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Role of the C-terminal basic amino acids and the lipid anchor of the $G\gamma_2$ protein in membrane interactions and cell localization

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ABSTRACT

Heterotrimeric G proteins are peripheral membrane proteins that frequently localize to the plasma membrane where their presence in molar excess over G protein coupled receptors permits signal amplification. Their distribution is regulated by protein-lipid interactions, which has a clear influence on their activity. Gβγ dimer drives the interaction between G protein heterotrimers with cell membranes. We focused our study on the role of the C-terminal region of the Gγ$_2$ protein in G protein interactions with cell membranes. The Gγ$_2$ subunit is modified at cysteine (Cys) 68 by the addition of an isoprenyl lipid, which is followed by the proteolytic removal of the last three residues that leaves an isoprenylated and carboxyl methylated Cys-68 as the terminal amino acid. The role of Cys isoprenylation of the CAAX box has been defined for other proteins, yet the importance of proteolysis and carboxyl methylation of isoprenylated proteins is less clear. Here, we showed that not only geranylgeranylation but also proteolysis and carboxyl methylation are essential for the correct localization of Gγ$_2$ in the plasma membrane. Moreover, we showed the importance of electrostatic interactions between the inner leaflet of the plasma membrane and the positively charged C-terminal domain of the Gγ$_2$ subunit (amino acids Arg-62, Lys-64 and Lys-65) as a second signal to reach the plasma membrane. Indeed, single or multiple point mutations at Gγ$_2$ C-terminal amino acids have a significant effect on Gγ$_2$ protein-plasma membrane interactions and its localization to charged Ld (liquid disordered) membrane microdomains.

1. INTRODUCTION

Guanine nucleotide-binding proteins (G proteins) are heterotrimers containing α, β, and γ subunits, and are responsible for propagating signals from G protein coupled receptors (GPCRs) to effectors that participate in olfaction, taste, vision, mood, hormone release, muscle contraction, cell migration, growth and differentiation (for review, see [1]). Ligand-induced stimulation of a GPCR activates a number of G protein α (αs, αi1-3, αq, αo, αolf, αz, αgust, αt1, αt2 α11-14, α16), β (β1-5) and γ (γ1-5, γ7-13), mediating the exchange of GDP for GTP in the G protein α subunit. GTP binding induces the dissociation of the heterotrimer into the monomeric Gα and dimeric Gβγ subunits that modify the activity of a great variety of specific effectors modulating the transduction signaling of a myriad of cellular events [2]. Moreover, the different subtypes of Gα, Gβ and Gγ subunits give rise to a wide variety of possible heterotrimeric combinations and effects [3]. These combinations are activated by different receptors and they can interact with distinct effectors depending on their milieu and the lipid composition of the membrane microdomains in a given cell. However, while G protein interactions with other proteins have been thoroughly studied, the interactions between heterotrimeric G proteins and membrane lipids remain largely unexplored, specially the role of the γ subunit alone.

The role of G protein posttranslational modifications and the involvement of certain amino acids in the localization of the heterotrimeric form is not fully understood. These subunits of the heterotrimeric form are known to be susceptible to five types of co/posttranslational modifications [4-5]. Gα subunits can be myristoylated and/or palmitoylated, and while Gβ subunits are not modified, they remain tightly associated with the Gγ subunit that can undergo sequential posttranslational processing that

includes prenylation, proteolysis and carboxyl methylation of its C-terminus [6].The lipid anchors and the adjacent basic residues on the Gα and Gγ subunits determine the localization of the distinct complexes (monomeric, dimeric and trimeric) and of the different Gα, β and γ subunits to specific cell membrane microdomains. For example, $G_{i/o}$ and G_q heterotrimers co-localize in the same microdomains, whereas their monomeric Gα subunits are distributed in different membrane microdomains [7]. This is probably so due to the fact that the Gβγ dimer drives the interaction between G protein heterotrimers and membranes [8]. Upon G protein activation, the Gα monomer dissociates from the other subunits and it associates with different membrane microdomains, where it interacts with target effector proteins [8-9]. Indeed, previous studies have shown that the Gβ subunit has only a weak influence on the interaction of G protein dimers and trimers with the plasma membrane (PM: [10-11] while the Gγ subunit might be responsible for the localization of Gβγ and Gαβγ proteins at the PM [10].

In fact, the prenylation of the Gγ subunit plays a major role in anchoring G protein dimers and trimers to the membrane [4, 8]. The C-terminus of the Gγ subunit contains a CAAX motif for prenylation (cysteine followed by two aliphatic amino acids and any amino acid), as well as for other sequential posttranslational processing events [6]. Accordingly, one farnesyl or geranylgeranyl moiety can be linked to the thiol group of the cysteine residue by a cytosolic protein isoprenyltransferase (farnesyltransferase -FTase- or geranylgeranyltransferase type I -GGTase I-, respectively) in function of the last amino acid of the CA_1A_2X motif (X: [12-13]. For example, the Gγ$_2$ subunit is specifically geranylgeranylated [14]. In this context, the A_1 position of the CA_1A_2X motif accepts a variety of aliphatic amino acids, whereas the A_2 accepts only a few hydrophobic amino acids that can be recognized by isoprenyltransferases [12-13].

Following isoprenylation, the last three amino acids of the Gγ protein subunit are cleaved by a specific endoprotease, the Ras-converting enzyme (Rce1: [15], which is associated with the endoplasmic reticulum (ER: [16]. Finally, the newly exposed isoprenylcysteine is methylated by an ER-associated methyltransferase, the isoprenylcysteine carboxyl methyltransferase (ICMT: [17-18]. Studies on the peripheral protein Ras have shown that FTase, GGTase and Rce1 enzymes are critical for its localization to the PM. Indeed, the absence of the activity of such enzymes or the deletion of the polybasic domain close to the CAAX motif is related to the mislocalization of Ras and its translocation from the PM to the cytosol or to internal membrane compartments [19-21].

The present study was designed to investigate the role of the C-terminal region of the Gγ$_2$ subunit in the cellular localization and PM interactions of oligomeric G proteins. For this purpose, we produced a chimeric protein containing the C-terminal 12 amino acids of the Gγ$_2$ subunit fused to the green fluorescent protein (GFP). Indeed, semi-quantitative analysis of the distribution of fluorescence indicated that this limited region of the small G protein subunit has a strong impact on the localization of G proteins. The Gγ subunit of G proteins has not formerly been considered relevant in the function of the protein heterotrimer. However, its proximity to the PM with respect to the other subunits and the relevance of the Gβγ dimer in the binding of the heterotrimer [4, 8], suggests that the Gγ subunit may fulfill more important roles than those contemplated previously. Moreover, the presence of an isoprenyl moiety at the C-terminal region of the Gγ$_2$ subunit and the fact that the Gβ subunit does not participate in this interaction suggest that the C-terminus of the Gγ subunit is important for the membrane association of the heterotrimer [10-11].

2. MATERIALS AND METHODS

2.1 Plasmid constructs

Double stranded synthetic DNA fragments that coded for the carboxy-terminal region of the $G\gamma_2$ protein (truncated wild type $G\gamma_2$ protein, $tG\gamma_2$-WT) and for 15 similar sequences carrying mutations were designed (Figure 1A), and they were generated by hybridization of reverse complementary primer sequences that produced cohesive ends for cloning purposes (Table S1). These constructs were cloned into the *Xho*I/*Eco*RI site of a GFP expression plasmid (pEGFP-C1 plasmid, Clontech, CA) to produce GFP fusion proteins with the $G\gamma_2$ protein C-terminal region ($tG\gamma_2$-WT) or with the 15 mutants (Figure 1A). In these, the $tG\gamma_2$-WT fusion protein has the identical C-terminal sequence (amino acids 60 to 71) as the $G\gamma_2$ protein, PFREKKFFCAIL, while the Arg-62-Gly, Lys-64-Gly, Lys-65-Gly and Cys-68-Ser mutations produced the $tG\gamma_2$-62, $tG\gamma_2$-64, $tG\gamma_2$-65 and $tG\gamma_2$-68 fusion proteins, respectively. We also produced fusion proteins with two, three and four mutations: $tG\gamma_2$-62/64, $tG\gamma_2$-62/65, $tG\gamma_2$-64/65, $tG\gamma_2$-62/64/65, $tG\gamma_2$-62/68, $tG\gamma_2$-64/68, $tG\gamma_2$-65/68, $tG\gamma_2$-62/64/68, $tG\gamma_2$-62/65/68, $tG\gamma_2$-64/65/68 and $tG\gamma_2$-62/64/65/68 (Figure 1A). Finally, we produced the negative control construct $tG\gamma_2$-70, PFREKKFFCASL, which prevents the deletion of the last three amino acids (Figure 2D) as the second aliphatic amino acid (A_2) of the CA_1A_2X motif at position 70 was mutated (Ile-70-Ser).

