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2023

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Investigating G-protein dual coupling in compartmentalized beta-adrenergic receptors

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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By

Evelyn Hernandez

*Para mis primeros maestros Adrián, Elvira, y Jacqueline, mi primera estudiante Ariana,
y para las dos mujeres que le dieron vida a toda mi familia y se despidieron durante mi
doctorado, Herlinda (mama Linda) y Consuelo (abuelita Chelo).*

Acknowledgements

First and foremost, I want to acknowledge that UCSF sits on unceded ancestral homeland of the Ramaytush Ohlone tribe who are the original inhabitants of the San Francisco Peninsula. It is important to start off with this statement as this is the land where I conducted all of the experiments involved in this dissertation.

I next want to honor my parents, Adrian and Elvira Hernandez, proud and hardworking immigrants from small towns in Jalisco, Mexico. Though they did not have the opportunity to pursue their education past middle school, they have been my biggest supporters in going to college and pursuing a higher degree. The discrimination that I have witnessed them experience, how gracefully they have dealt with it, and how they keep fighting like the *chingones* they are fueled my motivation and productive rage to achieve my goals and keep fighting for myself, others in my community, and those more marginalized than me. I also thank them for always making sure that I knew where I came from, to be proud of being Mexican, and keep my head up high when anyone had doubts of my place in science and higher education. I also want to thank my very first teacher, my elder sister Jacqueline Hernandez, who taught me how to read and write when I was three years old and with her brilliant and radical mind has taught me how to not be afraid to push back and challenge unjust institutional systems. I want to acknowledge my little sister Ariana Hernandez, my very first student, who I love so much and has taught me to have compassion for others with her big heart.

Next, I want to send my love to my very large extended family, those who have immigrated to the United States and to those still in Jalisco, Mexico. I especially want to thank the strong women in my family, my tias and abuelitas, who through their

stubbornness and resistance against the sexism in the Latin-American culture taught me to resist patriarchal ideas. I want to highlight my two grandmas, Mama Linda and Abuelita Chelo, who passed away in 2020 six months apart from each other, Mama Linda from COVID and Abuelita Chelo from heart complications. Like many biologists and researchers, I drew inspiration from their situations and focused my PhD on cardiac biology and I became involved in COVID-19 research with QBI Coronavirus Research Group as a team leader.

My formal educational journey began in Mrs. Hilda Alvarez's classroom in kindergarten, who was the first person to teach me to speak English. She always wanted us to try our best and not do anything half-done "no trochi mochi!" as she would say. Every time I wanted to slack a little bit on an experiment, or presentation, or assignment in undergrad and my PhD, I would always hear her voice saying "Evelyn, no trochi mochi!" and that was always a nice reminder that we should always try to do everything to the fullest of our abilities. My high school science teachers are the main reasons why I ever even became interested in science. Most importantly, my favorite teacher of all time (and awesome tennis player), Mr. Randall Rodgers, who taught me 9th grade biology, honors chemistry, and AP Biology. His sternness and dry humor always pushed me to go above and beyond in my science courses. Mr. Doug Dolter instructed anatomy and physiology and he taught me that science can be fun and did not always have to be so serious to have deep knowledge in a field. My physics teacher Mr. Shea put in the most effort in ensuring all his students understood complex topics. Our school was not very well funded but he would go out of his way in such creative ways to make sure we could perform cool

experiments in class that helped our understanding of physics. His class was one of the most useful ones in my undergrad courses at UCLA.

I also want to thank my high school tennis coaches: Coach Brodbeck, Coach Hart, and Coach Reid. Doing a PhD is like a very, very long singles tennis match. Though you practice for hours (read papers), have guidance from coaches (guidance from a PI), the match itself (the experiments) can be a very isolating experience. Like in tennis, in science you have to play the long mental game and be able talk yourself down from lost points (failed experiments) and move onto the next point (next experiment). Though I was not always the most patient at doing this as a high schooler nor as a scientist, the lessons I took from my coaches were always applied in the lab. Coach Brodbeck taught me that when you are playing the defensive game, sometimes all you have to do is get the ball over the net in whatever way possible, and it did not always have to be the prettiest shot. This taught me that sometimes, experiments will feel like they are going against you and the troubleshooting game is endless, but that no matter what, you have to show up the next day and try again. Coach Reid taught me to try to have a short-term memory in the failures and losses and just try to have fun in the process. Coach Hart, a biologist himself and bless his soul because he put up with some hot headedness from me on the court. He taught me that it is important to take a step back in those very heated moments and reassess the next step with a clearer mind.

Next, I want to thank the village that got me started in the academic research journey: Dr. Tama Hasson, Dr. Paul Barber, Dr. Devin Horton, Dr. Janet Goins, and Renay Panduro who all worked at the UCLA undergraduate research center. They worked endlessly to encourage historically marginalized students to pursue science

research and continue on to getting a PhD. I know for certain I would not be where I am today were it not for them. I also want to acknowledge Dr. Anne Andrews and Dr. Maureen Sampson, my very first principal investigator (PI) and graduate student mentor. Though I was only with them for one year at UCLA, I learned invaluable lessons from the work they did. I want to thank Dr. Jose A. Rodriguez, who is also from Jalisco, Mexico and showed me that people like us can succeed in scientific research. Not only did he spark my love for structural biology, but he also pushed me to be the very best and excel in ways that many undergrads are not pushed, and for that I thank him. From the Rodriguez lab, I also want to thank Dr. Maria Flores, also a proud Mexicana, whose camaraderie in the lab was invaluable when things got tough.

From UCSF, I of course want to thank a million times over Dr. Roshanak Irannejad. I came into UCSF thinking I would do my PhD in a structural biology lab but within one week of rotating in her lab, I knew she was worth the switch into cell biology. She has taught me more things than I can list here, science- and life-wise. Her infectious love and curiosity for science and the type of research she does fueled my love for G-protein coupled receptors. Furthermore, I cannot thank former Irannejad lab post-doc Dr. Quynh Nhu Mai enough for everything she has taught me. Quynh continues to be a mentor to me even after she left the lab, and she has been an amazing person to have to look up to. Quynh taught me nearly every cell biological and pharmacological technique that I know, so 100% this dissertation would not exist had it not been for her teachings and her incredible patience. Not only did she teach me hands on science techniques, but she always was also there to listen to my rants about science and life and provided the right words I needed to hear. Dr. D'Anne Duncan is the very best person, place, and everything

that exists at UCSF. I cannot stress enough that I would not have remained at UCSF had it not been for her immense understanding, care, love, and attentiveness to her students. She is the beating heart and soul of UCSF, especially for students of marginalized communities. I also want to thank Zach Smith, who I enjoyed working with so much as an S RTP mentor during summer 2022. He was a breath of fresh air for UCSF. Finally, I want to thank my qualifications and thesis committees, especially my chair Dr. Jeremy Reiter who not only was there to advise me in science but was also a great listener during the third-year slump. I want to thank my other committee member Dr. Shaeri Mukherjee who always reminded me to think critically at my thesis committee meetings.

Lastly, I want to thank the UCSF crew! Dr. Natasha Puri, my amazing lab mate and friend who I created so many amazing memories with, I miss her in the lab every day. She is an experienced cell biologist and always had the answers and provided me with calmness when I was in a panic. Dr. Jocy Lopez, my graduate school best bud, who added so much positivity in my life, from amazing travels together, F45 (gym workouts), crying sessions, San Francisco outings, and so much more. UCSF for me is unimaginable without Jocy in the picture. Harold Marin, who has become family for me, and with his scientific thoroughness and light-heartedness outside the lab showed me that you can always work hard and play hard as a scientist. Priscila Muñoz Sandoval, a beautiful and warm amiga who I was able to embrace my Mexican culture with while playing Latin music during our cooking sessions. Yewande Alabi, my most genuine friend, who I was able to always have much needed real talk with at any time of the day. Hayden Saunders, an awesome friend who I was able to share some of my favorite interests with such as futbol and tennis, and I also thank for being my tennis hitting partner. Gabriela Canales, an

incredible scientist and person, who fought tirelessly with me as co-diversity officer to try to make UCSF a better place for marginalized students that came after us. Richard Van, aka Coach Richy/Ricardo, who brought tennis back into my life in grad school. Last but absolutely not least, my bestest friend and life partner Andre Rodrigo Espinoza, who although is not a scientist nor a UCSF student, I met indirectly as a result of me coming to UCSF. He makes endings feel like beginnings, and I look forward to the next chapter of my life with him by my side.

Abstract

Investigating G-protein dual coupling in compartmentalized beta-adrenergic receptors

Evelyn Hernandez

Cell-to-cell communication is essential for multicellular organisms and the combined effects of multiple signals determine a cell's response. There are many ways that the cells that make up our tissues communicate with one another in order to send signals from one place in our bodies to another. One way is where molecules such as hormones travel through the bloodstream over longer distances to reach target cells that are located far away from the sending cell. Certain hormones are sensed by receptors that reside on the surfaces of cells, such as G protein coupled receptors (GPCRs). GPCRs respond to many stimuli that regulate smell, sight, mood, pain, and cardiovascular and nervous systems. As a result, these receptors are the targets of many drug development efforts and therefore make up about 30-40% of targeted receptors in clinical drugs. Previously, it was believed that GPCRs could only be activated at the plasma membrane and those residing inside of the cell were dormant or silent. However, some GPCRs can be activated within the cell. The beta-1 and beta-2 adrenergic receptors ($\beta 1AR$ and $\beta 2AR$, respectively) are GPCRs that regulate heart function and are activated by hormones released by the sympathetic system such as epinephrine and norepinephrine. $\beta 1AR$ and $\beta 2AR$ are located at the plasma membranes of cardiomyocytes but also have internal residences at organelles such as the Golgi apparatus for $\beta 1AR$ and endosomes for $\beta 2AR$. It was recently found that the receptors that live at the Golgi and endosomes contribute to distinct signaling responses and

provide different cellular outputs than cell surface receptors. This discovery is important because deciphering how the different receptor pools are regulated can aid in the development of appropriate drugs that target the correct receptor pool. Furthermore, β 1AR and β 2AR can couple to more than one G protein type, adding another layer of signaling dynamics that can occur in cardiovascular biology. The work detailed in chapter 2 provides a potential mechanism on how this dual coupling may be regulated. Chapter 3 focuses on deciphering the molecular determinants of β 1AR Golgi retention. Chapter 4 explores how G protein dual coupling may be manipulated by using drugs that bias β 1AR to one G protein over another. Lastly, chapter 5 sheds light on the distinct interactomes that arise when β 1AR is activated at the plasma membrane versus the Golgi apparatus using proximity labeling, as was previously done for β 2AR. This work reveals interesting pathways for β ARs that reside in distinct compartments and furthers our knowledge of cardiovascular function, and it can also reveal properties of other receptors in the GPCR family that can signal internally.

