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# Kinetic Changes of Ptdins (3,4,5) P3 within Fast and Slow Turnover Rates of Focal Adhesion

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## Abstract

***Background:*** The assembly and disassembly of the focal adhesions (FA) components occurs throughout life cycle of adhesion, with conservation of balance between removal and recruitment rate during temporal stages. Previous studies have demonstrated that phosphotidyilinositols play a role in regulating FA turnover. However, a little attention has been given to quantify the dynamics changes of Phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5) P3) within and during fast and slow turnover rates of FA.

***Methods:*** MDA-MB-231 breast cancer cell line was used as a model in this study due to high metastatic and motile. These cells were co-transfected with GFP- paxillin/vinculin, as FA marker, and the GFP/mCherry-Btk-PH, as a biosensor to visualize PtdIns (3,4,5) P3. Confocal time-lapse images were used to monitor changes or differences in the local generation of PtdIns (3,4,5) P3 within and during assembly and disassembly of FA. Following transfection, immunostaining was used to examine the spatial co-localization between FA and PtdIns (3,4,5) P3.

***Results:*** Our data demonstrated that PtdIns (3,4,5) P3 co-localized with FAs and increase during assembly and decline during disassembly of FA which exhibits slow turnover rates and was in a constant level during assembly and disassembly of FA that displays fast turnover rates.

***Conclusions:*** Our result suggested that the dynamic changes of PtdIns (3,4,5) P3, it may depend on components undergo turnover, such that early, nascent FA displays fast turnover rates and mature FA exhibits slow turnover rates. Thus, the local enrichment of PtdIns (3,4,5) P3 enhances FA assembly and disassembly activation.

**Keywords:** Cancer cell migration, Focal adhesion turnover, MDA-MB-231 cell line, PtdIns (3,4,5) P3.

## Introduction

Focal adhesion (FA) proteins are a set of proteins that are associated with the plasma membrane and aid in the linkage of the motile cell with the surrounding environment. They are known for their pivotal role in force transduction and modulate the cell signaling (1-6). They show different spatiotemporal polarity under different temporal stages (7,8,9). The group of FAs proteins include FAK, paxillin, vinculin, talin, α-actinin and zyxin. The FAK and paxillin are associated with integrin signaling while the vinculin and talin are associated with force transduction. The actin dynamics are regulated by α-actinin and zyxin (10,11). The integrin signaling recruits talin, vinculin and paxillin at the FA points and at the same time activate them (7,8, 12). There are multile proteins recruited to

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form the mature FAs that are involved in various structural and regulatory processes at the mature FAs (7,8). The protein complexes like actin-talin-integrin helps to stabilize the FAs and stress fibers. They recruit the extra FA partners like FAK (13,14), paxillin (15, 16) and Src-family kinases (SFKs) at the integrin tail (17,). There remains a dynamic balance between the removal and recruitment of the focal proteins over the cycle of cell adhesion. Different FAs show variable turnover rates over their life cycle (18-20). PtdIns(3,4,5)P3 is one of the key regulators of several signaling pathways (21, 22). It is generated by PI3K and associated with cell migration by activating AKT, PDK1 and various other cytosolic proteins (23-25). PtdIns(3,4,5)P3 restructures the FAs by deactivatingα-actinin and β-integrin interaction (24,25). The current study focuses on deciphering the lesser-known turnover dynamics of PtdIns(3,4,5)P3 during the nascent and mature FAs.

## Materials and Methods

### *Cell Lines and Culture*

MDA-MB-231 (ATCC HTB-26) cells from the American Type Culture Collection cell bank were acquired (Manassas, USA). MDA-MB-231 cells were cultured at 37 °C in a high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with L-glutamine, 10% (v/v) foetal bovine serum (FBS, Gibco), and 1% v/v penicillin/streptomycin environment with 95 percent air, 5% CO2, and 90% humidity. Mycoplasma testing was done on cells on a regular basis using an EZ-PCR Mycoplasma Test kit (Cat No. K1-0210, EZ-PCR Mycoplasma Test Kit, 20 tests, Geneflow, UK), as directed by the manufacturer.