2.2 Cell Culture and transfection

The human glioma cell line, SF-767, was obtained from the Brain Tumor Research Center Tissue Bank (University of California-San Francisco, Department of Neurological Surgery) and these cells were maintained at 37 ºC in a fully humidified

atmosphere with 5% CO_2. Monolayer cultures were grown in DMEM medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin. Spontaneously immortalized mouse embryonic fibroblasts (MEFs: $Rce^{flox/flox}$, $Rce^{-/-}$, $Icmt^{flox/flox}$, and $Icmt^{-/-}$) were prepared as described elsewhere [19-20] and they were cultured at 37 ºC in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, L-glutamine and β-mercaptoethanol 0,1 mM. Tissue culture medium and supplements were purchased from Sigma (Madrid, Spain), with the exception of β-mercaptoethanol, which was purchased from Scharlau (Barcelona, Spain).

The SF-767, Rce and Icmt cell lines were cultured to 60-70% confluence and on the following day, transfection was performed using polyethylenimine (PEI: Polysciences, Inc Warrington, PA) and 5 μg of DNA (DNA:PEI 1:3), as described elsewhere [22]. Inhibition of FTase and GGTase in transfected SF-767 cells was achieved by adding FTI-276 (5 μM: Farnesyltransferase and Geranylgeranyltransferase type I inhibitor, Calbiochem) to the culture medium for 2h 30 min.

2.3 Preparation of cellular fractions

Membrane and cytosolic fractions were prepared essentially as described elsewhere [23-24]. Briefly, after a 4 h transfection in 100 mm sterile culture dishes (Thermo fisher scientific, Spain), the cells were washed twice with PBS and harvested with a Teflon cell scraper in 225 μl subfractionation buffer: 250 mM Sucrose, 20 mM HEPES [pH 7.4], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and a phosphatase inhibitor cocktail (Roche Diagnostics,Mannheim, Germany). The cells were then disrupted by passing the suspension 25 times through a 25 gauge syringe and after centrifuging the homogenate at 1200 *g* for 10 min at 4 ºC, the pellet (nuclear

fraction) was discarded. The supernatant was then centrifuged at 100000 *g* for 1 h at 22 ºC, collecting the pellet (total membrane fraction) and supernatant (cytosol fraction) separately. The pellet was resuspended in 150 µl of subfractionation buffer with 0.1% SDS and it was homogenized by ultrasound (3 x10 s cycles at 50 W, 10% cycle) in a Braun Labsonic U sonicator (Melsungen, Germany). Subsequently, 10 µl aliquots of the resuspended membrane or cytosolic fractions were used for total protein quantification by the bicinchoninic acid method [25]. The remainder of these particulate or soluble samples was mixed with 10 x electrophoresis loading buffer (120 mM Tris-HCl [pH 6.8], 2% SDS, 50% glycerol, 0.1% bromophenol blue and 10% β-mercaptoethanol) and heated for 5 min at 95 ºC.

2.4 Electrophoresis (SDS/PAGE), Immunoblotting and Protein Quantification

For immunoblotting, total protein (50 µg) from the membrane and cytosolic fractions was resolved on SDS-polyacrylamide gels (11.5% polyacrylamide) and transferred to nitrocellulose membranes (Whatman, Schleicher and Schuell). The membranes were blocked for 1 h at room temperature (RT) with 5% non-fat dry milk in PBS and they were then incubated overnight at 4 ºC with the specific primary antibodies diluted in fresh blocking solution supplemented with 0.2% Tween 20: polyclonal rabbit anti-GFP IgG (1:1000; Abcam, Cambridge, UK), rabbit anti-Calpain 2 IgM and rabbit anti-EGFR IgG (1:1000; Cell Signaling, Danvers, MA, USA) and monoclonal mouse anti-Caveolin 1 IgM (1:1000; BD Transduction Laboratories, Heidelberg, Germany). After incubating with the primary antibody, the membranes were washed three times for 10 min with PBS containing 0.1% Tween 20 and incubated for 60 min at RT in the dark with a fluorescent-labeled secondary antibody diluted in fresh blocking solution containing 0.2% Tween 20 and 0.02% SDS: IRDye 800CW donkey anti-mouse IgG or IRDye

800CW donkey anti-rabbit IgG (1:5000; LI-COR, Inc., Lincoln, NE, USA). Finally, the membranes were washed three times for 10 minutes with PBS, and antibody binding to the proteins was visualized with the Odyssey Infrared Imaging System (LI-COR, Inc.) and quantified using the Quantity One software (Bio-Rad, Spain).

2.5 Confocal Microscopy

For confocal microscopy, cells were grown, transfected and visualized on Chambered Coverglasses (Lab-Tek^{TM} II, Thermo Fisher Scientific Inc.). After incubation with PEI (4 h at 37 ºC) the subcellular localization of the tGγ$_2$ constructs was studied *in vivo* by confocal microscopy on Leica TCS SP2 and TCS SPE confocal microscopes (630 x magnification). Digital images were analyzed using the LCS and LAS AF software to determine the intensity of GFP fluorescence in the different cellular regions of interest (ROIs) selected. The ROIs quantified were obtained as indicated in figure S1. Four regions of the cell were defined to determine the fluorescence intensities: plasma membrane and vicinity (PM), cytoplasm, endoplasmic reticulum (ER) and nucleus. For co-localization experiments, transfected cells were incubated for 30 min with 3 μM of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Invitrogen, Fischer Scientific, Spain) to label the liquid disordered (Ld) domains in the plasma membrane, and for 5 min with Hoechst 33342 (40 μg/ml, trihydrochloridetrihydrate) to label the nucleus. Both probes were purchased from Molecular Probes (Barcelona, Spain). For immunofluorescence experiments, human glioma cells were cultured as indicated above on Chambered Coverglasses. After washing twice with phosphate buffer (PB) 0.1 M (20 mM NaH_2PO_4, 80 mM Na_2HPO_4 [pH 7.4]) for 5 min, cells were fixed with 4% paraformaldehyde in PB for 30 min and washed again with PB. After washing once with Tris-buffered saline (TBS) buffer (137 mM NaCl, 2.7 mM, KCl, 25

mM, Tris-HCl [pH 7.4]), cells were incubated with 5% normal horse serum in TBS buffer for 1 h at room temperature and then immediately incubated overnight at 4 °C with a polyclonal anti-$G\gamma_2$ antibody (1:50, Santa Cruz Biotechnology, Inc., USA) in TBS buffer supplemented with 5% horse serum. Finally, the cells were washed with TBS buffer, incubated for 1 h with the secondary antibody (Alexa Fluor 594-labeled goat anti-rabbit IgG, 1:200, Molecular Probes; excitation at 543 nm and detection at 610–750 nm), and washed with TBS buffer. To label the nuclei, cells were incubated for 5 min with Hoecht 33342 (40 μg/ml). Images were acquired on a Leica TCS SPE spectral confocal microscope with 630x optical magnification, and they were analyzed with the LAS AF software.

2.6 Statistics

The data were expressed as the mean ± SEM value from at least three independent experiments. Statistical significance was determined using the Student's *t* test or an ANOVA test followed by the Bonferroni test. The asterisks indicate the significance with respect to the WT chimeric protein: $*P< 0.05$; $**P< 0.01$; $***P< 0.001$.

3. RESULTS

3.1 The C-terminal region of $G\gamma_2$ protein is a cell membrane localization signal.

We have studied here how the positively charged C-terminal amino acids, and the isoprenylated and carboxyl methylated Cys of the $G\gamma_2$ subunit interact with membranes. To this end , we cloned this region (the 12 terminal amino acids) of the $G\gamma_2$ protein (truncated $G\gamma_2$ protein, $tG\gamma_2$) into an EGFP expression vector in frame with the C-terminus of the GFP protein ($tG\gamma_2$-WT). We also produced a series of single and

multiple point mutants of the tGγ2 C-terminal region to modulate or suppress electrostatic interactions with anionic lipids (the Arg-62-Gly, Lys-64-Gly and Lys-65-Gly mutations, tGγ2-62, tGγ2-64, tGγ2-65, tGγ2-62/64, tGγ2-62/65, tGγ2-64/65 and tGγ2-62/64/65). Similarly, we eliminated the C-terminal geranylgeranyl moiety of the tGγ2 subunit (Cys-68-Ser mutation, tGγ2-68) and we generated fusion proteins with simultaneous mutations at the basic residues and at the cysteine (tGγ2-62/68, tGγ2-64/68, tGγ2-65/68, tGγ2-62/64/68, tGγ2-62/65/68, tGγ2-64/65/68 and tGγ2-62/64/65/68: Figure 1A).