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Chapter 1:
**G-protein coupled receptors, G proteins, and Beta-adrenergic
receptors**

Evelyn Hernandez

1.1 G-protein coupled receptors

Over 800 genes contain the code for G protein coupled receptors (GPCRs), the largest family of protein receptors in the mammalian genome, characterized by their signature seven alpha helices that traverse cell membranes. GPCRs dictate several biological functions in mammals from sight, olfaction, pain, heart and brain functions, and many more (Pierce, Premont, Lefkowitz, 2002). Cells can receive messages from one another through the signaling of GPCRs, which can be activated by several types of ligands, resulting in the coupling to a heterotrimeric G-protein, consisting of alpha, beta, and gamma subunits. Depending on the type of GPCR that is activated and the type of G alpha ($G\alpha$) protein that the receptor couples to, different types of signaling cascades occur that produce a wide range of biological responses.

1.2 GPCR compartmentalized signaling

Beta-1 and beta-2 adrenergic receptors ($\beta 1AR$ and $\beta 2AR$, respectively) are GPCRs activated by epinephrine and norepinephrine released by the sympathetic system and dictate important cardiovascular physiological responses. Epinephrine is known to activate $\beta 1AR$ and $\beta 2AR$ in cardiomyocytes and binds to both at similar affinities as shown by crystal structures (Xu et al, 2021; PDB: 7BTS for $\beta 1AR$ and 4LDO for $\beta 2AR$). Norepinephrine binds to both $\beta 1AR$ and $\beta 2AR$ more weakly than epinephrine, with a preference for $\beta 1AR$ over $\beta 2AR$ (Xu et al, 2021). In cardiomyocytes, $\beta 1AR$ and $\beta 2AR$ regulate cardiac function through activation of G_s - and G_i -mediated cAMP production or degradation, respectively. $\beta 1AR$ comprises ~80% of βAR s expressed in cardiomyocytes, and $\beta 2AR$ the remaining ~20% (Rohrer et al., 1999). Despite having similar effectors, $\beta 1AR$ and $\beta 2AR$ elicit significantly different effects on cardiac functions. For example,

β 1AR plays a dominant role in chronotropy (heart rate), inotropy (force of contraction) and lusitropy (relaxation) responses in healthy cardiomyocytes, whereas β 2AR produces modest chronotropic effect and no lusitropic responses (Rohrer et al., 1999). Interestingly, β 1AR and β 2AR have very distinct membrane localizations in cardiomyocytes. β 1AR is localized in the plasma membrane and perinuclear/Golgi regions, whereas β 2AR is localized in the T-tubules of adult cardiomyocytes and is not retained at the Golgi (Boivin et al., 2006). More importantly, our lab has shown that the plasma membrane- and Golgi-localized β 1ARs activate Gs-mediated cAMP responses, and β 2ARs activate Gs-mediated signaling from the plasma membrane and endosomes (Irannejad et al., 2013; Irannejad et al., 2017).

Epinephrine gains access to the internal pools of β ARs via the organic cation transporter 3 (OCT3) which is expressed at the plasma membrane of certain cells including cardiomyocytes (Irannejad et al., 2017). Overstimulation of β 1AR by epinephrine over time can lead to heart pathologies. Furthermore, the overstimulation of specifically Golgi- β 1AR causes hypertrophic phenotypes in rat cardiomyocytes (Nash et al., 2019). Other receptors, such as the dopamine 1 receptor (D1DR) and opioid receptors (delta and mu), also have consistent Golgi pools and can activate G-protein signaling from this internal location (Puri et al., 2022; Radoux-Mergault et al 2023). Downstream signaling effectors are distinct when activation of the delta-opioid receptor at the plasma membrane or the Golgi as shown by RNA-seq and phospho-proteomics (Radoux-Mergault et al 2023). Furthermore, β 2AR, Neurokinin-1 receptor (NK1R), thyroid stimulating hormone receptor (TSHR), and Vassopresin 2 receptor (V2R) at the plasma membrane and endosomes produce different transcriptional responses (Crilly and

Puthenveedu, 2020). Furthermore, depending on the location, receptor signaling can either be sustained or transient, thus producing different effects (Plouffe et al., 2020). Generally, sustained responses tend to originate from subcellular locations and transient responses from the plasma membrane, perhaps due to the proximity of internal organelles to the nucleus (Plouffe et al., 2020). Additionally, the importance of compartment-specific GPCR activation was illustrated with D1DR as local activation of Golgi-localized protein kinase A (PKA) was dependent on Golgi-D1DR specific stimulation (Puri et al., 2022).

1.3 G protein selectivity

There are over 800 types of GPCRs that relay their signals by coupling to sixteen types of G α proteins that are classified into four different families, Gs, Gi/o, Gq/11 and G12/13. How coupling selectivity is precisely determined is still unclear. The C-terminal domains of G α proteins have been classically attributed to determine G α protein coupling selectivity, however, the precise mechanism is not fully understood. Different modes of GPCRs binding to the G protein C-terminal domain have been shown structurally where the C-terminal domain of Gs seems to require a more open receptor core, while the Gi C-terminal domain engages a more closed structure (Van Eps et al., 2018). The C-terminal domain sequences from different G protein types (Gs, Gi/o, Gq, G12/13) are quite distinct especially in the last five amino acids. This is the region that embeds itself into the core of the receptor when interacting. While the C-terminal domain of G proteins is what has been classically attributed to providing the selectivity for GPCR binding, more recent evidence points to other parts of the G protein as also being important for coupling

selectivity (Flock et al., 2017; Okashah et al., 2019; Strohmman et al., 2019; Jang et al., 2023).

BRET analysis was used to study the engagement of several GPCRs, including β 2AR, and twelve G protein chimeras that were synthesized by swapping the C-terminal domains and N-terminal domains of Gs, Gi, Gq and G₁₂. It was shown that while the C-terminal domain was the most important contributor for selectivity, the N-terminal domain also contributed to determining selectivity as a slight rescue in recruitment of non-cognate G proteins containing the cognate G protein N-terminal domain was observed. Some receptors were less tolerant in accepting G protein chimeras than others, showing that not all receptors have the same ability to be flexible in binding to non-cognate G proteins (Okashah et al., 2019).

There are some receptors that couple to more than one G protein type. For example, β 2AR receptor and serotonin type 4 receptor (5HT₄) couple to Gs and Gi proteins (Daaka et al., 1997; Zamah et al., 2002; Ma et al., 2020; Huang et al., 2022), and the NK1R couples to Gs and Gq (Harris et al., 2022). Although β 1AR-Gs coupling has been more widely studied, there is evidence that it can also be Gi-coupled, where inhibition of Gi signaling using pertussis toxin influenced β 1AR stimulation readouts (Martin et al., 2004) as well as Gi signaling being required for β 1AR-arrestin signaling (Wang et al., 2017). Though there is a wide range of in vitro biochemical and structural analyses that begin to explain the molecular mechanisms of GPCR-G protein binding, how GPCRs are able to bind two different G proteins and what facilitates this dual coupling in cells is not very well understood. Moreover, why receptors evolved the ability

to couple to two antagonizing G proteins, as with Gs and Gi dual coupling, also remains an open question.

Others have attempted to find a 'selectivity barcode' or consensus sequence that dictates G protein coupling patterns (Flock et al., 2017). While a specific amino acid sequence for a selectivity barcode was not determined, amino acid properties such as electrostatic charge, hydrophobicity, and side chain size were found to be generally consistent in GPCR-G protein binding interfaces. Because there are more GPCRs than G proteins, the G protein sequence is under higher selectivity scrutiny, and this is seen in evolution where the G α subunit sequence has remained more conserved than that of receptors (Flock et al., 2017). Therefore, GPCRs most likely evolved features that allow it to overcome such high scrutiny to be able to bind to certain G proteins, such as modulating its expression levels in certain cell types, post-translational modifications, phosphorylation, and potential allostery provided by membrane phospholipids or phosphoinositides. These additional features help GPCRs fine-tune and in some cases, even alter their G protein selectivity patterns. Furthermore, it allows for adaptation to particular and more stressful environments where different stimuli might be present and for distinct signaling patterns across varying cell types (Flock et al., 2017).

The studies discussed thus far have been performed using nucleotide-decoupled G protein probes. In a recent study, researchers found that selectivity for G proteins can be broken down when G proteins are nucleotide-decoupled (Jang et al., 2023). This resulted in higher promiscuity in 24 out of 32 receptors that were tested, such as the muscarinic receptor 4 (M4R) and β 2AR, where they coupled a modest amount to all six G proteins that were tested (Gs, Gi, Gq, Go, G₁₂, G₁₃, G_{olf}). Decoupling G proteins from

nucleotide binding and exchange also caused ligand insensitivity, for example, nucleotide-decoupled Gs constitutively bound to $\beta 2AR$. However, a higher preference for cognate G protein binding persisted even when nucleotide-decoupled (Jang et al., 2023).

Another recent study found that the intracellular loop 3 regions (ICL3) of GPCRs are allosterically modulated to allow access to the GPCR core for G proteins to engage (Sadler et al., 2023). The ICL3 was shown to autoregulate its specificity away from G proteins that weakly bind to the receptor by physically blocking access to non-cognate G proteins. This was shown for $\beta 1AR$, $\beta 2AR$, and five other GPCRs including D1DR. This finding is quite interesting because the ICL regions of GPCRs are the most variable in sequence across all receptors and contain high structural flexibility, as the transmembrane regions must retain the rigid alpha-helical structure. Therefore, this has given receptors the ability to evolutionarily alter small segments of their ICL to fine-tune their signaling patterns.

