# Investigating the role of plasma membrane phosphoinositides in the regulation of the plasma membrane localization and function of Ras proteins

Ph.D. thesis

**Gergő Gulyás, M.D.**

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor: Péter Várnai

Reviewers: István Kenessey
Gabriella Czifra

Chairman of the comprehensive examination board:
László Tretter

Members of the comprehensive examination board:
Gábor Földes
László Homolya

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# Introduction

The interaction of intracellular signaling proteins with the hydrophobic membranes of eukaryotic cells is critical for the signaling competence of these proteins. In the case of the peripheral PM proteins, the interaction with the PM can occur via different mechanisms. Proteins that contain an amphipathic α-helix or hydrophobic loops can directly penetrate into the inner surface of the membrane. Others, however, do not contain enough clustered hydrophobic amino acids to maintain a stable membrane interaction. In this case, other mechanisms are necessary to attach the protein to the PM. Many signaling proteins undergo post-translational lipid modifications, attaching a myristoyl, palmitoyl or prenyl group on specific amino acid residues. These modifications provide the lipophilicity of the proteins ensuring proper PM localization. Multiple lipid modificated proteins can bind stably to the PM, but those that have only one terminal either myristoyl or prenyl group cannot maintain permanent PM connection. For stable localization, the presence of further ,,second signals" are necessary on the proteins. This ,,second signal" can be an additional lipid modification - in most cases palmitoylation-, or a complementary electrostatic interaction occurring between negatively charged phospholipids of the inner leaflet of the PM and basic amino acids of the proteins.

At cytoplasmic pH, the cytosolic face of the PM is enriched in two types of anionic phospholipids: phosphatidylserine (PS) is the most abundant anionic phospholipid, amounting to 3-10% of the total phospholipid pool of the cells found in the inner leaflet of the PM. The other is the various phosphoinositides (PPIns) that account less than 1% of total anionic phospholipids. Although PS is found in a relative high amount in the PM providing the bulk negativity for the inner leaflet of the membrane, its precise role in controlling cellular processes is vastly under-explored. In contrast, the impact of the PPIns in the regulation of variety of cellular processes is well established. The PPIns are a uniquely important class of phospholipids built from a diacylglycerol (DAG) backbone linked to an inositol ring via a phosphodiester linkage. There are seven distinct types of PPIns, in which the inositol ring is reversibly phosphorylated at positions 3, 4 and 5 by a plethora of lipid kinases and phosphatases. The PM of the eukaryotic cells contain three types of PPIns: phosphatidylinositol 4-

phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$) equally share the majority role, whereas phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)$P_3$) is present only in a very small amount even in stimulated cells.

PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ are well-known signaling molecules in G-protein coupled receptor and receptor tyrosine kinase pathways, but beside their triggered signaling events, these lipids have substantial roles in the regulation of ion channels, such as $K^+$-channels or store operated $Ca^{2+}$-entry (SOCE), cell motility, vesicular trafficking, protein synthesis, cell proliferation and they also represent molecular clues for targeting signaling proteins to membranes. These regulatory functions are achieved either via specific lipid-protein interactions, where the proteins contain preformed specific lipid-binding domains which are able to bind specific PPIns, or they could be managed due to non-specific electrostatic lipid-protein interactions occurring between the negatively charged phosphate groups of PPIns molecules and positively charged amino acids in the protein sequences.

Ras protein mutations are one of the most common causes in cancer and hence have attracted a lot of attention. These small globular proteins are molecular switches that change conformation upon GTP binding relative to their GDP-bound forms, therefore they can regulate the activity of numerous molecular partners, which control cell proliferation events. Their C-termini contain their localization signal providing for membrane attachment due to post-translational modifications. Numerous oncogenic mutations of Ras proteins have been identified in the N-terminal interacting and GDP/GTP binding portion of the protein, many of which affect GTP binding of these proteins, but naturally occurring mutations in the C-terminal anchoring domains have not been reported yet, showing the uniquely significant role of this conserved essential segment in the proper membrane localized Ras protein functions. H-Ras proteins undergo two lipid modifications: in addition to their C-terminal prenylation, they are also palmitoylated in their C-terminal tails. In contrast, K-Ras protein contain only the prenyl modification and their PM anchoring is highly dependent on a polybasic amino acid sequence as a „second signal” located in their C-terminal tail. Previous studies have shown that changing the positively charged amino acids to neutral amino acids by site directed mutagenesis can fully abolish the PM localization of the K-Ras protein.

Such mutated K-Ras proteins show non-specific endomembrane localization similarly to that of proteins that have only a prenyl or myristoyl group as targeting signals.