### *Construction of Biosensors*

Btk-PH-GFP (Addgene; Plasmid #51463) and mCherry-N1 (TAKARA; #632523) were cut using BamH1 and EcoR1 restriction enzymes to form the Btk-PH-mCherry reporter. The resultant Btk-PH fragment was fused to the mCherry-N1 construct (TAKARA #632523) at the restriction sites indicated above.

### *Live cell Imaging and Transfection*

In ibdi dishes, 2 mL of 1 105 MDA-MB-231 cells/mL were planted and covered with ECM collagen (BD Bioscience). 2-mg/mL non-pepsinised rat tail type 1 collagen (diluted in DMEM; pH adjusted to 7.0 with NaOH) for 15 minutes. At a w/w ratio of 1:1, 100 L of serum-free DMEM was used to dilute 3µg of plasmid DNA containing either vinculin-GFP (Addgene; plasmid #26720) or paxillin-GFP Btk-PH-(Addgene; Plasmid #51463) and mCherry (construct built in lab). The reaction was incubated for 15 minutes after adding 6µl of Polyethylenimine (PEI) (Sigma Aldrich). After that, the PEI/DNA combination was applied to the cells and incubated for 24 hours. A confocal microscope (Nikon Eclipse Ti Laser-scanner) and a heated chamber (37 °C) perfused with 5% CO2 were used to see live cells. GFP and RFP (or mCherry) fluorescence was observed at 488 nm/510 nm and 568 nm/590 nm, respectively (excitation/emission). The fluorescence signal-to-noise ratio was reduced by adjusting the laser intensity and exposure time.

### *Immunofluorescence Staining*

MDA-MB-231 cells were cultivated on glass coverslips with collagen cell surfaces and washed with PBS supplemented with Ca2+ and Mg2+ after transfection, then fixed for 20 minutes at room temperature with 4 percent PFA (diluted in PBS). The cells were then rinsed three times in PBS for ten minutes each time, followed by a permeabilization with 0.5 percent Triton-X 100 for ten minutes (diluted in PBS). The cells were treated for 1 hour with either an anti-vinculin antibody (Abcam; ab50391) (1:100) or an anti-paxillin antibody (Abcam; ab23510) (1:100), both diluted in 2 percent goat serum, after 30 minutes of blocking with 10 percent goat serum (diluted in 0.5 percent Triton-X 100). Excess primary antibodies were eliminated by washing the cells three times in PBS for ten minutes each time; following that, the cells were treated for one hour with an Alexa Fluor 488 conjugated

rabbit anti-IgG antibody (Cell Signalling; 4412; 1:100). The nuclei were labelled, and the cells were mounted onto glass slides with the fluoroshield mounting media containing DAPI after 3 successive washes (PBS; 10 min each time) to remove unbound secondary antibodies. Confocal microscopy was used to examine the cells (Nikon Eclipse Ti Laser-scanner). The Alexa Fluor 488 was detected at 488 nm/510 nm (excitation/emission) and the DAPI at 350 nm/470 nm (excitation/emission).

### *Quantification of PtdIns(3,4,5)P3 level within and during FA turnover*

On images produced from fixed and living cells, co-localization analysis between PtdIns(3,4,5)P3 and viculin/paxillin were done using Image J software. All photos were converted to 8-bit colour graphics, and each image was divided into two channels: one for fluorescence from GFP-FA, and the other for fluorescence from either mCherry/GFP-PtdIns(3,4,5)P3. Background fluorescence removal was used to improve the signal-to-noise ratio of each image. After magnification, individual FA was examined, and the co-localization coefficient was calculated usingthe Spearman's rank correlation coefficient. PtdIns(3,4,5)P3 and FA quantifications were carried out using Image J within a region of interest (ROI) defined in the vicinity of individual FA. RFP/GFP fluorescence in relation to FA and GFP/mCherry fluorescence in relation to PtdIns(3,4,5)P3 were studied in the ROI. Throughout the lifespan of FAs, the intensity of PtdIns(3,4,5)P3 fluorescence was monitored.

### *Statistical Analysis*

GraphPad Prism 5 was used to conduct all statistical analyses (GraphPad Software, San Diego, CA). All results are based on triplicates from at least three separate tests (N= 3).