The cellular distribution of these fusion proteins was studied in immunoblots of cytosolic and membrane fractions from SF-767 cells transiently transfected with the empty EGFP vector alone (GFP), or that containing the wild type or mutated tGγ2 (tGγ2-WT, tGγ2-62/64/65, tGγ2-68 and tGγ2-62/64/65/68). In these immunoblots, the tGγ2-WT fusion protein could be seen to be mainly distributed at the cell membrane, whereas the GFP protein accumulated in the soluble fraction (cytosolic: Figures 1B-C). Compared to the tGγ2-WT, Gγ2 protein mutants showed marked and significant reductions in the membrane-to-cytosol ratio (ca. 50% for tGγ2-62/64/65 and ca. 90% for the tGγ2-68 and the tGγ2-62/64/65/68 mutants: Figures 1B-C). Interestingly, not only removal of the lipid anchor but also mutation of the basic C-terminal amino acids caused a reduction in the membrane localization of tGγ2, demonstrating that the last 9 amino acids of this protein constitute an efficient membrane localization signal.

3.2 The influence of geranylgeranylation on the plasma membrane localization of Gγ2

Once analyzed their subcellular localization by immunoblots, we next investigated the distribution of the tGγ2 fusion proteins in SF-767 cells by confocal microscopy. Cells were first transiently transfected with the empty GFP plasmid, or with that expressing

the tGγ$_2$-WT or tGγ$_2$-68 fusion proteins, and the GFP fluorescence intensity was quantified in the regions of interest (ROIs, see Figure S1) associated with important cell compartments (plasma membrane, cytoplasm, ER and nucleus). The tGγ$_2$-WT fusion protein contains the last 9 amino acids of the mature protein and the C-terminal cysteine is geranylgeranylated and methylated. By contrast to GFP, this fusion protein mainly localized to membranes, whereas the fusion protein in which the final Cys residue was mutated (tGγ$_2$-68) adopted a similar distribution to that of GFP alone, with a marked and significant decrease in the proportion of protein associated with the membrane (Figures 2A-B). This result demonstrates the importance of the cysteine residue Cys-68 (and the geranylgeranyl moiety) for the distribution and membrane localization of this protein (Figure 2).

Some mutations in the CAAX motif impede the recognition of the C-terminal region by isoprenyltransferase and therefore, transfer of a geranylgeranyl moiety [26]. To investigate the role of posttranslational modification of the Gγ$_2$ C-terminus (proteolysis, geranylgeranylation or carboxyl methylation), we generated a construct carrying a mutation in the second aliphatic residue of the CAAX motif (A_2 position of the CA_1A_2X motif). When the Ile-70-Ser (tGγ$_2$-70) mutant was transiently transfected into SF-767 cells, it adopted a similar cell distribution to GFP and the tGγ$_2$-68 fusion protein *in vivo*, with no relevant subcellular differences (Figure 2D).

To obtain further evidence of how the isoprenyl moiety influences the distribution of the G protein gamma subunit (and therefore, the heterotrimeric form), we cultured cells in the presence or absence of the farnesyltransferase (FTase) and geranylgeranyltransferase type I (GGTase-I) inhibitor, FTI-276. When SF-767 cells were transiently transfected with tGγ$_2$-WT and tGγ$_2$-62/64/65, both containing the geranylgeranyl moiety and they were exposed to FTI-276, the Gγ$_2$ subunits adopted a

similar distribution to that in cells expressing the tGγ$_2$-68 or tGγ$_2$-70 fusion proteins. These results further support the importance of geranylgeranylation for the cellular distribution and PM localization of the Gγ$_2$ protein (Figure 2E). Taken together these results, we demonstrated that the CAAX motif and more particularly, geranylgeranylation of its cysteine residue (Cys-68), are essential to control the distribution and PM localization of the Gγ$_2$ protein.

3.3 Gγ$_2$ localization to the plasma membrane requires isoprenylation, C-terminal proteolysis and carboxyl methylation

Following geranylgeranylation, the last three residues of the Gγ$_2$ protein are cleaved by the Ras converting enzyme (Rce1) before the isoprenylcysteine carboxyl methyltransferase (ICMT) carboxyl methylates the newly exposed cysteine residue [17]. These two posttranslational modifications were further characterized in mouse embryonic fibroblasts (MEFs) expressing the Rce1 and Icmt enzymes (Rce$^{f/f}$ and Icmt$^{f/f}$ cells, respectively), and in the corresponding knock-out cells for Rce1 and Icmt enzymes (Rce$^{-/-}$ and Icmt$^{-/-}$ cells, respectively: [19-20]. In Rce$^{f/f}$ and Icmt$^{f/f}$ MEFs (cells expressing the Rce1 and Icmt enzymes) transiently transfected with the tGγ$_2$-WT or GFP plasmids, the tGγ$_2$-WT fusion protein mainly localized to the cell membranes when the distinct cell fractions were quantified in immunoblots, unlike GFP that preferentially accumulated in the cytosolic fraction of these cells. By contrast, when proteolysis (Rce$^{-/-}$ cells: Figures 3A-B) or carboxyl methylation (Icmt$^{-/-}$ cells: Figures 4A-B) was interrupted, there was a marked and significant decrease in the amount of membrane tGγ$_2$-WT compared to the Rce1$^{f/f}$ and Icmt$^{f/f}$ cells.

When we assessed the distribution of tGγ$_2$-WT by confocal microscopy, similar results were observed for the Rce (Figure 3C) and Icmt (Figure 4C) knock-out MEF cells.

Accordingly, the tGγ$_2$-WT fusion protein that was mainly present at the PM (arrows) and internal membranes, was in part translocated to the cytoplasm in cells that do not express the Rce1 or Icmt enzymes (Figures 3B and 4B). As proteolysis is necessary for carboxyl methylation to occur, these results suggest that the methyl group is important to localize the Gγ$_2$ constructs to the PM. Therefore, these results demonstrate that geranylgeranylation, proteolysis and carboxyl methylation are processes essential to preferentially direct Gγ$_2$ protein to cell membranes.

3.4 The Arg-62, Lys-64 and Lys-65 C-terminal basic amino acids regulate Gγ$_2$ protein-membrane interactions

We have shown that hydrophobic and electrostatic interactions were necessary for the interaction of the tGγ$_2$ fusion proteins tested here with cell membranes (Figure 1C). In order to assess the relevance of the basic amino acid residues in the electrostatic interactions between the Gγ$_2$ protein and the PM, we transiently transfected SF-767 cells with plasmids encoding fusion proteins with a mutation at one, two or three basic residues, alone or in association with the mutation of Cys 68. When their cellular distribution was assessed by confocal microscopy, the distribution of the tGγ$_2$-62 and tGγ$_2$-64 fusion proteins did not differ significantly to that of the tGγ$_2$-WT fusion protein (Figures 5A-B). By contrast, the plasma membrane and ER accumulation of the tGγ$_2$-65 and tGγ$_2$-62/64/65 proteins decreased significantly, while they concentrated more intensely in the nucleus. These results suggested that lysine 65 (Lys-65), the basic amino acid closest to Cys-68, fulfils an important role in the interaction of the Gγ$_2$ protein with the PM. In addition, mutation of Lys-65 in combination with mutations of Arg-62 and Lys-64 had a stronger effect on the cellular distribution of tGγ$_2$ (Figures 5A-B), suggesting that these additional residues also contribute to the electrostatic

interactions with the membrane. The cellular distribution of all the proteins with simultaneous mutations of the basic residues and Cys-68 (tGγ$_2$-62/68, tGγ$_2$-64/68, tGγ$_2$-65/68 and tGγ$_2$-62/64/65/68) was similar to that of the tGγ$_2$-68 fusion protein (Figures 5C-D).