1.4 Phospholipid environments

In vitro studies suggest that membrane lipid compositions may play a role in determining G protein coupling selectivity (Dawaliby et al., 2016; Yen et al., 2019; Janetzko et al., 2022). In particular, anionic phospholipids such as phosphoinositide 4,5 biphosphate (PI(4,5)P₂), which is highly enriched at the plasma membrane, promote Gs coupling via electrostatic interactions between anionic lipid head groups and positively charged amino acids in the core region of Gs (Strohman et al., 2019). Others have demonstrated that anionic phospholipids provide a priming mechanism for the receptor to engage signaling partners. Phospholipids were shown to embed themselves into the G protein binding site and stabilize the receptor in an open active conformation to facilitate

G protein coupling (Thakur et al., 2023). This mechanism suggests that negatively charged phospholipids directly prepare GPCRs to engage intracellular G proteins. This is reminiscent to previous models suggesting that non-cognate G proteins could synergistically prime GPCRs to bind to cognate G protein subtypes and enhance the signaling effect (Gupte et al., 2017).

We investigate what facilitates dual coupling for β 1AR and β 2AR to two opposing G proteins. We hypothesize that phosphoinositide composition at the plasma membrane plays a role in this switch as has been the case for other GPCRs.

Using conformational sensitive biosensors for activated GPCRs and G proteins, our lab and others have demonstrated that β ARs activate $G_{\alpha s}$ protein at the plasma membrane and the Golgi membranes for β 1AR (Irannejad et al., 2017; Nash et al., 2019) and at the plasma membrane and endosomes for β 2AR (Irannejad et al., 2013). The sensors we utilize, mini-G proteins, were curated for structural purposes and comprise of engineered GTPase Ras-like domains of G_{α} proteins that maintain the interactions formed between activated GPCR and its cognate G protein. We find that β 1AR and β 2AR couple to $G_{\alpha s}$ and $G_{\alpha i}$ distinctively when stimulated with isoproterenol. For β 1AR, we observed $G_{\alpha s}$ coupling at the Golgi but not $G_{\alpha i}$. Given the distinct lipid environment at the plasma membrane and the Golgi, we hypothesize that the phospholipid compositions at each of these subcellular locations may play a role in dictating the selectivity and the kinetics of G_{α} protein coupling.

1.5 Conclusion

To investigate how lipids regulate β AR function within specific subcellular environments, in chapter 2, we modified anionic phospholipids such as PI(4,5)P₂ that is

enriched on the plasma membrane by targeting phosphoinositide phosphatases at this compartment and testing whether this modification affects Gas and Gai coupling. We observe the loss of Gai coupling for $\beta 1AR$ and $\beta 2AR$ when PI(4,5)P₂ is depleted from the plasma membrane. We also observed a loss and reduction of Gai coupling for $\beta 1AR$ and $\beta 2AR$, respectively, when we tested miniG recruitment using receptor mutants that cannot bind PI(4,5)P₂. Additionally, while Gas coupling is observed at the Golgi, Gai coupling is not. The Golgi apparatus is a membrane structure devoid of PI(4,5)P₂ and enriched instead with PI(4)P, further suggesting that PI(4,5)P₂ may be acting as a facilitator for $\beta 1AR$ -Gai coupling. Finally, cAMP production is increased in cells expressing receptor mutants and those lacking PI(4,5)P₂ compared to cells expressing wildtype receptors. Together, these results suggest that PI(4,5)P₂ may be facilitating dual coupling for Gas and Gai to $\beta 1AR$ and $\beta 2AR$. These findings provide a deeper understanding of GPCRs/G α protein coupling selectivity and provide further evidence for the role of phosphoinositides in regulating GPCR activity at different subcellular compartments in cells.

In chapter 3, I address the conundrum of $\beta 1AR$ Golgi retention. I focus on its C-terminal domain and intracellular loops 3 of $\beta 1AR$ and $\beta 2AR$, which is where they diverge in sequence the most. Certain amino acid motifs in the C-terminal domain have been previously shown to retain other GPCR at the Golgi. Mutating these residues in $\beta 1AR$ did not seem to affect $\beta 1AR$ retention. However, when $\beta 1AR$ and $\beta 2AR$ had their intracellular loops 3 swapped, $\beta 1AR$ was retained significantly less at the Golgi, indicating that there may be a Golgi retention signal in this intracellular loop 3 region.

To better understand whether certain drugs activate specific G protein pathways, in chapter 4 I test the recruitment of Mini-G proteins when I stimulate $\beta 1AR$ with four drugs

that are commonly used in β AR laboratory research and in clinical settings. This study highlights the importance of understanding and identifying the downstream effectors that are activated when specific drugs are used especially when thinking about drug design that may eventually be prescribed as therapeutics for patients. Finally, in chapter 5 I utilize an unbiased approach using proximity labeling to further identify key players involved upon Golgi- β 1AR signaling to further elucidate the consequences of compartmentalized signaling of GPCRs. Given that GPCRs at the plasma membrane have been widely studied and several key downstream players have already been identified there, comparing the proteins that are activated in subcellular residences is important for understanding how receptors are regulated at different locations in the cell.

Altogether, this work furthers the field's knowledge of how compartmentalized signaling, a relatively novel concept, affects GPCR biology. This is crucial because GPCRs control several essential biological functions and because of this, they are targets for designing therapies and we must understand how novel drugs will affect GPCRs throughout the cell, not just at the plasma membrane.

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Chapter 2:

Anionic phospholipids determine G protein coupling selectivity at distinct subcellular membrane compartments

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2.1 Abstract

G protein-coupled receptors (GPCRs) activate different signaling pathways through coupling to heterotrimeric G proteins consisting of four types of G alpha ($G\alpha$) subunits and the beta gamma ($\beta\gamma$) subunits. The C-terminal regions of $G\alpha$ proteins is thought to determine coupling selectivity. However, there are several reports indicating that some receptors are promiscuous and can couple to multiple $G\alpha$ proteins. The precise mechanism promoting promiscuous coupling is not fully understood. Here we show that anionic phospholipids such as phosphoinositide 4,5 bisphosphate (PI(4,5)P₂) promote electrostatic interactions between anionic lipid head groups and positively charged amino acids in the transmembrane 4 (TM4) region of beta adrenergic receptors (β ARs) to stabilize active conformation of receptor and prime coupling of the cognate $G_{\alpha s}$ and non-cognate $G_{\alpha i}$ proteins. The promiscuous coupling can only occur at the plasma membrane, a membrane compartment enriched in PI(4,5)P₂, while receptors can couple only to its cognate $G_{\alpha s}$ protein in compartments that are enriched in less negatively charged phosphoinositides such as the Golgi membranes. We took advantage of the rapamycin dimerization system to rapidly and inducibly deplete PI(4,5)P₂ by recruiting a phosphatase to the plasma membrane and found that in the absence of anionic phospholipids, β ARs couple only to their cognate $G_{\alpha s}$ protein. Finally, we found that mutating β ARs PI(4,5)P₂ binding motifs or depleting PI(4,5)P₂ results in enhanced β ARs-mediated cAMP response. Together, these findings reveal a role for negatively charged phosphoinositides in regulating GPCR activity at different subcellular compartments.

2.2 Introduction

There are over 800 types of GPCRs that relay their signals by coupling to sixteen types of Gα proteins, classified into four different families of Gas, Gai/o, Gαq/11 and Gα12/13 (Pierce et al., 2002). How coupling selectivity is precisely determined is still unclear. The C-terminal domains of Gα proteins have been classically attributed to determine Gα protein coupling selectivity. The C-terminal domains of different Gα protein types are distinct especially in the last ten amino acids. This is the region that embeds itself into the core of the receptor in the open active conformation (Oldham et al., 2008; Marinissen et al., 2001; Flock et al., 2017; Wess 1998). While the significance of the Gα protein C-terminal domain for coupling selectivity has been extensively studied, more recent evidence highlights the importance of the N-terminal region of the Gα protein (Flock et al., 2017; Sandhu et al., 2022; Okashah et al., 2019; Strohmman et al., 2019). By analyzing the coupling efficiency of various types of Gα protein chimeras that contained a combination of C- and N-terminal domains of Gas, Gai, Gαq and Gα₁₂, it was shown that while the C-terminal domain plays a significant role in determining the coupling selectivity, the N-terminal core region of the Gα proteins also contributed to determining selectivity (Okashah et al., 2019).

A wide range of, *in vitro*, biochemical, and structural analyses began to reveal the molecular mechanisms determining GPCR-Gα protein coupling selectivity. A comprehensive analysis of GPCR and Gα protein sequences unveiled a 'selectivity barcode' that dictates Gα protein coupling patterns (Flock et al., 2017). While a specific amino acid sequence for a selectivity barcode was not determined, amino acid properties such as electrostatic charge, hydrophobicity, and side chain size were found to be the

determining factor in GPCR-G α protein binding interfaces. In addition, post-translational modifications, phosphorylation, and potential allostery provided by membrane phospholipids or phosphoinositides were indicated to determine selectivity (Flock et al., 2017). A more recent study found that the intracellular loop 3 regions (ICL3) of GPCRs are allosterically modulated to allow access to the GPCR core for G α proteins coupling (Sadler et al., 2023).

As integral membrane proteins, GPCRs are intimately associated with lipids, but the role of lipids in protein function and signaling is poorly understood. *In vitro* studies suggest that membrane lipid compositions may play a role in determining G α protein coupling selectivity (Dawaliby et al., 2016; Yen et al., 2018; Thakur et al., 2023). In particular, anionic phospholipids such as phosphoinositide 4,5 bisphosphate (PI(4,5)P₂), which are highly enriched at the plasma membrane, promote G α s coupling via electrostatic interactions between anionic lipid head groups and positively charged amino acids in the core region of G α s (Strohman et al., 2019). Other groups have demonstrated that anionic phospholipids provide a priming mechanism for the receptor to engage signaling partners (Thakur et al., 2023). Phospholipids were shown to embed themselves into the G protein binding site and stabilize the receptor in an open active conformation to facilitate G α protein coupling (Thakur et al., 2023). This mechanism suggests that negatively charged phospholipids directly prepare GPCRs to engage intracellular G α proteins. This is reminiscent to previous models suggesting that non-cognate G α proteins could synergistically prime GPCRs to bind to cognate G α protein subtypes and enhance the signaling effect (Gupte et al., 2017).