There are many examples in the literature for controversial experiments, which demonstrated an intracellular Ras protein mislocalization upon decreasing the negative surface charge of the inner leaflet of the PM. Based on these studies, PM PPIns appear to be important in K-Ras PM recruitment, although the detailed exploration of the importance of the different types of PPIns molecules, the examination of the route through which the protein rearrangement occurs and the determination of the target endomembrane systems remains unclear. Besides the significance of the PPIns in the PM anchoring process, the role of PtdSer on K-Ras localization has been reported. Although PS only carries a single negative charge it is present in the inner leaflet of the PM in relatively high amount, therefore, could contribute to the localization of proteins containing basic surface patches.

The proliferating effect of the Ras proteins depends on the activation of three distinct pathways: 1.) active Ras can elevate PM PtdIns(3,4,5)$P_3$ levels due to activation of PI3K enzymes and therefore activates Akt signaling 2.) it can directly activate the MAPK cascade via activation of Raf proteins and 3.) it can activate the Ral-GDS protein, which than triggers the conversation of GDP bound inactive Ral proteins to GTP bound active form. Although this latter pathway is less established compared to the plenty of literature on the first two pathways, its significance is increasing as a major factor in the transforming ability of the Ras proteins.

There are many examples in the literature that small G-proteins are capable of initiating signaling events from endomembranes. It has been reported that Ras proteins can be activated at the Golgi and are able to initiate proliferation signals through the activation of ERK. These reports, however, referred to H- and N-Ras, but not K-Ras4B, since during its intracellular trafficking K-Ras is not found in the Golgi. However, a potential significance of the translocation of K-Ras from the PM to the endomembranes could be that Ras signaling might be switched on in these other organelles, if Ras modulating proteins can control their activation state.

The store operated $Ca^{2+}$-entry (SOCE) plays an important role in the physiological $Ca^{2+}$-homeostasis of the cells. This process is triggered through the depletion of the ER $Ca^{2+}$-stores which is evoked physiologically upon activation of PLC

enzymes by numerous surface receptors. After the stimulation, the enzyme hydrolyzes PtdIns(4,5)$P_2$ molecules of the PM, and therefore generate inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$) in remarkable extent which than binds to its ER localized receptor resulting elevated $Ca^{2+}$-permeability of the organelle. The initiation of the SOCE process occurs through a $Ca^{2+}$-depletion-evoked conformational change on the luminal portion of the ER resident STIM1 protein, which then dimerizes and oligomerizes before travels to the ER-PM contact sites to activate the other important component of this current, the Orai1 channels. Previous studies showed, that PM PPIns could play a regulatory role in the activation and maintain of this process, but the precise mechanism, and the detailed function of the different PM PPIns molecules through which the regulation occurs, remains undiscovered.

The major aim of my Ph.D. work was to investigate how the rapid depletion of PM PPIns can regulate the intracellular distribution and function of lipid anchored peripheral PM proteins, especially in the case of the K- and H-Ras proteins, and how this lipid modification can influence the SOCE process.

# Objectives

However, several previous works showed, that PM PPIns molecules play an important role either in the Ras protein membrane association and function, or in the regulation of the SOCE process, the involvement of the distinct PM PPIns in these processes is not fully explored. Our research group and our collaborators developed and successfully employed a method, the rapamycin induced PM lipid-depletion system in the past years, with which is possible to occur a rapid and specific reduction of the distinct PM PPIns pools. Using this system, we wanted to examine the precise PM PPIns dependence of the two above-mentioned cellular processes.

The major aims of my Ph.D. work were the following:

- to develop a quantitative method, which is capable to follow the intracellular rearrangements of peripheral PM proteins, especially in the case of the Ras proteins
- to investigate the intracellular redistribution and its route of the peripheral PM proteins including Ras proteins after PM PPIns depletion
- to examine the role of distinct PM PPIns in the stabilizing process of peripheral PM proteins
- to explore physiological processes which could provoke the rapid intracellular redistribution of the Ras proteins
- to study the functional consequences of the PM PPIns depletion induced Ras protein rearrangements
- to investigate the regulatory role of the PM PPIns molecules in the SOCE process

# Methods

## DNA constructs

Wild type human $M_3$ cholinergic receptor (M3R) was purchased from S&T cDNA Resource Center. The human EGF receptor (EGFR), and the non-internalizing rat type-I angiotensin receptor (AT1R-Δ319) were described earlier by our group.