## Results

### *Spatiotemporal organization of PtdIns(3,4,5)P3 during fast and low FA dynamics rates*

To evaluate the potential co-localization value between PtdIns(3,4,5)P3 and vinculin/paxillin, MDA-MB-231 cells cultured on collagen and co-transfected Btk-PH-GFP/mCherry biosensors and vinculin/paxillin-GFP. The coefficients resulting from the spatial co-localization was 0.55±0.08 (Figs. 1A and B).

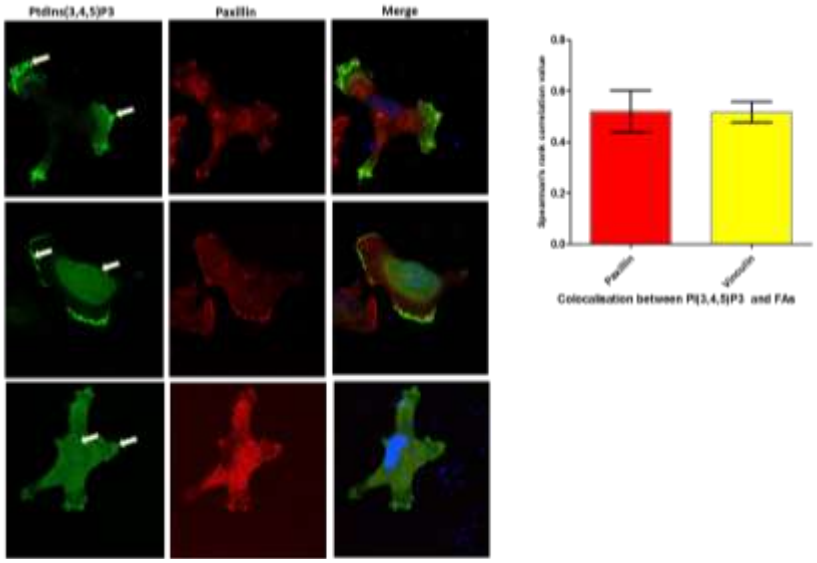


**Fig. 1.** Spatial co-localized distribution of PtdIns(3,4,5)P3 and FAs. (A) The spatial interaction between PtdIns(3,4,5)P3 and vinculin/paxillin were visualized using MD-MB-231 cells that were transfected with Btk-PH-GFP(green) and immunostained for vinculin and paxillin (red). The co-localization value between ROI of PtdIns(3,4,5)P3 and vinculin/paxillin was obtained by doing the Spearman's (rho) correlation coefficient analysis which was found to be moderate. The data shown in the representative image represents the means ± SD of three independent experiments in which n= 30 cells, and 60 single FAs per cell were measured. Scale bars= 6 µm.

Then we went on to directly examine the dynamic change of PtdIns(3,4,5)P3 within and during on FA displays high and slow turnover rates. MDA-MB-231 cells were co-transfected with GFP-vinculin/talin and Btk-PH-GFP/mCherry seeded on collagen-coated chambered slides and time-lapse sequences were recorded, over a period of 10 minutes. Cells expressing the construct displayed slow and high rates of FA turnover. The intensities of fluorescence of PtdIns(3,4,5) P3 was measured within the ROI of vinculin/paxillin throughout the course of the complete lifetime (turnover cycle) of the vinculin/paxillin (Figs. 2A and 3A). Our data found that PtdIns(3,4,5) P3 increase during assembly and decline during disassembly of FA shows slow rates of turnover while it was in constant level during FA show high rates of turnover (Figs. 2C-D and 3C-D).