While many of these studies have focused on coupling selectivity, there are also several studies that suggest some receptors can couple to non-cognate G α proteins in addition to their cognate G α proteins. For example, β 2 adrenergic receptor (β 2AR) and muscarinic receptor 4 have been reported to couple to multiple G α proteins with various efficiencies (Martin et al., 2004; Jang et al., 2023) serotonin type 4 receptor couples to Gas and Gai proteins (Huang et al., 2022), and the Neurokinin-1 receptor couples to Gas and G α q (Harris et al., 2022). Although β 1AR coupling to Gas protein has been widely studied, there is evidence that it can also be Gai-coupled, where inhibition of Gai signaling by pertussis toxin influenced β 1AR-mediated signaling (Martin et al, 2004; Wang et al., 2017). What promotes the promiscuous coupling is not fully understood. A recent study suggests that most GPCRs interact with all four families of nucleotide-decoupled G proteins, however, these interactions do not necessarily lead to GDP release of the noncognate G α proteins and they retain a preference for cognate G proteins (Jang et al., 2023).

In the current study, we investigate the role of phospholipids in regulating coupling selectivity of an exemplary receptor, β 1AR, to two opposing G α proteins. We found that PI(4,5)P2 at the plasma membrane plays a role in stabilizing an active conformation of the receptors, allowing for both Gas and Gai protein coupling, while β 1AR can only couple to Gas at the Golgi membranes that are enriched in PI(4)P. We found that mutating the positively charged residues of β 1AR at TM4 that have been reported to interact with PI(4,5)P2 (Yen et al., 2018), disrupts this dual coupling and biases receptor coupling to Gas only. Similarly, depleting anionic phospholipids such as PI(4,5)P2, by rapidly recruiting a specific phosphatase at the plasma membrane using the rapamycin

dimerization strategy, results in $\beta 1AR$ coupling only to its cognate G αs protein. This dual coupling mechanism is not unique to $\beta 1AR$, as we have also observed it for another receptor with similar PI(4,5)P₂ binding motifs such as $\beta 2AR$. Finally, we demonstrate that isoproterenol-mediated cAMP production is enhanced in cells expressing βAR PI(4,5)P₂ binding mutants or when PI(4,5)P₂ is depleted. Altogether, these results suggest that PI(4,5)P₂ plays a role in priming the active conformation of receptors and facilitating promiscuous G α protein coupling to $\beta 1AR$ and $\beta 2AR$ at the plasma membrane. These findings also provide evidence for the role of phosphoinositides in regulating GPCR coupling selectivity at different subcellular compartments.

2.3 Results

$\beta 1AR$ couples to G αs and G αi proteins with distinct efficiency and kinetics

Several studies suggest that GPCRs can couple to multiple G α subunits. What contributes to this promiscuous coupling is not fully understood. The C-terminal domains of G α proteins have been thought to play a major role in determining G α protein coupling selectivity but more recent evidence suggest that the core region of G α proteins may also play a role in promoting GPCR-G protein coupling (Flock et al., 2017; Okashah et al., 2019; Strohman et al., 2019). This has been shown particularly for G αs involving the electrostatic interactions between the positively charged residues on the intracellular loops of GPCRs with anionic phospholipids such as PI(4,5)P₂ and the positively charged amino acids in the core region of G αs (Yen et al., 2018; Strohman et al., 2019). To begin to test the role of these electrostatic interactions, we first focused on the $\beta 1AR$ because it not only couples to its cognate G αs protein, but evidence suggests that $\beta 1AR$ -mediated cAMP response is sensitive to pertussis toxin, indicating involvement of G αi coupling

(Martin et al, 2004; Wang et al., 2017). To assess whether β 1AR can couple to both Gas and Gai, we employed Mini-G proteins, which are engineered GTPase Ras-like domains of Ga proteins that conserve the associations formed between activated GPCR and its respective Ga protein (Wan et al., 2018; Nehme et al., 2017). These Mini-G proteins are designed to only interact with activate conformations of the GPCR and are found to uncouple nucleotide binding and receptor association (Figure 2.1a) (Nehme et al., 2017; Carpenter et al., 2016). Due to stability issues during the purification process for structural studies, the Mini-G tool used to represent Gai protein was made as a chimera of Gas and Gai sequences containing the C-terminus of Gai and the N-terminus and core regions of Gas, thus we refer to this tool as Mini-G(s)i (Nehme et al., 2017). To monitor the interaction of Mini-G proteins to activated β 1AR, we first used bioluminescence resonance energy transfer (BRET) in HeLa cells expressing β 1AR fused to NanoLuc luciferase (Flag- β 1AR-NanoLuc) with mVenus tagged to either Mini-Gs and Mini-G(s)i. Stimulation of HeLa cells with 10 mM isoproterenol resulted in a pronounced increased in energy transfer between β 1AR and Mini-Gs. Interestingly, we also observed BRET between β 1AR and Mini-G(s)i, although less efficiently (Figure 2.1b). These data suggest that β 1AR could couple to both Gas and Gai.

To better monitor the cellular location where this dual coupling is occurring, we then expressed SNAP-tagged β 1AR and Mini-Gs fused to mApple (mApple-Mini-Gs) in HeLa cells. In unstimulated cells, mApple-Mini-Gs was diffuse throughout the cytoplasm (Figure 2.1c, top panels; 0 min). Upon stimulation of cells with 10 μ M Isoproterenol, a β AR full agonist, mApple-Mini-Gs was recruited to the plasma membrane starting at ~300 seconds (Figure 2.1c, top panel and 2.1d, blue line). HeLa cells expressing SNAP-tagged

β 1AR and Mini-G(s)i fused to mApple (mApple-Mini-G(s)i), also showed recruitment to the plasma membrane, however, at a later time point, starting at ~400 seconds (Figure 2.1c, bottom panels and 2.1d, red line).

Importantly, this dual coupling was only observed at the plasma membrane when cells were stimulated with membrane impermeant full agonist, isoproterenol. We have previously shown that β 1AR can activate G α s protein-mediated signaling from the plasma membrane as well as the Golgi (Irannejad et al., 2017; Nash et al., 2019; Lin and Mai et al., 2023). To monitor whether this dual coupling can occur at the Golgi, HeLa cells were stimulated with epinephrine, another full agonist of β ARs that is able to reach Golgi-localized β 1AR. Epinephrine is a substrate for an organic cation transporter (OCT3) therefore it can be transported to the Golgi membranes (Irannejad et al., 2017; Nash et al., 2019; Lin and Mai et al., 2023). Stimulating β 1AR expressing HeLa cells with 10 μ M epinephrine resulted in the recruitment of both mApple-Mini-Gs and mApple-Mini-G(s)i to the plasma membrane like what we observed with isoproterenol. However, epinephrine stimulation resulted in the recruitment of mApple-Mini-Gs only to Golgi-localized β 1AR (Supplementary Figure 2.1a and b). When HeLa cells were stimulated with dobutamine, a β 1AR partial agonist that can also reach the Golgi membrane due to its hydrophobicity, we found that coupling was biased towards G α s only, at both the plasma as well as the Golgi membranes (Supplementary Figure 2.2). Altogether, these data suggest that full agonists (epinephrine and isoproterenol) can stabilize an active conformation of β 1AR at the plasma membrane, allowing for coupling of both the cognate and non-cognate G α proteins, while dobutamine, a partial agonist, biases this coupling toward the cognate G protein, G α s. Moreover, this dual coupling is only observed at the plasma membrane, a

membrane compartment enriched in negatively charged phospholipids such as PI(4,5)P₂ and not at the Golgi membrane, which is enriched in PI(4)P (Hammond et al., 2012), suggesting that the lipid composition of subcellular membrane compartments could play a role in modulating β 1AR-G α protein coupling promiscuity.

Positively charged residues at the intracellular TM4 region of β 1AR promote dual Gas and Gai coupling

The lack of promiscuous coupling of Gas and Gai to activated β 1AR at the Golgi membranes (Supplementary Figure 2.1) suggested the involvement of negatively charged phosphoinositides such as PI(4,5)P₂ in determining β 1AR-G α dual coupling. Anionic phospholipids have been shown to facilitate GPCR-G protein coupling and signaling through bridging interactions between cationic residues on receptor and Gas protein surfaces and directing allostery of GPCR activity (Dawaliby et al., 2016; Yen et al., 2018; Strohman et al., 2019; Janetzko et al., 2022). Certain class A GPCRs that preferentially couple to Gas interact with anionic phospholipids PI(4,5)P₂, which are highly enriched at plasma membranes, over other phosphoinositides such as PI(3)P and PI(4)P that are found at the endosomes and Golgi membranes, respectively (Yen et al., 2018; Hammond et al., 2012). The core region of Gas contains positively charged residues that have been shown to form electrostatic interactions with anionic phospholipids and the positively charged residues on the intracellular loops of class A GPCRs (Strohman et al., 2019) (Supplementary Figure 2.3a). The mApple-Mini-G(s)i is a chimera of the core region of Gas and the C-terminus of Gai protein (Supplementary Figure 2.3a and 2.4). Thus, to assess the effect of these positively charged residues (KDKQ) on the recruitment of mApple-Mini-G(s)i chimera to activated β 1AR at the plasma

membrane, we mutated the positively charged residues (KDKQ) to the negative residues (EDGE) that are present in G α i proteins (Radoux-Mergault et al., 2023) (Supplementary Figure 2.3a and 2.4). Stimulating HeLa cells with isoproterenol resulted in mApple-Mini-Gi EDGE recruitment to β 1AR at the plasma membrane, however with less efficiency and starting ~500 seconds (Supplementary Figure 2.2b and d). Interestingly, this weak recruitment at the plasma membrane was significantly enhanced when mGi EDGE C-terminus was swapped with G α s C-terminus (Mini-Gi EDGE w/ Gs CTD) (Supplementary Figure 2.3c and d), suggesting that the core and the C-terminal regions of G α proteins are both involved in coupling to β 1AR. Importantly, the positively charged residues on the core region of G α s protein contribute to faster and more efficient coupling of the cognate G α s protein to activated β 1AR at the plasma membrane.