To create the various PM targeted Venus constructs, the targeting sequences coding DNA-fragments of the following proteins were synthetized as DNA-oligo (Sigma): the N-terminal 14 amino acids (AA) of Lyn ($Lyn_{1-14}$), the N-terminal 10 AA of Lck ($Lck_{1-10}$), the N-terminal 15 AA of c-Src ($c\text{-}Src_{1-15}$), and the C-terminal 22 AA both of K-Ras4B, and H-Ras proteins (K-Ras-CAAX and H-Ras-CAAX). The fragments were then inserted into monomeric Venus encoding N1 vector using the NheI and BglII enzymes in the case of the N-terminal targeting sequences, or into C1 plasmid using the EcoRI and BamHI sites in the case of the C-terminal Ras sequences. The fluorescent Citrine tagged full-length K-Ras4B G12V and the wild type or G12V mutant H-Ras encoding plasmids were a kind gift from John F Hancock. To create our monomeric Venus tagged versions, the sequences of the Ras proteins were amplified using PCR, and the fragments were cloned into monomeric Venus containing C3 vectors. The missing wild type and mutant versions of the Ras proteins (K-Ras wt, K-Ras S17N, and H-Ras S17N) were created using site directed mutagenesis.

To create the various endomembrane targeted Luciferase constructs, the coding DNA-fragments of targeting sequences or the full-length forms of the following proteins were used: TOM 70, TGN38, Sac1 and EEA for mitochondria, Golgi, endoplasmic reticulum and early endosome targeting, respectively. The N-terminal DNA-coding sequence of the first 30 AA of the TOM 70 protein was synthetized as DNA-oligo, and then was ligated into super Renilla luciferase containing N1 plasmid, using the NheI and BglII enzyme pairs. The sequences of the TGN38, Sac1 or EEA1 were used earlier. The proper fragments of these proteins were amplified with polymerase chain reaction, then were inserted into super Renilla Luciferase containing N1 vector using NheI and BglII enzymes in the case of the TGN38-Luciferase construct or into super Renilla Luciferase containing C1 vector using EcoRI and BamHI sites by Luc-ER and Luciferase-EEA1-FYVE constructs.

The rapamycin inducible lipid depletion system, applied for the PM PPIns depletion, and the TGN38-FRB-mRFP, which were used for recruiting our cytoplasmic phosphatase to the Golgi, were described earlier. The specific BRET-based lipid-, Ins(1,4,5)$P_3$-(R265K) and $Ca^{2+}$-sensors, were also previously used.

## Cell culture

HEK 293T, COS-7 and HeLa cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a 5% humidified CO2 incubator at 37°C in 10 cm tissue culture plastic dishes.

## Bioluminescence Resonance Energy Transfer (BRET) measurements

For BRET measurements HEK 293T cells were trypsinized and plated on poly-L-lysine-pretreated (0.001%, 1 hour) white 96-well plates at a density of $7x10^4$ cells/well together with the indicated DNA constructs (0.15-0.2 µg total DNA/well) and the cell transfection reagent (1.5 µl/well GeneCellin) in Opti-MEM reduced serum medium (Gibco). After 6 hours 100 µl/well DMEM containing serum and antibiotics was added. Measurements were performed 25-26 hours after transfection. Before the measurements, the medium over the cells was changed to a mosified Krebs–Ringer buffer (50 µl). Measurements were performed at 37°C using a Thermoscientific Varioskan Flash Reader (PerkinElmer). The measurements started with the addition of the cell permeable luciferase substrate coelenterazine h (40 µl, final concentration of 5 µM). The reagents were also dissolved in modified Krebs–Ringer buffer and were added manually in 10 µl. All measurements were done in 2-4 biological replicates. By the measurements of the PM lipid levels, BRET ratios were calculated by dividing the 530 nm and 485 nm intensities and normalized to the baseline. Since the absolute ratio values depended on the expression of the sensors in case of the intermolecular inositol lipid sensors the resting levels were considered as 100%, whereas the 0% was determined from values of those experiments where cytoplasmic Renilla luciferase construct was expressed alone. In the case of the interaction experiments between the Venus tagged PM-targeted constructs and Luciferase tagged endomembrane targeted constructs, the difference of the BRET ratios was calculated between rapamycin (300 nM) or carbachol (10 µM) treated and vehicle (DMSO, or distillated water) treated

cells, respectively. In the case of the cytoplasmic Ins(1,4,5)$P_3$ and $Ca^{2+}$-sensors, the values of the stimulated points were normalized to the sporadic changes of the values by the unstimulated points. After that, the values were also normalized to the mean values of the last three points before the stimulation.