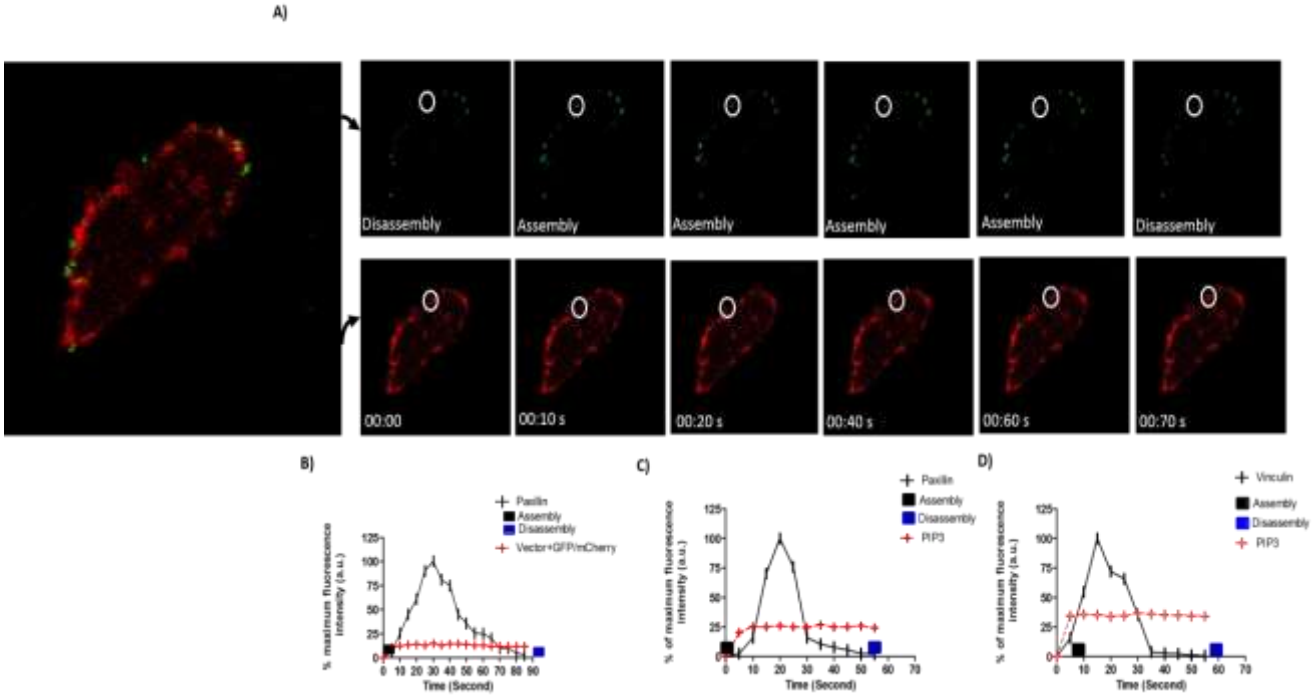


**Fig. 2.** Quantitative assessment of the % change in the PtdIns(3,4,5)P3 intensity in the cells expressing GFP-vinculin/paxillin with faster turnover rates. The MDA-MB-231 cells were co-transfected with Btk-PH-mCherry and GFP-vinculin/paxillin. The confocal time lapse images were taken for 10 minutes with an interval of 15 seconds, indicating the % change of PtdIns(3,4,5)P3 in the average intensity of selected FA with respect to the time frame of the study. The paxillin/vinculin assembly and disassembly of FA is represented by the first row while the level of PtdIns(3,4,5)P3 within the FAs turnover area is indicated by the second row. (A) The highlighted region marks the area of interest representing localization and levels of PtdIns(3,4,5)P3 over the selected FAs. Note: The selected FA used for the analysis should be absent in the beginning and the end of the time-lapse interval under consideration. (B) The intensity level of vinculin/paxillin assembly and disassembly is marked by the black curve for the time course of 70 seconds. The intensity levels of vector + mCherry remained constant for the time course of 70 seconds during the vinculin/paxillin assembly and disassembly as marked by the red curve. (C and D) The quantitative assessment of the change in the local levels of Btk-PH-mCherry was assessed during the assembly and disassembly of FA. The intensity of vinculin/paxillin assembly and disassembly was measured for the time course of 70 seconds, which is indicated by the black curve while the local of PtdIns(3,4,5)P3 level was also at constant level within the vinculin/paxillin assembly and disassembly measured for the time course of 70 seconds is indicated by the red curve.