PI(4,5)P₂ binding sites were identified for a variety of G α s coupled class A GPCRs such as neurotensin receptor 1 (NTSR1) and turkey β 1AR (Yen et al., 2018). These binding sites include primarily amino acid residues with positive charges such as lysine (K) and arginine (R). The sites that provided the largest disruption to phosphoinositide interaction were enriched in the TM4 region of NTSR1 (Yen et al., 2018). To identify similar amino acids in human β 1AR, we aligned human β 1AR sequence with NTSR1 and turkey β 1AR and found the corresponding and homologous residues (Supplementary Figure 2.5). We mutated these positively charged amino acids in the TM4 of SNAP-tagged human β 1AR (RARAR) to neutral residues (IATAL) (Figure 2.2a). To assess the consequence of disrupting the electrostatic interactions between the receptor and PI(4,5)P₂ in coupling promiscuity, we compared mGs and mG(s)i recruitments to β 1AR IATAL mutant. We matched the expression level of wild type β 1AR and β 1AR IATAL

mutant (Supplementary Figure 2.6a). We found that $\beta 1AR$ IATL mutant impedes recruitment of mG(s)i to the plasma membrane (Figure 2.2b-d). These data further suggest that PI(4,5)P2 stabilizes an active conformation of $\beta 1AR$ at the plasma membrane via electrostatic interaction with TM4 region, thus allowing dual coupling of G αs and G αi .

PI(4,5)P2 contributes to βAR -G α protein promiscuous coupling at the plasma membrane

To further investigate how PI(4,5)P2 regulates $\beta 1AR$ /G α protein dual coupling at the plasma membrane, we next depleted PI(4,5)P2 by targeting phosphoinositide phosphatases and testing whether this modification affects G α protein coupling selectivity. Using the FKBP-FRB rapamycin system, we specifically targeted FKBP to the plasma membrane using a known plasma membrane targeting sequence (first 11 amino acids of Lyn) (PM-FKPB) and cytosolically expressed the FRB fragment fused to mVenus and a chimeric PI(4)P (Sac1) and PI(5)P (INP55E) phosphatase, named Pseudojanin (mVenus-FRB-Pseudojanin) that depletes phosphate 4 and 5 groups from PI(4,5)P2 (Hammond et al., 2012) (Figure 2.3a). Upon addition of 1 μM rapamycin, mVenus-FRB-Pseudojanin was recruited to the plasma membrane, depleting PI(4,5)P2, as detected by a decrease in PH-PLC δ localization, a PI(4,5)P2 maker (Figure 2.3a-c). We then expressed PM-FKBP and mVenus-FRB- Pseudojanin to assess the kinetics of Mini-Gs and Mini-G(s)i recruitment to wild-type $\beta 1AR$ (Figure 2.3d). We pre-incubated these cells with 1 μM rapamycin for 10 min and looked for cells where PI(4,5)P2 were depleted. Upon depletion of PI(4,5)P2, mApple-Mini-Gs was recruited to the activated $\beta 1AR$ at the plasma membrane (Figure 2.3d, top panel and 2.3e, green line) while mApple-Mini-G(s)i was not

(Figure 2.3d, bottom panel, 3e orange line), consistent with what we observed with $\beta 1$ AR INTAL mutant (Figure 2.2).

To further confirm the role of PI(4,5)P₂ in promoting coupling promiscuity, we next focused on $\beta 2$ AR, another closely related adrenergic receptor that has been shown to couple to both G α s and G α i (Daaka et al., 1997; Zamah et al., 2002; Ma et al., 2020). We first confirmed this dual coupling using mApple-Mini-Gs and mApple-Mini-G(s)i and similarly found faster kinetics in the G α s coupling followed by G α i coupling in response to 10 μ M isoproterenol (Figure 2.4a-c). To identify similar amino acids that were shown to bind PI(4,5)P₂ in NRTS1 and $\beta 1$ AR, we aligned human $\beta 1$ AR sequence with $\beta 2$ AR and found the corresponding and homologous residues (Supplementary Figure 2.7). We then mutated these residues to neutral residues (INTAL) (Figure 2.4d). To assess the consequence of disrupting the electrostatic interactions between $\beta 2$ AR and PI(4,5)P₂ in coupling promiscuity, we compared Mini-Gs and Mini-G(s)i recruitments to $\beta 2$ AR INTAL mutant. We matched the expression level of wild type $\beta 2$ AR and $\beta 2$ AR INTAL mutant (Supplementary Figure 2.6b). Similar to what we found for $\beta 1$ AR PI(4,5)P₂ binding mutant, $\beta 2$ AR INTAL also impedes recruitment of Mini-G(s)i to the plasma membrane (Supplementary Figure 2.8a-b, Figure 2.4e). Depleting PI(4,5)P₂ using the Pseudojanin rapamycin system at the plasma membrane resulted in decreased $\beta 2$ AR endocytosis (Supplementary Figure 2.8c) (Gaidarov et al., 1999; Eichel et al., 2018; Barsi-Rhyne et al., 2022). Nonetheless, depleting PI(4,5)P₂ biased $\beta 2$ AR coupling to G α s at the plasma membrane (Supplementary Figure 2.8d). Altogether, these data further confirm that PI(4,5)P₂ stabilizes an active conformation of receptors at the plasma

membrane via electrostatic interactions with the TM4 region, thus allowing dual coupling of Gas and Gai.

PI(4,5)P2 allosterically modulates GPCR-mediated cAMP signaling

We next asked whether receptor coupling to opposing Gas and Gai proteins has any significant effect on modulating the cAMP response. We compared isoproterenol-mediated cAMP response in wild-type β 1AR with PI(4,5)P2 binding mutants, β 1AR IATAL. We first checked the surface expression of wild-type β 1AR and β 1AR IATAL and found that surface expression β 1AR IATAL is substantially lower than wild-type β 1AR (Figure 2.5a). Stimulating HeLa cells expressing wild type β 1AR and β 1AR IATAL with comparable surface expression, with 10 μ M isoproterenol resulted in significantly higher cAMP accumulation in β 1AR IATAL (Figure 2.5b). We then took advantage of our pseudojanin rapamycin system to assess the effect of rapidly and inducibly depleting PI(4,5)P2 on β 1AR-mediated cAMP response. Rapamycin itself had no significant effect on isoproterenol-mediated cAMP response in β 1AR expressing HeLa cells (Supplementary Figure 2.9a). However, depleting PI(4,5)P2 in PM-FKBP and mVenus-FRB-pseudojanin expressing HeLa cells resulted in significantly higher cAMP accumulation in response to 10 μ M isoproterenol (Figure 2.5c and d). Importantly, depleting PI(4,5)P2 had no significant effect on β 1AR-mediated cAMP signaling when HeLa cells were stimulated with 10 μ M dobutamine (Supplementary Figure 2.5b). This is because β 1AR can only couple to Gas when stimulated with dobutamine. These results suggest that PI(4,5)P2 can allosterically modulate β ARs coupling to opposing Gas and Gai on the plasma membrane thus modulating cAMP response in that location.

2.4 Discussion

The mechanisms determining GPCR- $G\alpha$ protein selectivity has been studied for decades. Given that there are more GPCRs than $G\alpha$ proteins, it has been proposed that GPCRs are likely evolved to overcome such high scrutiny to couple to specific types of $G\alpha$ proteins. Various biochemical and structural studies have identified regions within the N- and C-terminal regions of $G\alpha$ protein to play key roles in determining coupling selectivity (Pierce et al., 2002; Oldham et al., 2008; Marinissen et al., 2001; Flock et al., 2017). One such mechanism proposed that GPCRs binding to the C-terminal domain of $G_{\alpha s}$ seems to require a more open receptor core, while the $G_{\alpha i}$ C-terminal domain engages a more closed conformation (Van Eps et al., 2018) and that $G_{\alpha i}$ -coupled receptors generally reject $G_{\alpha s}$ proteins due to the bulky C terminus of $G_{\alpha s}$ (Rose et al., 2014). However, a more recent study rejects this model, suggesting that there are no structural barriers to forming a complex between most GPCR- $G\alpha$ protein pairs and argues the importance of conformational plasticity and highly variable selectivity mechanisms across GPCRs (Jang et al., 2023). There are other evolved features that could also play role in determining selectivity. These include post-translational modifications, phosphorylation, and potential allostery provided by membrane phospholipids or phosphoinositides. These additional features help GPCRs fine-tune and in some cases, even alter their G protein selectivity patterns (Flock et al., 2017).

In this study, we found that the presence of negatively charged phospholipids, such as PI(4,5)P₂, allows a conformational state of the receptor that can promote promiscuous coupling to cognate and non-cognate $G\alpha$ proteins. This non-selective coupling only occurs at the plasma membrane, a membrane compartment enriched in PI(4,5)P₂.

Furthermore, receptors were able to selectively couple to their cognate G α proteins at the Golgi membranes, which are enriched in less negatively charged phospholipids, PI(4)P. These observations support the notion that selectivity can also be determined by the lipid environment where receptors reside.

We also tested whether this promiscuous coupling affects receptor activity. We found that depleting PI(4,5)P₂ at the plasma membrane or mutating the predicted PI(4,5)P₂ binding sites on exemplary class A GPCRs, β 1AR, results in an increased β 1AR-mediated cAMP response. Importantly, dual coupling and changes in cAMP levels were only observed when receptors were activated by full agonists (Iso and Epi) and not a partial agonist such as dobutamine. These data further suggest that a fully activated conformation of GPCR is required to promote promiscuous coupling. Whether β ARs coupling to its non-cognate G α i protein promotes nucleotide exchange and activation of G α i protein is unclear. We believe our data support a model where receptor coupling to non-cognate G α proteins, can nevertheless, fine-tune receptor/cAMP-mediated signaling at the plasma membrane by occupying the β AR-G protein interface and presumably inhibiting nucleotide exchange on the cognate G α s protein.