## Confocal microscopy

HEK 293T cells at a density of $3x10^4$ cells/well or COS-7 cells at a density of $2x10^4$ cells/well were cultured on IBIDI 8 well μ-Slides in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C. After one day, the culture medium was changed to 200 μl transfection solution containing the indicated DNA constructs (0.2 μg total DNA/well) and 0.33 μl/dish Lipofectamine 2000. After 6 hours, the transfection solution was changed to 300 μl supplemented DMEM culture medium. Confocal measurements were performed 24-26 hours after transfection at room temperature in a modified Krebs-Ringer buffer, using a Zeiss LSM 710 scanning confocal microscope and a 63x/1.4 oil-immersion objective. Post-acquisition picture analysis was performed using Fiji and Photoshop (Adobe) software's to expand to the full dynamic range but only linear changes were allowed.

## Permeabilization measurements

HeLa cells were cultured on 25 mm No. 1.5 glass coverslips (2×105 cells/dish) and transfected with Venus-K-Ras-CAAX or Venus-H-Ras-CAAX constructs (0.5 μg DNA/dish) using 2 μl/dish Lipofectamine 2000 for 24 h. Confocal measurements were performed at room temperature in an intracellular medium, using a Zeiss LSM 710 confocal microscope and a 63x/1.4 oil-immersion objective. For the permeabilization 25 μg/ml digitonin was applied.

## [3H]-Leucine incorporation assay

The effect of PM lipid depletion on protein synthesis in COS-7 cells was evaluated by a [$^3$H]-Leucine incorporation assay. $5×10^4$ cells were seeded in poly-L-lysine-pretreated (0.001%, 1 hour) 24-well plates and left to adhere overnight at 37°C. The cells transfected with plasmids and after 1 day the cells were incubated with serum-free DMEM for 6 hours. The quiescent COS-7 cells were further incubated in serum-

free DMEM containing 1 μCi/ml [3H]-Leucine (American Radiolabeled Chemicals), with or without 100 nM rapamycin (repeated in every 8 hours). After 24 hours of incubation the plates were placed on ice, the cells were washed twice with ice-cold PBS, and incubated for 30 min in cold trichloroacetic acid (5%). The fixed cells were then washed with PBS and dissolved in 0.5 M NaOH for 30 min. Finally, the lysed cells were transferred to scintillation vials containing 10 ml of OptiPhase HiSafe3 (Perkin Elmer). The radioactivity was counted by Beckman LS5000 TD liquid scintillation counter.

## Statistical analysis

For the statistical analysis of ΔBRET ratio data, paired t-test was used to compare the mean of the last three data points before and after the stimulation of the individual curves. When two separate curves were compared, unpaired t-test was also performed on the means of the last three data points of the curves after the stimulation. When three separate curves were compared, one-way ANOVA was used followed by Holm-Sidak all pairwise multiple comparison method on the means of the last three data points of the curves after the stimulation. To investigate the effects of the different Ras mutants and/or the PM PPIns depletion system in the [$^3$H]-Leucine incorporation assay, two-way ANOVA followed by Holm-Sidak all pairwise multiple comparison method was used. To calculate the half-time values ($\tau$) of the Ins(1,4,5)$P_3$- or $Ca^{2+}$-sensors' decay phase, a curve fitting procedure was applied to the values in each individual experiment using the 3 parametric exponential decay equation of ($y=y_0+ae^{-bx}$). $\tau$ values were then averaged and subjected to a t-test. Statistical analysis was performed using SigmaStat 3.5 program (Systat Software Inc.).

# Results

## Energy transfer-based monitoring of the intracellular movement of peripheral proteins bound to the cytoplasmic surface of the PM

To follow the intracellular rearrangements of peripheral PM proteins, first we designed several constructs to target the Venus protein to the inner leaflet of the PM using targeting signal sequences either added to the N- or the C-termini of the fluorescent protein. These included the N-terminal 14 amino acids of the Lyn kinase, which has a single palmitoyl and a single myristoyl modification ($Lyn_{1-14}$-Venus), the N-terminal 10 amino acids of the Lck kinase, which has a double palmitoyl and a single myristoyl residue ($Lck_{1-10}$-Venus), and the N-terminal 15 amino acids of the c-Src kinase, which contains only a single myristoyl (c-$Src_{1-15}$-Venus) group. As for C-terminal signal sequences, we used the C-terminal 22 residues of K-Ras modified by prenylation (Venus-K-Ras-CAAX), or the C-terminal 22 amino acid signal sequence of the H-Ras, which possesses double palmitoylation and prenylation (Venus-H-Ras-CAAX). Notably, in addition to the lipid modification, the targeting sequences of c-Src and K-Ras also contained a number of positively charged amino acids. As expected and showed before by various reports, transient expression of all of these constructs in HEK 293T cells labeled the PM.

