interference. Interestingly the second column, Ago2 is observed being localized within the nucleus, indicated by red fluorescence. This nuclear Ago2 suggests a potential role in nuclear RNA processing or other nuclear functions under low PLCβ1 conditions. Lastly, in the third column, where TRBP and Ago2 signals are merged, colocalization in certain regions of the cell is observed, indicating potential interactions between the two molecules.

Figure 3 presents images of PC12 cells under high PLCβ1 conditions. The first column of the figure shows TRBP staining exciting a green fluorescent color, again showing a widespread distribution throughout the cell, with a shift towards increased nuclear activity in comparison to low PLCβ1 levels. Through this observation, it is suggested that PLCβ1 plays a regulatory role in controlling TRBP localization which can potentially impact its functional interactions within the cell. In the second column, Ago2 localization is more evenly distributed throughout the cell with no significant nuclear activity compared to low PLCβ1 levels. This shift of Ago2's activity implies a strong response of Ago2 to changes in PLCβ1 levels, which suggests further investigation into the regulatory mechanisms. Furthermore, the last column indicates an alteration between TRBP-Ago2 interactions in comparison to low PLCβ1 levels, as Ago2's activity shows no fluorescence intensity within the nucleus and a more challenging detection of its activity within the cell. While these results provide valuable insight into the cellular dynamics of PC12 cells, specifically for Ago2 and TRBP, quantifying the fluorescence intensity would result in more accurate analysis and thus a better understanding of the interactions within the cell.

To build upon this research and foster a better understanding of the subcellular localization of Ago2 and TRBP while monitoring PLCβ1 modulation, following are the suggested recommendations.

Chapter 4 Conclusion and Recommendations

4.1 Recommendations about Further Research Directions

More research needs to be conducted on investigating the molecular mechanisms behind the subcellular localization of Ago2 and TRBP in response to PLCβ1 expression in PC12 cells. While the results of this study stemmed from eye observations, to build off this research and, ultimately, continue the work, a future researcher could quantify the fluorescence intensity of Ago2 and TRBP staining to provide more precise and accurate analysis. Furthermore, it would be beneficial to further explore the functional consequences of Ago2 and TRBP localization on RNA interference processes, and cellular responses in PC12 cells, beyond the context of PLCβ1 expression. This could be achieved through assays which can assess the impact of Ago2 and TRBP localization on RNA interference processes and explore cellular responses by cell proliferation measurements such as cell counting. This process of cell counting can indicate a potential role of Ago2 and TRBP activity in promoting cell growth. Additionally, fluorescence staining techniques can be used to detect PLCβ1's activity in PC12 cells that can help to effectively visualize and identify PLCβ1 activity when cell imaging. Lastly, in addition to staining for Ago2 and TRBP, DAPI staining can be used to visualize the cells' nuclei. In that way, both TRBP and Ago2 nuclear activity can more easily be identified, thus, allowing a better and more precise analysis of their subcellular localization dynamics and functions in the cell.

By addressing these recommendations, we can gain a deeper understanding of cell dynamics between PLCβ1 expression and Ago2/TRBP localization, potentially leading to the identification of novel therapeutic targets and the development of targeted interventions for neurological disorders and cancer.

4.2 Conclusion

In summary, this study investigated the localization of Argonaute-2 (Ago2) and TAR RNA-binding protein (TRBP) while monitoring PLCβ1 expression in differentiated/ transfected PC12 cells. When PLCβ1 expression is low, Ago2 was shown to localize in the nucleus, while TRBP showed a more diffuse distribution throughout the cell. Conversely, upon induction of PLCβ1 expression in differentiated cells, TRBP showed increased fluorescence intensity shifting to a more nuclear activity. Meanwhile, Ago2 activity decreased in the nucleus while becoming more distributed throughout the cell. The findings of this research do not support the working model shown in figure 1. That is because the working model depicted indicates a cytosolic activity of the complex formed when there is low level of PLCβ1 activity, compared to the results that show an increased Ago2 nuclear activity in that same enzymatic level. Furthermore, while TRBP does show to have a role in the cytosol when the PLCβ1 levels are low, as figure 1 depicts, it can arguably be said that when PLCβ1 levels are high TRBP shifts towards a nuclear activity which is not depicted on figure 1's working model. However, Ago2's activity does show

a more even distribution throughout the cell, that confirms its role in the cytosol when PLCβ1 levels are high.

The results of the study showed a better understanding of PLCβ1 signaling and its significance for transmitting extracellular signals within the cell. Both, Ago2 and TRBP, are essential components of RNA interference (RNAi), which plays an important role in post-transcriptional gene regulation. These proteins are also shown to impact cellular processes such as neuronal differentiation and potential cancer progression. Overall, by expanding our knowledge on the functional roles of PLCβ1, Ago2, and TRBP in cellular processes, we can gain a deeper understanding on disease mechanisms and the development of neurological disorders and cancer, and potentially identify therapeutic targets.

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