Compartmentalized GPCR signaling have been reported for several other receptors at various subcellular organelles, such as the endosome and the Golgi membranes, it would be important to know whether such coupling selectivity exist for other reported G α i or G α q-coupled GPCRs at these subcellular locations (Harris et al., 2022; Radoux-Mergault et al., 2023; Stoeber et al., 2018; Jensen et al., 2017).

2.5 Figures

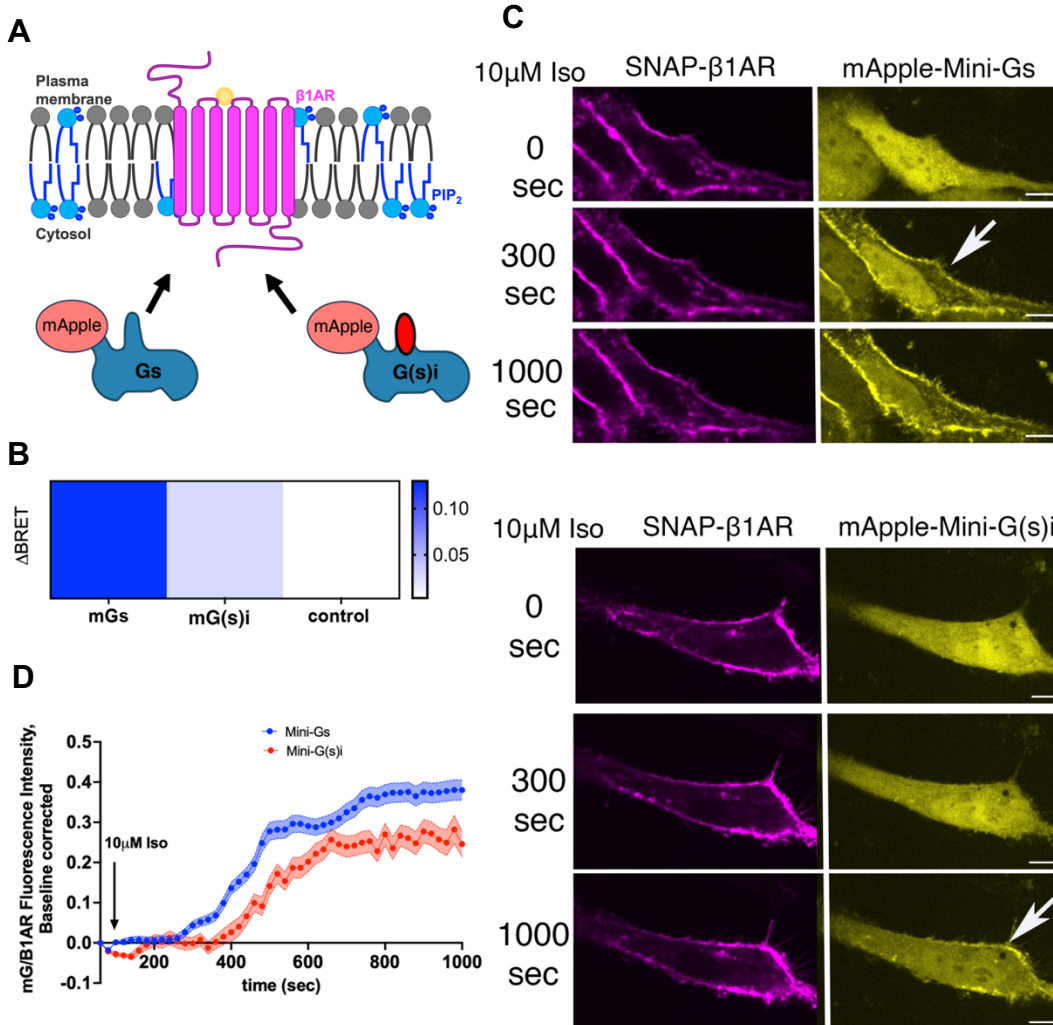
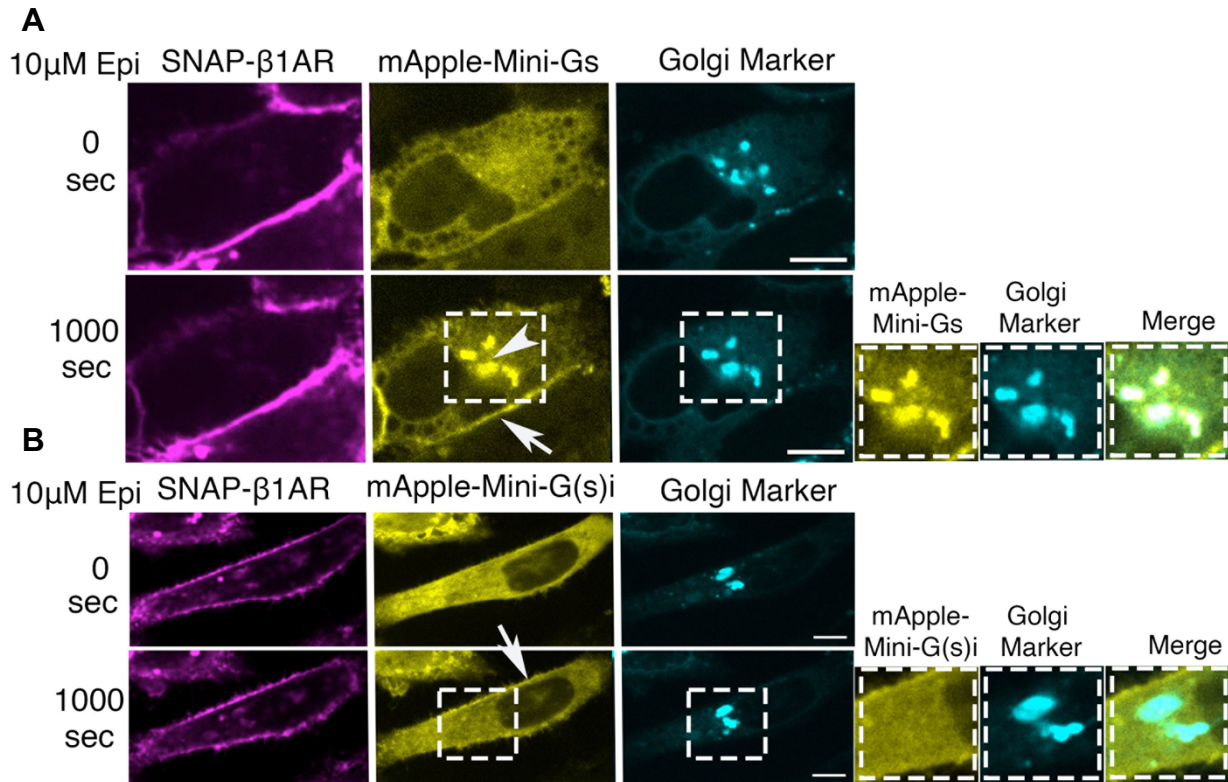
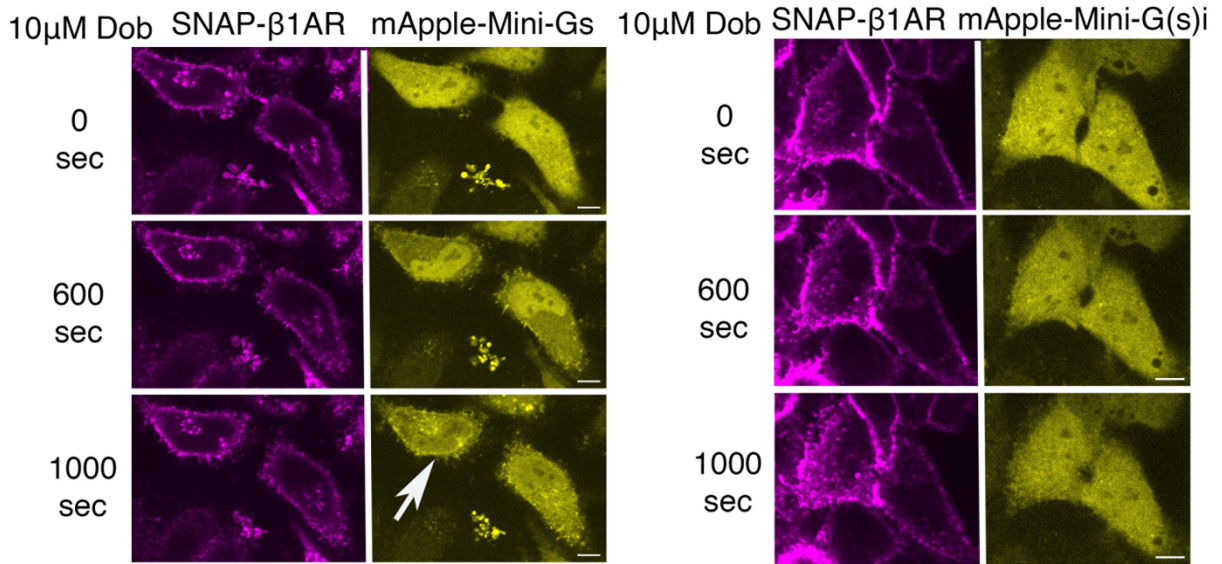
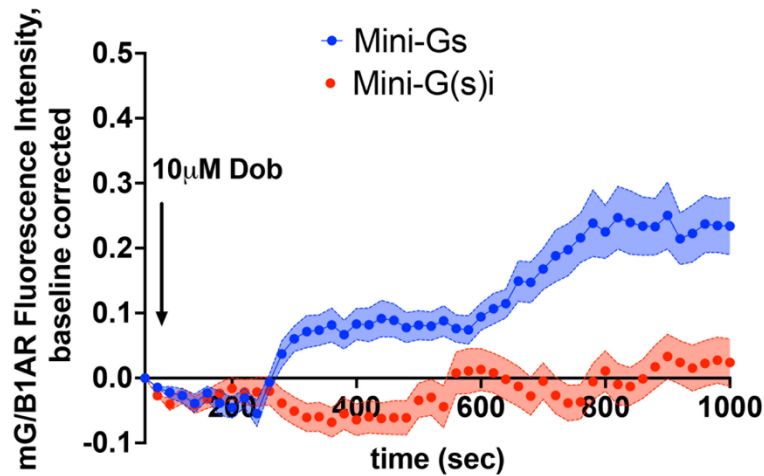