Recently we developed a bioluminescent energy transfer (BRET)-based method monitoring the proximity of luciferase-tagged PM receptors and the Venus protein targeted to the surface of various intracellular organelles. To investigate the dynamic changes of the membrane localization of peripheral membrane proteins that bind the PM, and follow their intracellular fates, we utilized the same approach with some modifications. The most important modification was that now we targeted the luciferase enzyme itself to the cytoplasmic surface of the different intracellular organelles to serve as donor and used the Venus protein tagged with the various PM-targeting sequences as the acceptor.

For targeting the luciferase enzyme to different intracellular organelles, we used targeting sequences or full-length forms of the following proteins: TGN38 (N-terminal target of the full length protein) for Golgi, TOM70 (N-terminal 1-30 amino acids) for mitochondria, Sac1 (C-terminal 521-587 amino acids) for ER, and EEA1 (1253-1411 amino acids that corresponds its FYVE-domain) for early endosomes.

## Acute manipulation of PM PPIns pools

To achieve the PM PPIns depletion, a previously described rapamycin-induced heterodimerization system was used. In most of the experiments, the FKBP-fused version of the bifunctional phosphatase, Pseudojanin (PJ) containing a 4- and a 5-phosphatase domain. FRB was targeted to the PM using the targeting sequence of the Lck protein. When the role of PtdIns(3,4,5)$P_3$ was studied, low (100 nM) concentration of wortmannin was used to inhibit Class I PtdIns 3-kinase activity.

Effects of these manipulations on PM PPIns levels were validated either in confocal or BRET measurements, using specific biosensors based on the following lipid binding domains: SidM-2xP4M as the PtdIns4*P* sensor, PLC$\delta_1$-PH as the PtdIns(4,5)$P_2$ sensor and Btk-PH as the PtdIns(3,4,5)$P_3$ sensor. PM recruitment of PJ enzymes of various activities with 300 nM rapamycin resulted in a rapid decrease of the PM PtdIns4*P*, PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$. Wortmannin pretreatment did not affect the PM PtdIns4*P*, PtdIns(4,5)$P_2$, but induced maximal depletion of PM PtdIns(3,4,5)$P_3$. Notably, wortmannin treatment reduced PM PtdIns(3,4,5)$P_3$ to a lower level than the rapamycin-induced depletion of its precursor, PtdIns(4,5)$P_2$, indicating that the removal of the PtdIns(3,4,5)$P_3$ precursor may not have been complete and the inhibition of the PI3Ks enzyme is more effective under these conditions.

## PM PPIns depletion evokes the redistribution of the polybasic peripheral proteins from the PM to the Golgi

Previous studies showed, that peripheral proteins containing positively charged amino acids are released from the PM after combined PtdIns4P and PtdIns(4,5)$P_2$ depletion. To investigate this phenomenon in further details using the various PM-targeted Venus constructs, we initially performed confocal measurements. Depletion of PM PtdIns4P, PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ levels by activation of our lipid depletion system reduced the PM localization only of Venus-K-Ras-CAAX and c-Src$_{1-15}$-Venus, while the localization of the other PM targeted Venus constructs (Lyn$_{1-14}$-Venus, Lck$_{1-10}$-Venus, Venus-H-Ras-CAAX) remained unchanged.

To obtain more information about the fate of the proteins after their release from the PM, we next performed BRET measurements between the proteins and various intracellular organelles. First, we used HEK 293T cells co-expressing Luc-ER on top of

the expression of the components of the lipid-depletion system and the PM–targeted Venus proteins. Since the ER has a large surface, we reasoned that proteins released from the PM would generate a signal with the ER-targeted luciferase. Upon PM PPIns depletion we did detect increased energy transfer only in cells expressing either the Venus-K-Ras-CAAX or c-Src$_{1-15}$-Venus constructs.

Next, we investigated whether these two targeting motifs can generate energy transfer with luciferase targeted to the surface of other organelles. Releasing Venus-K-Ras-CAAX from the PM has generated a remarkable significant increase in the BRET signal in the Golgi; a very small, but still significant increase in the case of the mitochondria; but not in early endosomes. c-Src$_{1-15}$-tagged Venus resulted in similar change. Importantly, we did not detect any interaction between Golgi-Luc and Venus-H-Ras-CAAX after PM PPIns depletion.