To further characterize these changes in the cellular distribution of the tGγ$_2$ fusion proteins, we compared their relative abundance in the PM region alone. The PM levels of the Gγ$_2$-GFP fusion proteins decreased significantly with the introduction of single mutations to the basic residues Lys-64 and Lys-65 alone, a reduction that was more pronounced in double and triple mutants (Figure 6A). In addition, the PM levels of the fusion protein carrying simultaneous mutations of the geranylgeranylated residue and the basic residues were similar to those of the tGγ$_2$-68 fusion protein (Figure 6B).

The importance of the basic residues in the tGγ$_2$-membrane interaction was Lys-65>Lys-64>Arg-62, most likely due to the spatial organization of these residues with respect to the PM. The 3D structure of the protein (adapted from the PDB code 2BCJ structure) indicates that lysines 64 and 65 are close enough to the membrane to interact with the lipid bilayer (Figure 6C). By contrast, arginine 62 is not so close to the membrane and thus, it is likely to contribute less than the other two positively charged amino acids to the electrostatic interactions with negatively charged phospholipids (e.g., phosphatidylserine or phosphatydilinositol). In any case, the simultaneous mutation of two or three basic residues caused further mislocalization of the tGγ$_2$ fusion proteins, indicating that they are all important for the localization of tGγ$_2$ at the PM and possibly, for its distribution to specific membrane microdomains.

Finally, to further study the involvement of residues 62, 64 and 65 in the localization of tGγ$_2$ fusion proteins at the PM, we used DiI to label the liquid disordered (Ld) lipid domains of the membrane to which GPCRs, as well as heterotrimeric and dimeric G

proteins localize [8]. While the tGγ2-WT fusion protein co-localized tightly with DiI in the PM, the triple mutant tGγ2-62/64/65 was not present in the Ld domains of the PM labeled with DiI (Figure 6D). In summary, we demonstrated that correct tGγ2 protein localization at the plasma membrane required both the hydrophobicity provided by the geranylgeranyl moiety and the carboxyl methyl group at cysteine 68, and the positive charges provided by arginine 62, lysine 64 and lysine 65.

4. DISCUSSION

The data presented here show that the C-terminal region of the Gγ2 subunit is an efficient localization signal that drives the protein to cell membranes and that it could be involved in the microdomain sub-localization of heterotrimeric G proteins. This subcellular distribution is produced by two signal sequences: a CAAX box and a short polybasic domain flanking this sequence. The CAAX box identified as a target for post-transductional modification by lipids at the end of last century [21] is a motif subjected to three posttranslational modifications that generate a hydrophobic anchor in the C-terminal tail of the Gγ2 protein: isoprenylation, proteolysis and carboxyl methylation. In addition, we showed that the basic amino acid stretch provides positive charges that facilitate the interaction with negatively charged phospholipids (e.g., phosphatidylserine, phosphatidic acid, phosphatidylinositol), which are abundant in the cytoplasmic leaflet of the PM.

In contrast to GFP, tGγ2-WT accumulated at the PM, indicating that the C-terminal tail of the Gγ2 subunit is sufficient to direct the hybrid protein to this structure and it keeps the same subcellular distribution as the native Gγ2 (Figure S2). Moreover, we showed that it co-localized with DiI in Ld membrane domains and this co-localization was lost by any of the alterations in the bipartite signal here described. This result is in

agreement with the preference of Gγ$_2$ subunit-bearing G proteins (Gβγ and Gαβγ) for nonlamellar-prone lipids, which mainly segregate to Ld membrane regions [8]. In previous studies the Gγ$_2$ subunit alone did not appear to localize well to the PM [27], although this may reflect the instability of this protein unless it is permanently bound to the Gβ subunit. To circumvent this problem, we focused on the C-terminal region of Gγ$_2$, which we previously found to be critical for the binding of G proteins to membranes [10] and we used a soluble reporter protein (GFP) to demonstrate that the tGγ$_2$-WT fusion protein accumulates significantly at the cell membrane. It has been proposed that Gα and Gβ act as a signal to localize Gγ$_2$ to the PM, although the relevance of the α and β subunits in the binding of heterotrimeric G proteins to membranes remains unclear [8, 10-11]. Here, the short basic amino acid motif formed by Arg-62, Lys-64 and Lys-65 appeared to act as a signal necessary for Gγ$_2$ to localize to the PM, as evident through the failure of the triple mutant tGγ$_2$-62/64/65 fusion protein to co-localize with DiI at the membrane to disordered (Ld) membrane microdomains. However, it should be noted that Gα subunits prefer ordered (Lo) membrane microdomains when they dissociate from the Gβγ dimer due to the different types of membrane anchors monomeric G protein subunits contain (myristic and/or palmitic acid). In addition, palmitoylation of the Gα subunit appears to require its interaction with Gβγ [28], and Gγ$_2$ geranylgeranylation has also been reported to require further Gα myristoylation to target G proteins to the PM [29].

The farnesyl moiety of the Gγ subunit (a 15 carbon isoprenoid) quantitatively lowers the lipid bilayer-binding energy and it might be required for interactions with the Gα protein, as well as for PM localization. By contrast, the 20 carbon geranylgeranyl isoprenoid that associates in the tGγ$_2$-WT fusion protein can stably anchor a protein to the PM and increase the Gβγ complex's affinity for membranes, also dictating the Gβγ

membrane orientation [30-31]. When the tGγ$_2$ protein is not geranylgeranylated it is not specifically targeted within the cell and it does not localize to the PM. Consistent with our results, Gγ$_2$ that is not geranylgeranylated does not attach to the membrane, the Gβγ dimer accumulating in the nucleus [11, 32]. We also found that the A_2 residue in the CA_1A_2X motif is not only essential for the contact with FTase [13, 33] but also with the GGTase, as the Ile-70-Ser mutation caused a similar effect as the loss of the geranylgeranyl moiety. Indeed, geranylgeranylation is also required for carboxyl methylation [34].

The influence of geranylgeranylation and carboxyl methylation on protein localization was considered together and separately here. Methylation of the cysteine carboxyl group would eliminate the negative charge of this group of prenylated proteins and in addition, it makes the C-terminus of the protein more hydrophobic. This process is relevant for farnesylated Gγ$_1$ and Ras GTPases, although it appears to be more subtle for geranylgeranylated Gγ$_2$, Rab and Rho GTPases [20, 27, 35-36]. Our experiments with Rce$^{-/-}$ and Icmt$^{-/-}$ cells allowed us to evaluate the effects of geranylgeranylation and carboxyl methylation on tGγ$_2$-WT separately. We found that carboxyl methylation is required for the efficient localization of tGγ$_2$-WT to the PM even when the protein is geranylgeranylated. These results are consistent with the hypothesis that carboxyl methylation of Gγ$_2$ is associated with the activation of G proteins and the attachment of Gγ$_2$ to the PM [37]. Although geranylgeranylation appears to be sufficient to stably anchor the protein to the membrane [38-39], both the lack of isoprenylation or carboxyl methylation produced similar mislocalization to that described for Ras in Rce$^{-/-}$ or Icmt$^{-/-}$ cells [19-20].

In addition, for the first time we also describe here the critical role of a short polybasic domain (Arg-62, Lys-64 and Lys-65) in Gγ$_2$-membrane interactions and localization.

All Gγ subtypes have 2 or 3 conserved basic residues at the C-terminus and indeed, other membrane signaling proteins use a similar strategy, combining electrostatic and hydrophobic interactions to bind to the membrane (e.g., K-Ras, the myristoylated alanine-rich C kinase substrate -MARCKS-, the non-receptor tyrosine kinase Src, [40]). The basic C-terminal residues provide a positively charged domain that can interact with the anionic phospholipids (mainly but not exclusively phosphatidylserine) that are abundant in the cytoplasmic leaflet of the PM [41]. Such electrostatic interactions depend on both the net membrane and protein charges [42]. In fact, electrostatic interactions of peripheral proteins with the membrane increase sharply as the proportion of acidic lipids increases up to the physiological range [43], as well as with the positive charge on the protein [44].