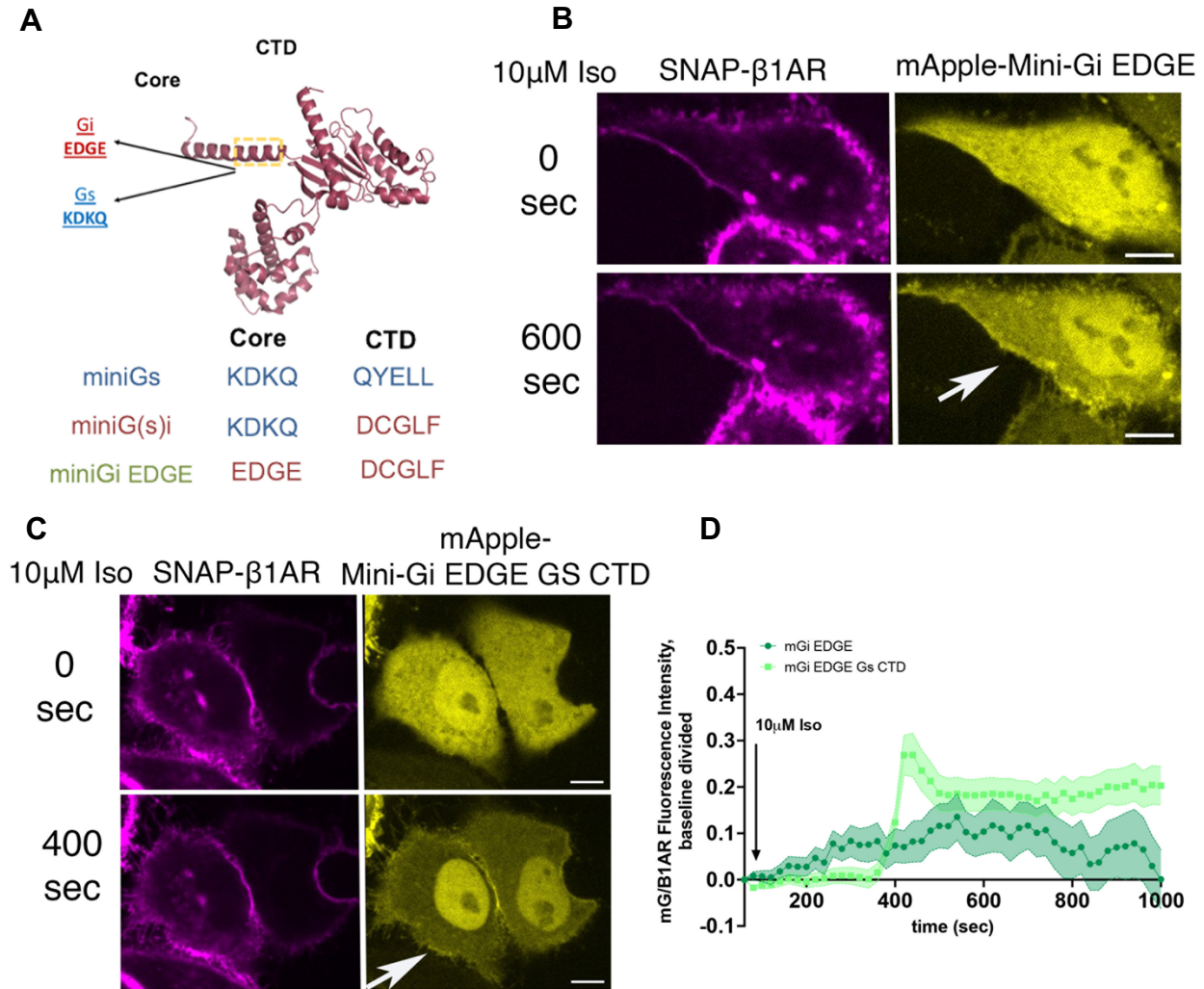
Figure 2.1. β 1AR couples to $G_{\alpha s}$ and $G_{\alpha i}$ proteins at the plasma membrane upon isoproterenol (Iso) stimulation, as detected by Mini- $G_{\alpha s}$ and Mini- $G_{\alpha i}$ recruitment. (a) Schematic representation of Mini- $G_{\alpha s}$ and Mini- $G_{\alpha i}$ coupling to β 1AR at the plasma membrane. Red oval represents G_i C-terminal sequence. (b) Heat-map representation of BRET analysis of Flag- β 1AR-NanoLuc with mVenus tagged Mini- $G_{\alpha s}$ and Mini- $G_{\alpha i}$. Each value represents the mean normalized Δ BRET over 20 minutes. N= 3 biological replicates. (c) Confocal images of representative SNAP-tagged β 1AR-expressing HeLa cells with mApple-Mini- $G_{\alpha s}$ (top panels) and mApple-Mini- $G_{\alpha i}$ (bottom panels) expression before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in the recruitment of Mini- $G_{\alpha s}$ (n= 38 cells, 3 biological replicates) and Mini- $G_{\alpha i}$ (n= 26 cells, 5 biological replicates) to active β 1AR at the plasma membrane in HeLa cells. Arrows indicate recruited Mini- $G_{\alpha s}$ and Mini- $G_{\alpha i}$ at plasma membrane; Scale bar = 10 μ m. (d) Quantification of Mini- G_{α} recruitments to activated β 1AR at the plasma membrane in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini- G probes at plasma membrane relative to SNAP-tagged- β 1AR. Quantifications were baseline corrected.



Supplementary figure 2.1. β 1AR couples to Gas proteins only at the Golgi membrane upon epinephrine (Epi) stimulation, as detected by Mini-Gs recruitment. (a) Confocal images of representative SNAP-tagged β 1AR-expressing HeLa cells (left panels) with mApple-Mini-Gs (middle panels) before and after 10 μ M Epi addition. Stimulation with 10 μ M Epi results in recruitment of Mini-Gs to active β 1AR at the Golgi apparatus, indicated by the Golgi marker (right panels) in HeLa cells (n= 26 cells, 3 biological replicates). Arrow and arrowhead indicate Mini-Gs recruitment at the plasma membrane and Golgi, respectively; Scale bar = 10 μ m. Right panels show zoomed images of insets for Mini-Gs, Golgi marker, and merge. (b) Confocal images of representative SNAP-tagged β 1AR-expressing HeLa cells (left panels) with mApple-Mini-G(s)i (middle panels) before and after 10 μ M Epi addition. Stimulation with 10 μ M Epi does not result in the recruitment of Mini-G(s)i to active β 1AR at the Golgi membranes (n= 14 cells, 3 biological replicates). Arrow indicates recruited Mini-G(s)i at the plasma membrane only; Scale bar = 10 μ m. Right panels show zoomed images of insets for Mini-G(s)i, Golgi marker, and merge.

A**B**

Supplementary figure 2.2. $\beta 1AR$ couples to G α_s proteins only at the plasma membrane and Golgi membrane upon dobutamine (Dob) stimulation, as detected by Mini-Gs recruitment. (a) Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (left panels) with mApple-Mini-Gs (top right panels) and mApple-Mini-G(s)i (bottom right panels) expression before and after 10 μM Dob addition. Stimulation with 10 μM Dob results in recruitment of Mini-Gs ($n = 17$ cells, one biological replicate) but not Mini-G(s)i ($n = 15$ cells, one biological replicate) to active $\beta 1AR$ at the plasma membrane in HeLa cells. Arrows indicate recruited Mini-Gs and unrecruited Mini-G(s)i at plasma membrane; Scale bar = 10 μm . (b) Quantification of Mini-G α recruitments to activated $\beta 1AR$ at the plasma membrane in HeLa cells after 10 μM Dob addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- $\beta 1AR$. Quantifications were baseline corrected after addition of Iso.



Supplementary figure 2.3. N-terminal and core region of Gas promote efficient coupling to activated β 1AR upon isoproterenol stimulation. (a) The N-terminal core region of Mini-G(s)i was mutated from its positive sequence (KDKQ) to negative sequence (EDGE) to create G α i-like biosensor, Mini-Gi EDGE. (b) Confocal images of representative SNAP-tagged β 1AR-expressing HeLa cells (left panels) with mApple-Mini-Gi EDGE (right panels) expression before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in recruitment of Mini-Gi EDGE (n= 14 cells, 3 biological replicates) to active β 1AR at the plasma membrane in HeLa cells. Arrows indicate recruited Mini-Gi EDGE at plasma membrane; Scale bar = 10 μ m. (c) Confocal images of representative SNAP-tagged β 1AR-expressing HeLa cells (left panels) with mApple-Mini-Gi EDGE with Gs CTD (right panels) before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in recruitment of Mini-Gi EDGE with Gs CTD (n= 24 cells, 1 biological replicate) to active β 1AR at the plasma membrane in HeLa cells. Arrows indicate recruited Mini-Gi EDGE with Gs CTD at plasma membrane; Scale bar = 10 μ m. (d) Quantification of Mini-G α recruitments to activated β 1AR at the plasma membrane in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini-Gi EDGE and Mini-Gi EDGE with Gs CTD probes at plasma membrane relative to SNAP-tagged- β 1AR. Quantifications were baseline corrected.