## Translocation of Venus-K-Ras-CAAX from the PM to the Golgi happens through the cytoplasm and depends on the Golgi PtdIns4P level

Based on previous studies, K-Ras proteins travel between intracellular membranes through binding to "solubilizing factors" such as Galectin-3 and PDEδ to prevent random attachment to the endomembranes, rather than using vesicle trafficking. Structural analysis showed, that these transport proteins provide with a hydrophobic grove suitable to adopt the hydrophobic farnesyl group on the K-Ras protein, thereby increasing its water solubility. To test how this process affects the translocation of the protein from the PM to the Golgi, we pretreated cells with deltarasin, a compound that competes to Ras binding on these molecules by mimicking the structure of farnesyl groups. Deltarasin treatment significantly reduced the appearance of Venus-K-Ras-CAAX at the Golgi in BRET measurements upon PM PPIns depletion. Confocal experiments on deltarasin pretreated COS-7 cells strengthened these results, moreover they showed, that Venus-K-Ras-CAAX remained bound to intracellular vesicular structures, instead of the Golgi rearrangement.

To confirm that Venus-K-Ras-CAAX can exist in the cytoplasm not in a membrane bound, but in free, solubilized form, we carried out a simple experiment. Cells that expressed Venus-K-Ras-CAAX were treated with the permeabilization agent digitonin allowing the release of small cytoplasmic proteins from the cell. Upon the

permeabilization Venus-K-Ras-CAAX also appeared in the membrane of the other, non-transfected cells of the culture, supporting the hypothesis of the membrane-independent cytoplasmic trafficking. This phenomenon was never noticed when H-Ras-CAAX was used instead of the K-RAS-CAAX in the experiments.

To examine whether it is the Golgi PtdIns4*P* that recruits the Venus-K-Ras-CAAX during its Golgi translocation, we performed BRET measurements in which in addition to depletion of PM PPIns, the PtdIns4*P* pool of the Golgi was also reduced. To do this, we co-transfected HEK 293T cells with plasmids that targeted FRB both to the PM and the Golgi for the rapamycin-dependent PJ translocation to both compartments. Concomitant Golgi PtdIns4*P* depletion resulted in a significantly lower translocation of the K-Ras-CAAX tagged Venus, compared to the case where PPIns depletion occurred only at the PM.

## PM binding of Venus-K-Ras-CAAX depends on electrostatic interactions rather than binding to specific lipids

Rapamycin-induced PJ-recruitment led to acute depletion of PtdIns4P, PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ in the PM. To investigate whether any of these lipids has a specific role keeping the K-Ras-CAAX-tagged Venus in the PM, we depleted these PPIns individually.

First, we examined the effect of the wortmannin-evoked robust PM PtdIns(3,4,5)$P_3$ depletion on the Venus-K-Ras-CAAX translocation, which did not affect the other PM PPIns pools in previous experiments. This manipulation did not cause Venus-K-Ras-CAAX to appear in the Golgi compartment, nor did it affect the Golgi translocation response following PJ recruitment to the PM. This suggests that PM PtdIns(3,4,5)$P_3$ plays a negligible role in the stabilization of the Venus-K-Ras-CAAX protein at the PM.

Next, we depleted PM PtdIns4*P* and PtdIns(4,5)$P_2$ separately to examine the contribution of these lipids individually to the PM localization of the K-Ras. For this, we used PJ enzymes inactivated in either its 5-phosphatase (FKBP-PJ-4ptase) or 4-phosphatase (FKBP-PJ-5ptase) activities. The recruitment of the FKBP-PJ-5ptase, which reduced only the PM PtdIns(4,5)$P_2$ level, caused an about 40% reduction of Venus-K-Ras-CAAX translocation to the Golgi when compared to the effect of FKBP-

PJ. Recruitment of the FKBP-PJ-4ptase, which reduced only PM PtdIns4*P* was less effective (85% reduction), but still caused a significant increase of K-Ras-CAAX Golgi appearance.

## The effect of PM PPIns depletion on the intracellular trafficking of full-length K- and H-Ras proteins

In the previous experiments, we examined the isolated targeting sequences of the Ras proteins. To study the behavior of the full-length Ras proteins, we created N-terminally Venus-tagged versions of the full-length, constitutively active G12V forms of K- and H-Ras proteins, and investigated their movements under conditions of PPIns manipulations. The full-length proteins showed the same changes in response to PPIns depletion as their isolated targeting sequences examined both by confocal microscopy and BRET in HEK 293T cells. Wild type or dominant negative forms of the Ras proteins, behaved the same way indicating that the translocation process is independent of the activity state of these proteins.