Likewise, the polybasic domain has also been shown to be important for the nanoscale distribution of the protein (i.e., for its subcellular localization to specific membrane microdomains) which strongly influences its activity [45]. Ras proteins represent a well-known example of the importance of basic residues and of posttranslational modifications for the differential nanodomain localization of proteins. The K-Ras4b protein, which contains a farnesyl moiety and a basic cluster at its C-terminus, localizes to distinct microdomains than H-Ras, which contains a farnesylation and two palmitoylation sites at its C-terminus [46-47]. In this context, the reduced membrane binding of isoprenylated tGγ$_2$ constructs lacking one or more basic residues, and the failure of the polybasic domain to affect non-geranylated constructs, suggests that these amino acids may fulfill an important role in the PM sub-localization of G proteins to specific microdomains. Moreover, the heterogeneous distribution of the tGγ$_2$-WT fusion protein in the PM further supports its preferential distribution to certain microdomains, a phenomenon that is mostly lost in the triple mutant lacking all the basic amino acid

residues (tGγ$_2$-62/64/65). Co-localization of tGγ$_2$-WT with DiI in Ld domains is consistent with our previous studies showing that heterotrimeric Gαβγ proteins and Gαβ dimers prefer membranes with a high phosphatidylethanolamine content (also with high nonlamellar phase propensity and low surface packing pressure: [8, 48-49]). Accordingly, the preferred membrane microdomains for G proteins containing the Gγ$_2$ subunit would be rich in phosphatidylethanolamine and phosphatidylserine. Indeed, various proteins bearing a hydrophobic anchor with adjacent positive charges not only prefer phosphatidylethanolamine-rich microdomains but also those containing phospholipids with negative charge [42, 50-51].

In summary, we showed that two signals are necessary to direct the cellular localization of Gγ$_2$ (and most likely oligomeric G proteins) to specific microdomains in the PM or to other membranes. The first is provided by the CAAX motif, which undergoes geranylgeranylation, proteolytic cleavage and carboxyl methylation, while the second signal is provided by a polybasic domain close to the C-terminus. Because the cellular localization of a protein in part determines its role in cell signaling, the results here presented shed new light on certain pathophysiological processes and possible, on future therapeutic approaches. In fact, nutraceutical or pharmaceutical interventions can regulate the membrane lipid composition, and the present results explain in part the molecular base underlying cell signaling modulation by membrane lipid therapy approaches [52].

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FIGURE LEGENDS

Figure 1. The C-terminal region of the Gγ2 subunit is critical for its localization.

A Schematic representation that shows the Gγ2 protein (upper diagram) and tGγ2-GFP fusion protein sequences. The amino acid sequence of the WT construct corresponds to the mature (posttranslationally processed) Gγ2 protein with the geranylgeranyl moiety indicated on Cys 68. The residues studied are indicated in *purple italic* in the WT and the mutated residues are in **bold magenta**. The geranylgeranyl moiety is absent on the right hand side constructs due to the mutation of Cys 68, and the positive charge provided by basic amino acids is indicated for each construct. The various constructs produced are identified by the number corresponding to the amino acid in the original Gγ2 protein sequence. **B-C** SF-767 cells were transiently transfected with the fusion proteins indicated (tGγ2-WT, tGγ2-64/65/68, tGγ2-68 and tGγ2-62/64/65/68), the membrane and cytosolic fractions were obtained from the cell lysates and the proteins were analyzed quantitatively in immunoblots. **B** Representative immunoblots showing the distribution of the tGγ2 fusion proteins between the membrane (M) and cytosolic (C) fractions, using GFP to detect the fusion proteins. Calpain and caveolin were used as cytosolic and membrane markers, respectively. **C** Bars correspond to the membrane:cytosolic ratio (mean ± SEM values of three independent experiments): *,$P < 0.05$; **, $P < 0.01$; ***, $P< 0.001$.

Figure 2. Geranylgeranylation is necessary for an appropriate protein distribution within cells and for localization to the PM.

A Geranylgeranylation deficient tGγ2 fusion protein (tGγ2-68: 68) displayed a non-specific distribution within the cell and like GFP alone, it showed no preference for

membranes. By contrast, the tGγ$_2$-WT fusion protein showed a preference for membranes, including the PM. **B-C** Relative (semi-quantitative) distribution of the fusion proteins shown in A (mean ± SEM values of five independent experiments): **, P< 0.01; ***, P< 0.001. **D** Mutation (Ile-70-Ser) in the CAAX motif causes mislocalization of the fusion protein. The confocal microscopy image is representative of five independent experiments. **E** Pharmacological inhibition of geranylgeranylation induces tGγ$_2$ fusion protein mislocalization. SF-767 cells were transfected tGγ$_2$-WT or tGγ$_2$-62/64/65, treated with FTI (FTase and GGTase-I inhibitor). Images are representative of three independent confocal microscopy experiments in live cells. Scale bar= 15 μm.

Figure 3. C-terminal proteolysis is necessary for tGγ$_2$ PM targeting.

A-B Absence of Rce1 (i.e., of proteolysis) diminished the tGγ$_2$-WT fusion protein localized at the cell membranes. Rce$^{flox/flox}$ (f/f) and Rce$^{-/-}$ (-/-) cells transfected with GFP alone or tGγ$_2$-WT. Cell lysates were centrifuged to obtain the membrane (M) or cytosolic (C) fractions, and the proteins were separated by SDS-PAGE and quantified in immunoblots. **A** Representative immunoblots. **B** Quantification of the relative amount of protein in each fraction: tGγ$_2$-WT (open bars) and GFP (solid bars) in transfected Rce$^{f/f}$ (100% of GFP fluorescence) and Rce$^{-/-}$ cells. The values represent the mean ± SEM of four independent experiments: **, P< 0.01. **C** Confocal microscopy images of Rce$^{f/f}$ and Rce$^{-/-}$ cells transfected with tGγ$_2$-WT. Nuclei were labeled in blue with Hoechst. The results represent the means and are representative of three independent experiments. Scale bar= 15 μm.

Figure 4. Cysteine carboxyl methylation is essential for tGγ$_2$ membrane targeting.

A-B Absence of Icmt (i.e., methylation) impaired the localization of the tGγ$_2$-WT fusion protein to the cell membrane. Icmt$^{flox/flox}$ (f/f) and Icmt$^{-/-}$ (-/-) cells transfected with GFP alone or with tGγ$_2$-WT. The membrane (M) or cytosolic (C) fractions were obtained from the cell lysates, separated by SDS-PAGE and quantified in immunoblots. **A** Representative immunoblots. **B** Quantification of the relative amount of protein in each fraction: tGγ$_2$-WT (open bars) and GFP (solid bars) in transfected Icmt$^{f/f}$ (100% of GFP fluorescence) and Icmt$^{-/-}$ cells. The values represent the mean ± SEM values of four independent experiments: **, $P< 0.01$. **C** Confocal microscopy images of Icmt$^{f/f}$ and Icmt$^{-/-}$ cells transfected with tGγ$_2$-WT. The fusion protein has a distinct distribution *in vivo* in cells with or without the Icmt enzyme (nuclei were labeled in blue with Hoechst). The results were the means and are representative of three independent experiments. Scale bar= 15 μm.

Figure 5. Gγ$_2$ protein C-terminal region: structure and cell localization.

SF-767 cells were transfected with the fusion proteins containing the mutation indicated in each panel, and their distribution was analyzed by semi-quantitative confocal microscopy (see Materials and Methods, and the supporting information for more details). **A-B** Confocal microscopy images (A) and relative cell distribution (B) of tGγ$_2$ mutated at the basic residues but not the terminal Cys (carboxyl methylated and geranylgeranylated). **C-D** Confocal microscopy images (C) and relative cell distribution (D) of tGγ$_2$ mutated at the basic residues and at the terminal Cys (carboxyl methylated and geranylgeranylated). The bars correspond to the mean ± SEM values from 12 cells from five independent experiments, and 2 measurements per cell (a total of 24 measurements per mutant): *, $P < 0.05$; **, $P< 0.01$; ***, $P< 0.001$ indicates the

statistical significance using a two way ANOVA followed by the Bonferroni test and compared to the WT. Scale bar= 15 μm.

Figure 6. Role of the C-terminal region of the Gγ2 protein in PM localization.