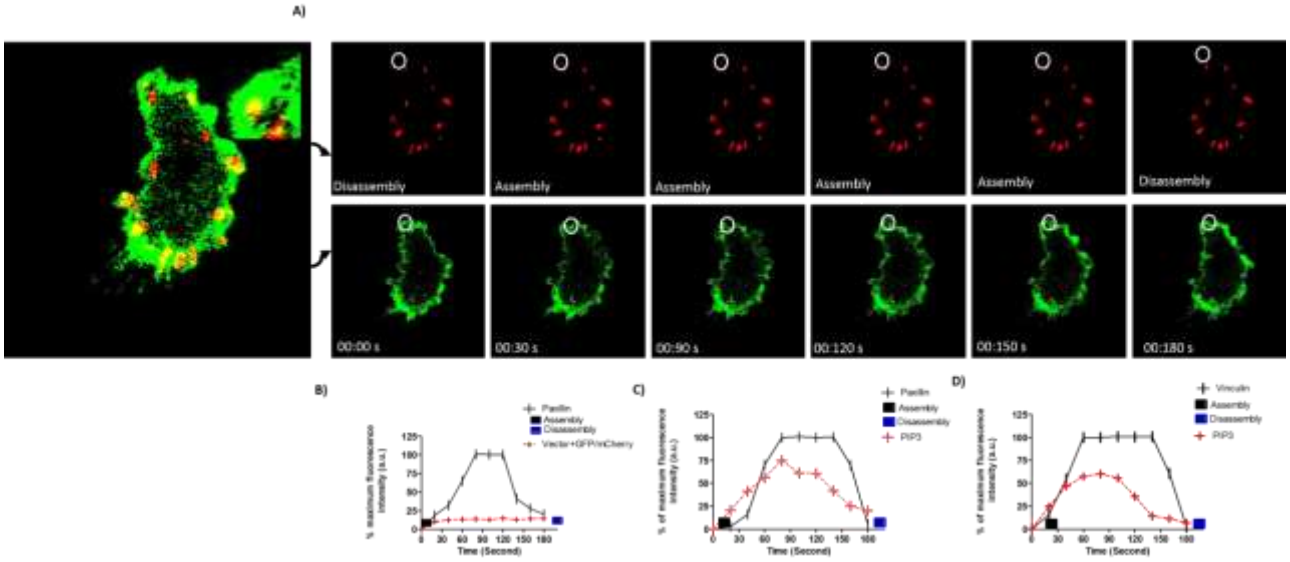


**Fig. 3**. Quantitative assessment of the % change in the PtdIns(3,4,5)P3 intensity in the cells expressing GFP-vinculin/paxillin with slower turnover rates. The MDA-MB-231 cells were co-transfected with Btk-PH-GFP and RFP-vinculin/paxillin. The confocal time lapse images were taken for 10 minutes with an interval of 15 seconds, indicating the % change of PtdIns(3,4,5)P3 in the average intensity of selected FA with respect to the time frame of the study. The paxillin/vinculin assembly and disassembly of FA is represented by first rows while the levels of PtdIns(3,4,5)P3 within the FAs turnover areas are indicated by the second rows. (A) The highlighted region marks the area of interest representing localization and levels of PtdIns(3,4,5)P3 over the selected FAs. Note: The selected FA used for the analysis should be absent in the beginning and the end of the time-lapse interval under consideration. (B) The intensity level of vinculin/paxillin assembly and disassembly is marked by the black curve for the time course of 180seconds. The intensity levels of vector + GFP were remained constant during the vinculin/paxillin assembly and disassembly as marked by the red curve. (C,D) The quantitative assessment of the change in the local levels of Btk-PH-GFP was assessed during the assembly and disassembly of FA. The intensity of vinculin/paxillin assembly and disassembly was measured for the time course of 180 seconds, which is indicated by the black curve while the local increase and reduction in the levels of PtdIns(3,4,5)P3 within the vinculin/paxillin assembly and disassembly over a time course of 180 seconds is indicated by the red curve.