## K-Ras moves to the Golgi during $G_q$-coupled or EGF receptor activation

After testing Ras distribution using the artificial PPIns manipulations, we wanted to determine how PPIns changes that occur during more physiological conditions, namely during Gq-coupled receptor or EGF-receptor (EGFR) activation affect the intracellular distribution of the K-Ras protein. For this, M3 muscarinic receptors (M3R) or EGFRs were expressed in HEK 293T cells, together with either Venus-K-Ras-CAAX or Venus-K-Ras G12V constructs and the Golgi-Luc protein to examine K-Ras translocation to the Golgi membrane in BRET measurements. The M3R agonist, carbachol, or EGF were used to stimulate PLC activation. In the case of the M3R stimulation, the distribution of the Venus-K-Ras-CAAX construct was also examined with confocal microscopy, using transiently transfected COS-7 cells. Carbachol treatment caused a moderate transient elevation in the Golgi appearance of either Venus-K-Ras-CAAX or the full Venus-K-Ras G12V protein, which was more pronounced with the Venus-K-Ras-CAAX construct. Pretreatment of the cells with A1, an inhibitor of the type-III PI4Kα kinase (PI4KA), which prevents PtdIns4*P* and

PtdIns(4,5)$P_2$ resynthesis, resulted in a more sustained effects. Similar changes were observed in COS-7 cells using confocal microscopy.

Previously we showed that stimulation of HEK 293T cells with EGF decreases PM PtdIns(4,5)$P_2$ level by activating the generation of PtdIns(3,4,5)$P_3$ and IP3 by PI3 kinases and PLCγ, respectively. Stimulation of overexpressed, but not endogenous EGF receptors of HEK cells by 100 ng/ml EGF caused translocation of Venus-K-Ras-CAAX from the PM to the Golgi in a similar way, as did by the M3R stimulation. Similarly, this effect was even larger when PI4KA was inhibited with A1 consistent with the notion that depletion of plasma membrane PtdIns4*P* and PtdIns(4,5)$P_2$ together liberates K-Ras, which is then recruited to the Golgi by PtdIns4*P* in that compartment.

## PM PPIns depletion attenuates K-Ras but not H-Ras induced cell proliferation

Ras proteins control numerous cellular processes ranging from cell growth to differentiation and cell survival. To investigate the functional consequences of K-Ras translocation to the Golgi after PM PPIns depletion, we carried out a 24 hour [$^3$H]-Leucine incorporation assay to evaluate the rate of the protein synthesis as marker of cell proliferation.

First, the proliferative effect of the constitutive active mutation of Ras was examined. For this, constitutive active G12V and dominant negative S17N forms of the K- and H-Ras proteins were expressed transiently in COS-7 cells. As expected, cells expressing the activating Ras mutants showed significantly larger Leucine incorporation compared to those expressing the dominant negative forms. Next, we examined how the PM PPIns depletion affects the ability of active Ras to induce proliferation. Again, PM PPIns depletion was achieved using recruitment of FKBP-PJ. Since rapamycin, an mTOR inhibitor was used for recruitment; we used the catalytically inactivated FKBP-PJ enzyme as control. Active K-Ras increased the [$^3$H]-Leucine incorporation even in the presence of 100 nM rapamycin, although its effect was reduced. PM PPIns depletion did not affect the already low [$^3$H]-Leucine incorporation in cells expressing the dominant negative form of K-Ras S17N, but had a significant inhibitory effect in cells expressing the active form of K-Ras G12V. Notably, PPIns depletion did not change the proliferative effect of H-Ras G12V.

## Joint depletion of the PM PtdIns4P and PtdIns(4,5)$P_2$ lipid pools abrogate the SOCE process

To investigate the PPIns dependence of SOCE process, we applied two different approaches. First, we investigated how the PM PPIns pools can regulate the SOCE process upon hormonal stimulation of non-internalizing AT1 receptor mutant (AT1R-Δ319). Activation of the receptor leads not only to an elevation in the cytoplasmic Ins(1,4,5)$P_3$ and $Ca^{2+}$-levels, but decreases the PM PtdIns(4,5)$P_2$ lipid pools, which have a possible regulatory effect in the SOCE process. For this, we performed parallel measurements of Ins(1,4,5)$P_3$ and $Ca^{2+}$-levels on transiently transfected HEK293T cells, which were containing our BRET-based $Ca^{2+}$- or Ins(1,4,5)$P_3$-biosensors, and the rapamycin-inducible PM PtdIns(4,5)$P_2$-depletion system. Stimulation with Ang II resulted in a large and sustained increase in cytoplasmic Ins(1,4,5)$P_3$ levels, which was rapidly terminated upon PtdIns(4,5)$P_2$ depletion evoked by rapamycin addition. The decay of the Ins(1,4,5)$P_3$ signal correlated well with the decline of the cytoplasmic $Ca^{2+}$-level, showing that the termination of the SOCE occurred because of the reduced Ins(1,4,5)$P_3$ levels, and not due to direct inhibition of the current upon PM PtdIns(4,5)$P_2$ depletion. When the replenishment of the PM lipid pools was hindered by inhibition PI4KA with either wortmannin (10 μM) or A1 (10 nM) pretreatment, the decay in Ins(1,4,5)$P_3$-level was much slower than the decline in the $Ca^{2+}$-levels, showing the direct inhibitory effect of the PM PPIns depletion in the SOCE process.