A Quantification of the relative impact of mutating the basic amino acids on the PM localization of the geranylgeranylated: tGγ2-WT, GFP alone, one point mutants (tGγ2-62, tGγ2-64, tGγ2-65), two point mutants (tGγ2-62/64, tGγ2-62/65, tGγ2-64/65) and three point mutants (tGγ2-62/64/65). **B** Quantification of the relative distribution at the PM of tGγ2-WT, GFP alone, and of the two, three and four point mutants lacking the geranylgeranyl moiety on Cys-68 (tGγ2-62/68, tGγ2-64/68, tGγ2-65/68, tGγ2-62/64/68, tGγ2-62/65/68, tGγ2-64/65/68 and tGγ2-62/64/65/68). The bars correspond to the mean ± SEM values from 12 cells from five independent experiments (a total of 24 measurements per each mutant): *, $P < 0.05$; **, $P< 0.01$; ***, $P< 0.001$. **C** 3D-structure of the Gβ1γ2 dimer (adapted from 2BCJ structure of PDB code), indicating the Lys-65 (green), Lys-64 (purple) and Arg-62 (yellow) residues at the C-terminal extreme of the Gγ2 subunit. **D** tGγ2-WT fusion protein (green, first column) and DiI (yellow, Ld membrane domains, second column) co-localize at the plasma membrane (white, fifth column). The nuclei were labeled with Hoechst (third column). Scale bar= 5 μm.