## Discussion

In the current study, an attempt was made to visualize and understand the dynamics of PtdIns(3,4,5)P3 in MDA-MB-231 cell membrane. Different biosensors of PtdIns(3,4,5)P3 are available like the cytosolic regulator adenyl cyclase (CRAC) protein, Akt-PH domain (26, 27), and Btk-PH domain (28,29). Akt-PH and CRAC are known to interact with PtdIns(3,4,5)P3 and PtdIns(4,5)P2 both (30). In this study we used Btk-PH as the PtdIns(3,4,5)P3 sensor, as it is selective and specific for PtdIns(3,4,5)P3 only and binds it with high affinity (28,29). In our study, we found different recruitment pattern of Btk-PH based on its concentration in the cell. A very few cells showed limited localization of Btk-PH-GFP in the MDA-MB-231 cell membranes when the expression of Btk-PH-GFP was low in the transfected cells (Fig. 1A) while the cells that expressed higher concentration of Btk-PH-GFP after transfection showed localization of Btk-PH-GFP in the cell membrane as well as in the cytosol (Figs. 1B and 1C). The probable reason for this selective localization could be its dependence on the enrichment of PtdIns(3,4,5)P3 at the MDA-MB-213 cell membrane. The available PtdIns(3,4,5)P3 in the cell membrane gets saturated with Btk-PH-GFP while the remaining Btk-PH-GFP interacts with other types of inositides in the cytosol (29). It has been observed that the overexpression of PH domain influences the specificity and affinity of kinases with inosities and modulate the cellular metabolism of the cell (29) and make them the ideal candidate for studying the dynamics of phosphotidylinositol in live cells (29, 31). Looking at the other side of the coin, the synthesis and concentration of the

PtdIns(3,4,5)P3 is itself dependent on certain stimuli. The levels of PtdIns(3,4,5)P3 gets upregulated on the stimulation of PI3K at the cell membrane by EGF (25, 29), while the synthesis of PtdIns(3,4,5)P3 gets downregulated when the PTEN is recruited at the cell membrane that dephosphorylates the PtdIns(3,4,5)P3 to PtdIns(4,5)P2 (32). These interactions with PI3K and PTEN might affect the local concentrations of the PtdIns(3,4,5)P3 at the cell membrane. Our results also demonstrated the co-localization of PtdIns(3,4,5)P3 with FA (Fig. 1D), where the levels of PtdIns(3,4,5)P3 increased during the assembly and decreased during the disassembly of mature FA that followed slow turnover time rates (Fig. 2). However, the levels of PtdIns(3,4,5)P3 remained constant during the assembly and disassembly of nascent FA that followed the fast turnover time rates. This variation in the concentration of PtdIns(3,4,5)P3 between the dynamics of nascent and mature FA can be explained based on the other interacting partners recruited at the FA during its lifecycle. Several enzymes like PI3K, PLC and PTEN are recruited at the mature FA that upregulates the synthesis and hydrolysis of the PtdIns(3,4,5)P3, while these enzymes are absent in the nascent FA. It has been reported by multiple groups that the initiation of FA is mediated by the interaction of talin and integrin, which in-turn recruits other intracellular proteins like vinculin and α-actinin to form nascent FAs. The fate of FA to remain in the nascent state or to grow in size and form mature FAs depends on the recruitment of many cellular proteins that respond to the mechanical force generated from actomyosin and the ECM. The difference in assembly and disassembly rate at the FA may transform the mechanical signal to chemical signals inside the cells (18, 33-35). Hence, it can be said that the local activation of PI3K and the production of PtdIns(3,4,5)P3 in FA-dependent manner mediates the formation of mature FAs on the cell membrane. We observed the enrichment of PtdIns(3,4,5)P3 precedes the FA assembly activation, and its levels are detectable in mature FAs and undetectable in the nascent FAs. The concentration of PtdIns(4,5)P2 at the cell membrane is much higher than the PtdIns(3,4,5)P3 and could be the reason for the undetectable change in the local concentration of PtdIns(3,4,5)P3 at the nascent FA. PtdIns(3,4,5)P3 are speculated to indirectly control the cell migration by activating the signaling pathways involved in the cell migration (36). PtdIns(3,4,5)P3 might regulate the FA dynamics by recruiting different kinases and strengthen the kink between FAs and integrin molecules (37, 38). Other groups have showed the role of PtdIns(3,4,5)P3 in restructuring the FAs by its association with α-actinin and deactivating the interaction between α-actinin and β integrin (39).PtdIns(3,4,5)P3 might be involved in other aspects of cancer migration and suspected to be non-fundamental in FA turnover (40).

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