In the second approach, the depletion of the ER $Ca^{2+}$-stores was achieved with the ER $Ca^{2+}$-pump inhibitor thapsigargin (Tg). In this case, we could clearly investigate the PM PPIns dependence of the SOCE, because of the lacking perturbation of the PLC activation as we have seen at the hormonal stimulation. In these experiments, we used transiently transfected HEK293T cells which were containing our inducible lipid-depletion system with various phosphatase activities (FKBP-PJ, FKBP-PJ-4ptase, FKBP-PJ-5ptase or FKBP-PJ-dead) and our BRET-based $Ca^{2+}$-biosensor. After 200 nM Tg treatment, a remarkable $Ca^{2+}$-signal was detected in the cells, which could be only inhibited by joint depletion of PM PtdIns4*P* and PtdIns(4,5)$P_2$ molecules, the separate depletion was totally ineffective in the abrogation of this process.

# Conclusions

According to our aims, we sum up the following conclusions:

- Based on our results, those peripheral PM-proteins, which contain positively charged amino acids in their sequence detach from the PM upon PM PtdIns4*P* és PtdIns(4,5)$P_2$ depletion
- The PM PtdIns(3,4,5)$P_3$ pool plays just a little if any role in the PM attachment process of positively charged amino acid containing peripheral PM-proteins
- The K-Ras protein redistribute from the PM mostly to the Golgi, in which the organelle's PtdIns4*P* pool is a crucial determinant
- The intracellular redistribution of the K-Ras occurs in the cytoplasm, where it travels in solubilized form by specific chaperones
- The observed intracellular rearrangement occurs also upon physiological stimuli induced PM PPIns depletion, which was evoked by activation of $G_q$-coupled M3R or EGFR
- The proliferative effect of the constitutively active K-Ras is reduced upon PM PPIns depletion, likely because of the observed Golgi translocation upon these circumstances
- We demonstrated, that the existence of the PM PtdIns4*P* or PtdIns(4,5)$P_2$ molecules are necessary for the physiological function of the SOCE, since depletion of both molecules on the PM led to the inhibition of the process

# List of publications

## The PhD thesis is based on the following publications:

**Gulyas, G.**, Radvanszki, G., Matuska, R., Balla, A., Hunyady, L., Balla, T., and Varnai, P. (2017) Plasma membrane phosphatidylinositol 4-phosphate and 4,5-bisphosphate determine the distribution and function of K-Ras4B but not H-Ras proteins. J Biol Chem 292: pp. 18862-18877 IF: 4,125

**Gulyas, G.***, Toth, J. T.*, Toth, D. J., Kurucz, I., Hunyady, L., Balla, T., and Varnai, P. (2015) Measurement of inositol 1,4,5-trisphosphate in living cells using an improved set of resonance energy transfer-based biosensors. PLoS One 10, e0125601 IF: 3,057
*(* These authors contributed equally)*

## Other publications:

Várnai P., **Gulyás G.**, Tóth D.J., Sohn M., Sengupta N., Balla T.
Quantifying lipid changes in various membrane compartments using lipid binding protein domains. Cell Calcium 64: pp. 72-82. (2017) IF: 3,707
(Review)

Toth J.T., **Gulyas G.**, Toth D.J., Balla A., Hammond G., Hunyady L., Balla T., Varnai P. (2016) BRET-monitoring of the dynamics changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4P increase upon EGF and M3 receptor activation. Biochim Biophys Acta. 1861(3):177-187. IF: 5,547

Toth D.J., Toth J.T., **Gulyas G.**, Balla A., Balla T., Hunyady L., Varnai P. (2012) Acute depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate impairs specific steps in endocytosis of the G-protein-coupled receptor. *J Cell Sci*, 125: 2185-97. IF: 5,877