Fig.1

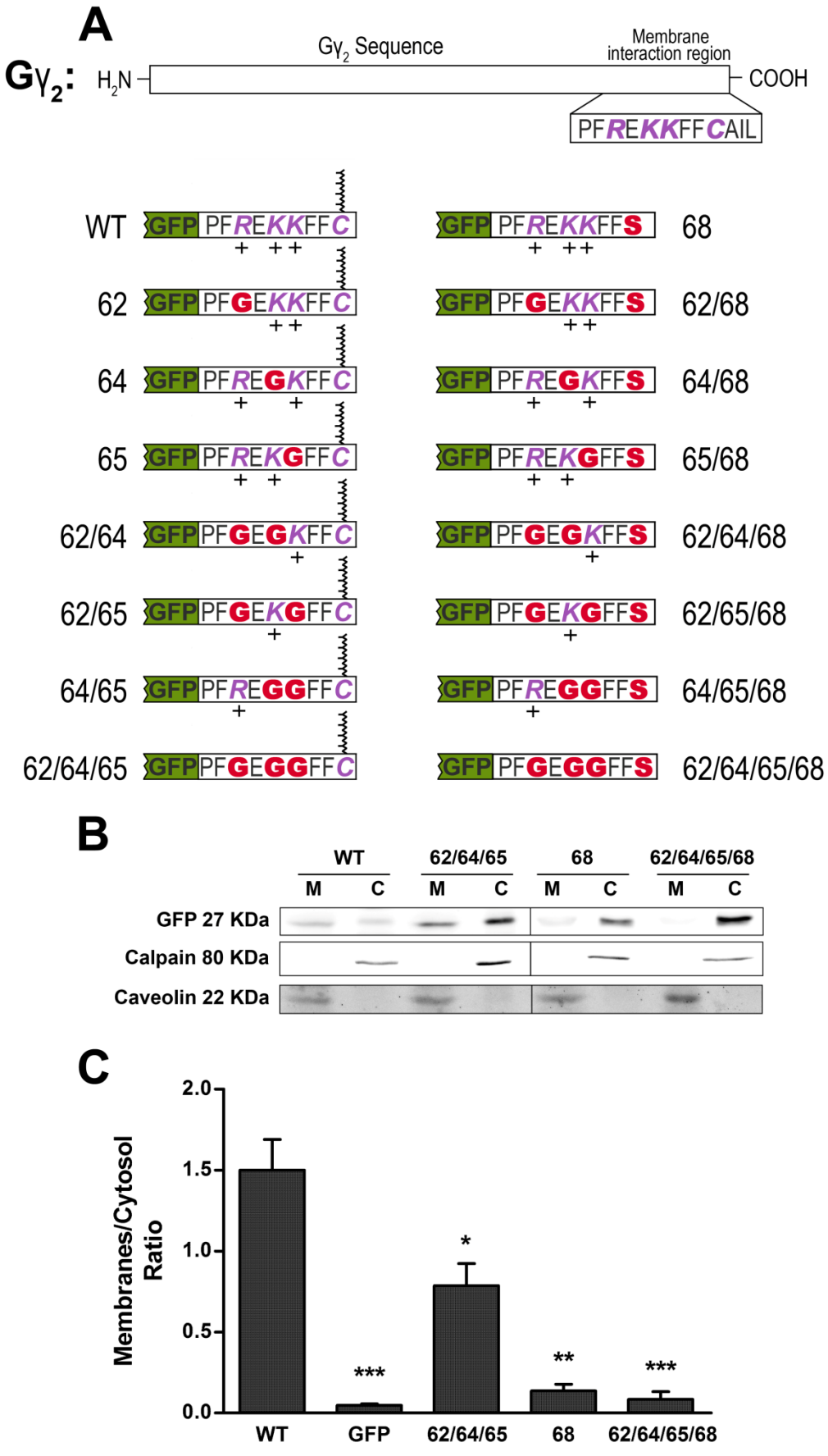


Fig.2

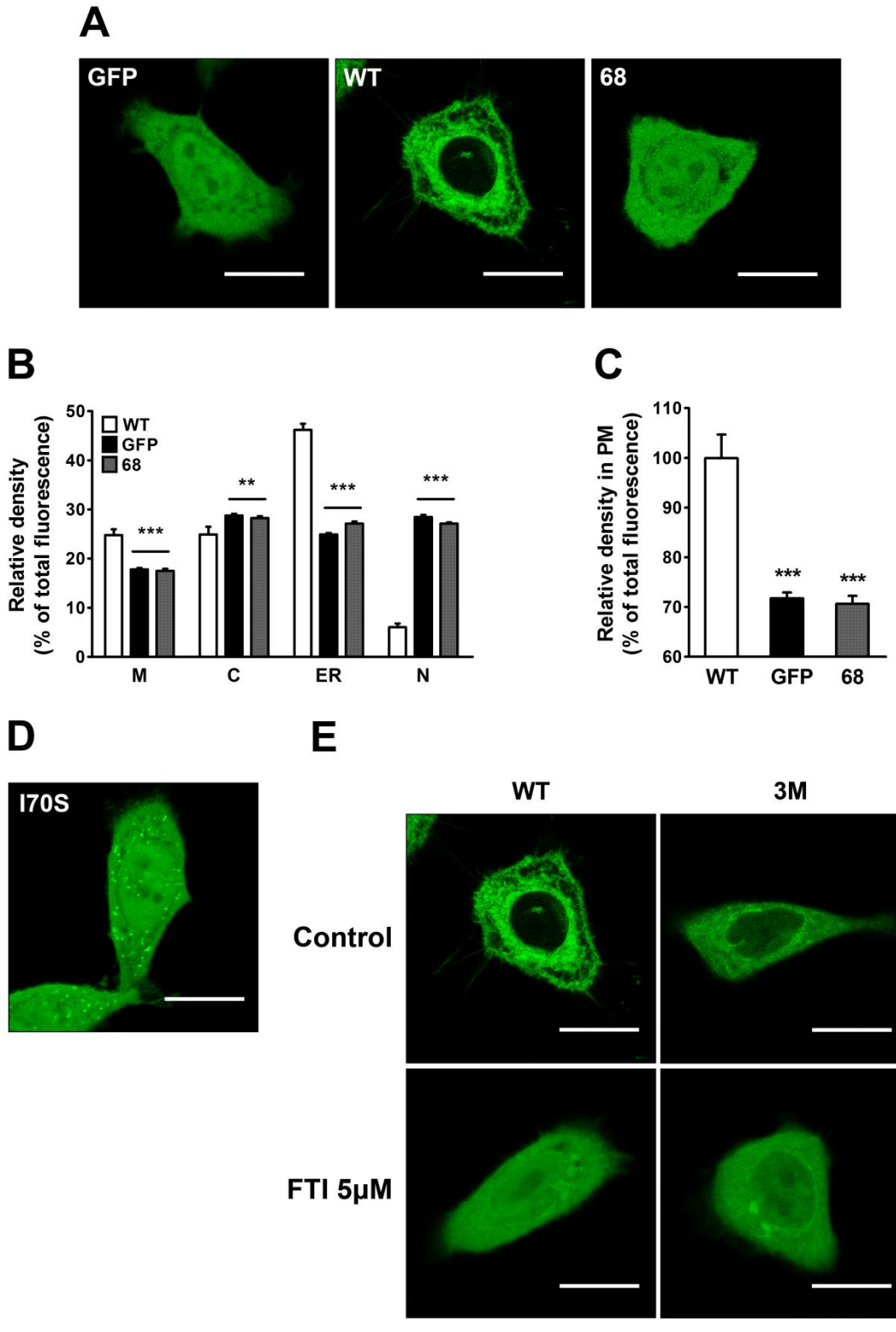


Fig.3

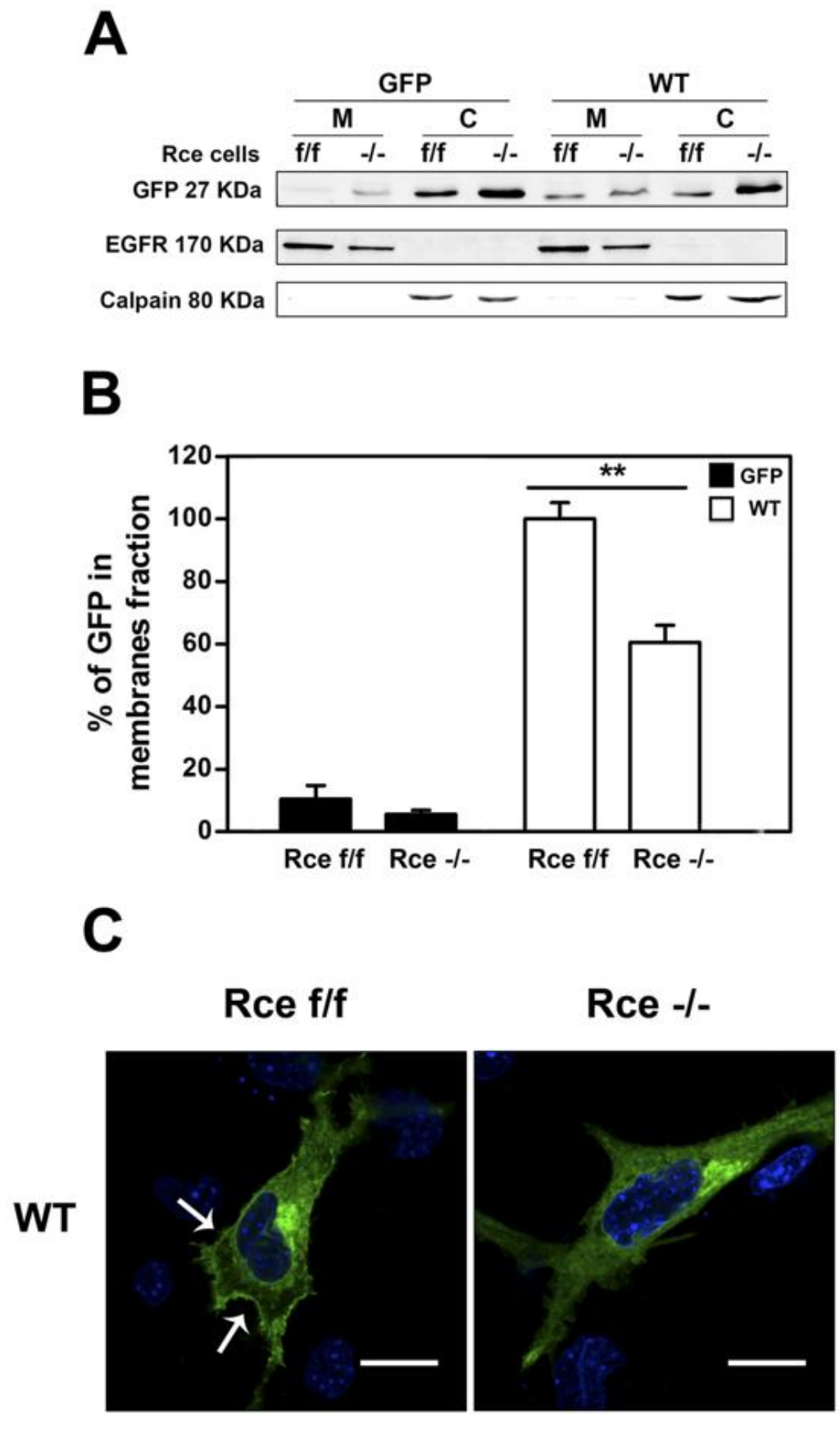


Fig.4

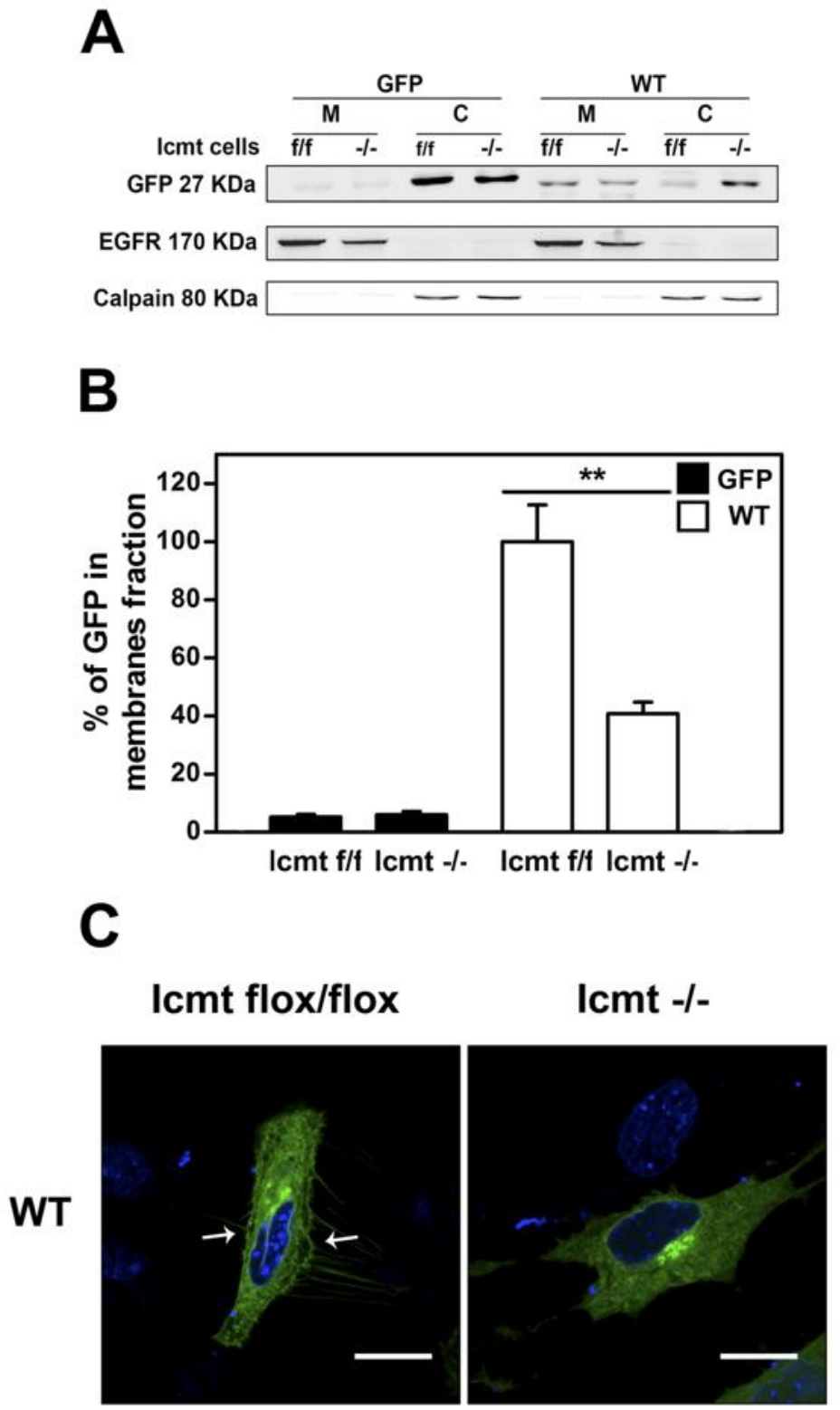


Fig.5

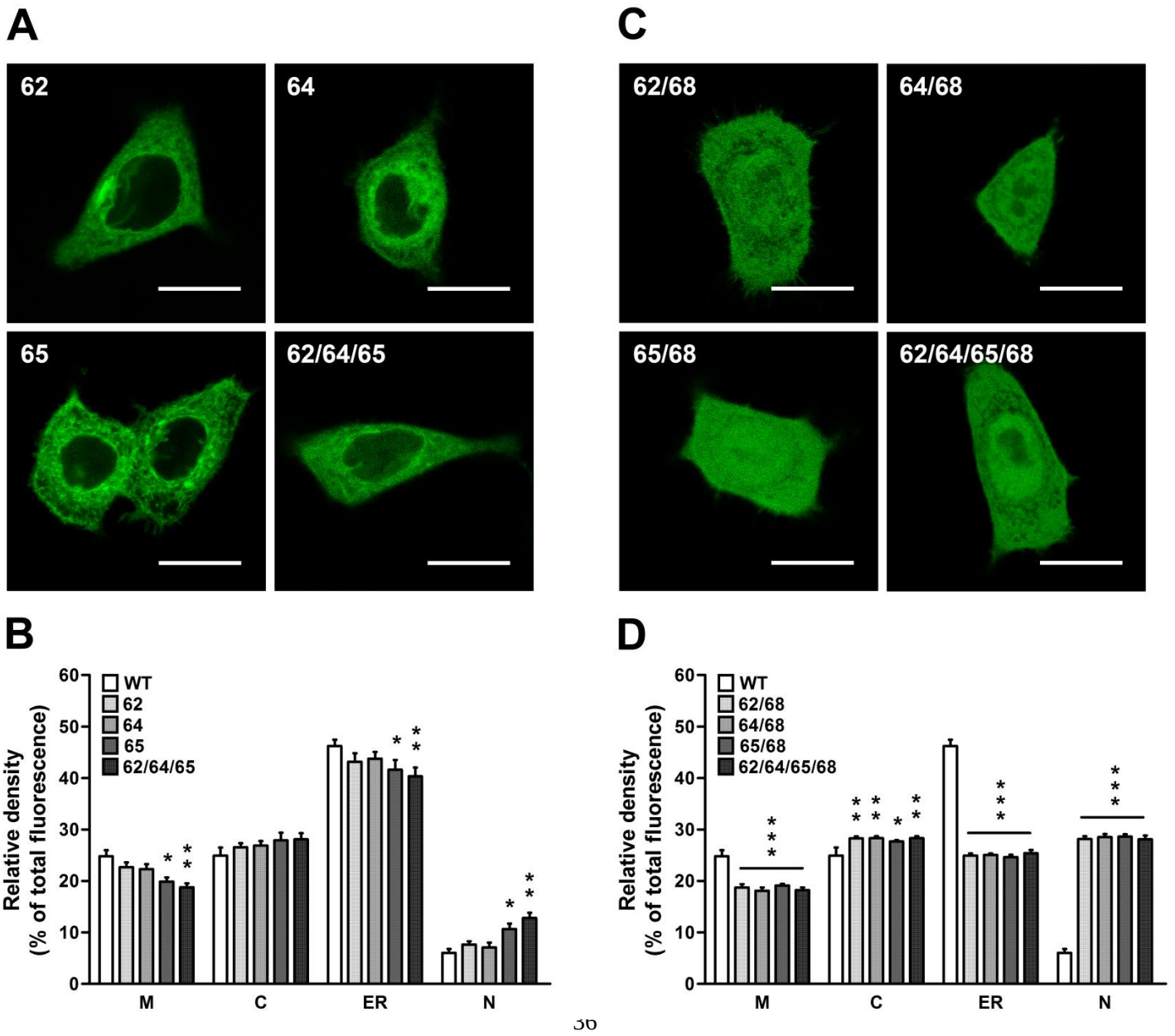
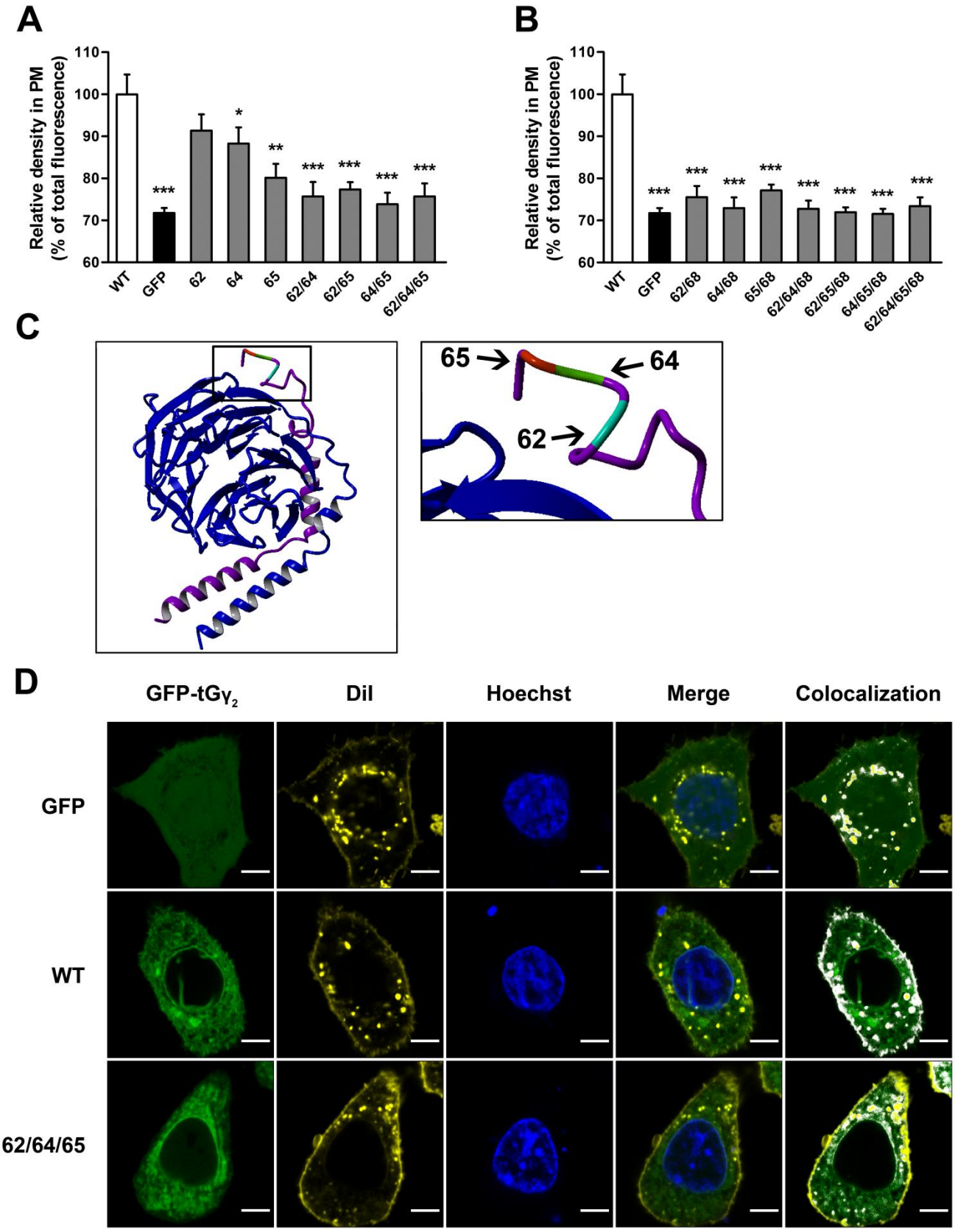


Fig 6



Highlights

Heterotrimeric G proteins are peripheral membrane proteins that frequently localize to the plasma membrane where their presence in molar excess over G protein coupled receptors permits signal amplification. Their distribution is regulated by protein-lipid interactions, which has a clear influence on their activity. G□□ dimer drives the interaction between G protein heterotrimers with cell membranes.

We focused our study on the role of the C-terminal region of the Gγ2 protein in G protein interactions with cell membranes. The Gγ2 subunit is modified at cysteine (Cys) 68 by the addition of an isoprenyl lipid, which is followed by the proteolytic removal of the last three residues that leaves an isoprenylated and carboxyl methylated Cys-68 as the terminal amino acid. The role of Cys isoprenylation of the CAAX box has been defined for other proteins, yet the importance of proteolysis and carboxyl methylation of isoprenylated proteins is less clear.

Here, we showed that not only geranylgeranylation but also proteolysis and carboxyl methylation are essential for the correct localization of Gγ2 in the plasma membrane.

Moreover, we showed the importance of electrostatic interactions between the inner leaflet of the plasma membrane and the positively charged C-terminal domain of the Gγ2 subunit (amino acids Arg-62, Lys-64 and Lys-65) as a second signal to reach the plasma membrane. Indeed, single or multiple point mutations at Gγ2 C-terminal amino acids have a significant effect on Gγ2 protein-plasma membrane interactions and its localization to charged Ld (liquid disordered) membrane microdomains.