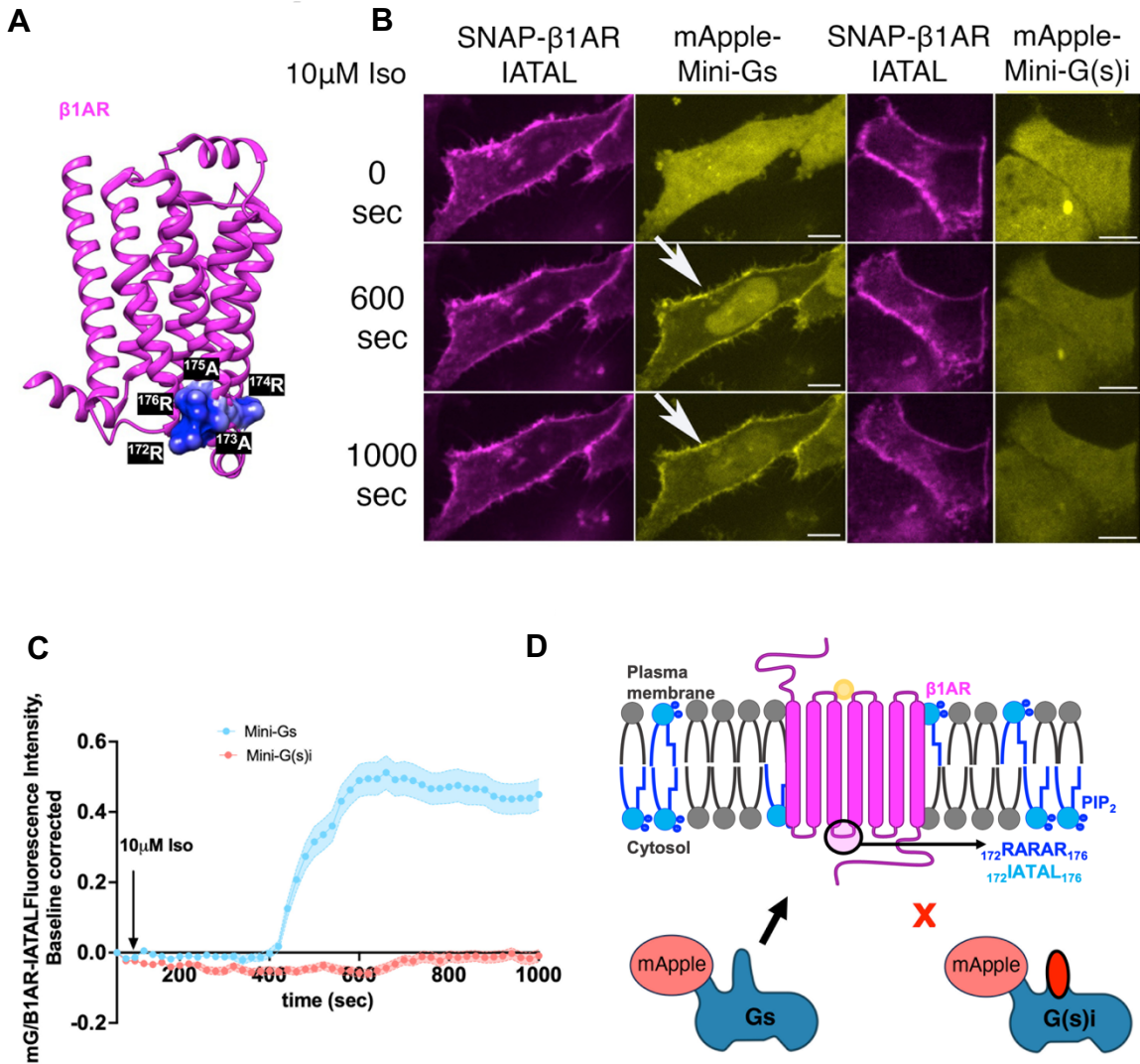


Figure 2.2. $\beta 1AR$ PI(4,5)P₂ binding mutant cannot recruit Mini-G(s)i. (a) $\beta 1AR$ crystal structure (PDB: 7BTS) with predicted PI(4,5)P₂ binding motif at residues 172-176 (RARAR) showing positive electrostatic charge. (b) Confocal images of representative SNAP-tagged mutant $\beta 1AR$ IATAL-expressing HeLa cells (magenta panels) with mApple-Mini-Gs and mApple-Mini-G(s)i (yellow panels) expression before and after 10 μM Iso addition. Stimulation with 10 μM Iso results in recruitment of Mini-Gs but not Mini-G(s)i to active mutant $\beta 1AR$ IATAL at the plasma membrane in HeLa cells (Mini-Gs: n = 44 cells, 4 biological replicates; Mini-G(s)i: n = 30 cells, 4 biological replicates). Arrow indicates recruited Mini-Gs at plasma membrane; Scale bar = 10 μm . (c) Quantification of Mini-G recruitments to activated $\beta 1AR$ IATAL at the plasma membrane in HeLa cells after 10 μM Iso addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- $\beta 1AR$. Quantifications were baseline corrected. (d) Schematic representation of Mini-Gs coupling to the mutated $\beta 1AR$. Residues 172-176 were mutated from their positive RARAR to neutral residues IATAL. Red oval represents G_i C-terminal sequence.

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human      MGAGVLVLGASEPGNLSSAAPLPDGAATAARLLVPASPASLLPPASESPEPLSQQWTAG 60
turkey     MGDGWLPPDCG-PHNRS-----GGGGAT-----AAPTGSRQVSAELLSQQWEAG 43
          ** * *   . . . * * *       . *.**           *:      . * * * * * * **

human      MGLLMALIVLLIVAGNVLVIVAIKTPRIQLTLTNLFIMSLASADLVMGLLVVPFGATIVV 120
turkey     MSLLMALVLLIVAGNVLVIAAIGRTQRLQTLTNLFITSLACADLVMGLLVVPFGATLVV 103
          * .*****:*****.***.:* ***** ** .*****:***

human      WGRWEYGSFFCELWTSVDVLCVTASIELTLCVIALDRYLAITSPFRYQSLT172RARAF176GLVC 180
turkey     RGTWLWGSFLCECWTSLDVLCVTASIELTLCVIAIDRYLAITSPFRYQSLMTRARAKVIIC 163
          * * :***:*** **.:*****:*****:*****:*****:***:***:***
                                     185 189

human      TVWAI172SALVSFLPILMHWRAESDEARRCYNDPKCCDFVTNRAYAIASSVVSFYVPLCIM 240
turkey     TVWAI172SALVSFLPIMMHWWRDED176PQALKCYQDPGCCDFVTNRAYAIASSIISFYIPLIM 223
          *****:***** * . :* :***:*** *****:***:*** **

human      AFVYLRFVREAQKQVKKIDSCERRFLGGPARPPSPSPVPAPAPPPGPPRPA172AAAAATAP 300
turkey     IFVYLRFVREAKEQIRKIDRCEGRFYGSQE176QPP---PLP-----QHQP185I 266
          *****:***:***:*** ** * * . :* * * *:

human      LANGRAGKRRPSRLVALRE172QKALKTLGIIMGVFTLCWL176PFFLANVVKAFHRELVPDRLEFV 360
turkey     LGNGRASKRKTSRVMAMREHKALKTLGIIMGVFTLCWL176PFFLVNIVNVFNRLVDPDWLEFV 326
          * .***.***: **.:***:*****:*****:***:***:*** **

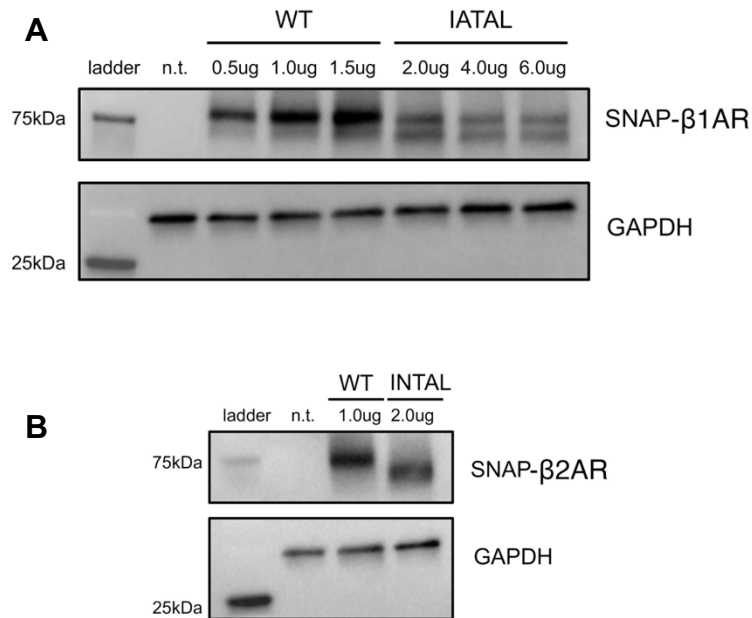
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turkey     FFNWLG172YANS176AFNP172IYCRSPDFRKA176FKRL172CFPRKADRLHAGGQAPLPGGFISTLGS 386
          *****:*****:*** *:* ** * * * . * : : *

human      PPSPGAASDDDDDDVVGATPPARLLEPWAGCNGGAAADSDSSLDEPCRPGFAS172ESKV--- 477
turkey     PE-----HSPGGTWSDCNGGTRGGSESSLEERHSKTSRSES176KMERE 427
          *                               *: .*****: .*:***:*** *****:

human      ----- 477
turkey     KNILATTRFYCTFLGNGDKAVFCTVLRIVKLFEDATCTCPH172THKLKMKWRFKQHQA 483

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Supplementary figure 2.5. Protein sequence alignments of human and turkey β 1AR. Segments in blue represent PI(4,5)P2 binding region previously identified for turkey β 1AR. Alignments were performed using Clustal Omega Multiple Sequence Alignment.



Supplementary figure 2.6. Protein expression levels of wild type β AR compared to PI(4,5)P2 binding mutants. (a) Representative western blots of HeLa cells expressing wild type SNAP-tagged- β 1AR and mutant SNAP-tagged- β 1AR IATAL, detected by anti-SNAP antibody. Lanes containing 0.5 μ g WT β 1AR and 2.0 μ g mutant β 1AR IATAL DNA transfection in HeLa cells showed the closest expression levels. (b) Representative western blots of HeLa cells expressing wild type SNAP-tagged- β 2AR and mutant SNAP-tagged- β 2AR INTAL, detected by anti-SNAP antibody. Lanes containing 1.0 μ g WT SNAP-tagged- β 2AR and 2.0 μ g mutant SNAP-tagged- β 2AR INTAL DNA transfection in HeLa cells showed the closest expression levels. GAPDH antibody was used as a loading control.

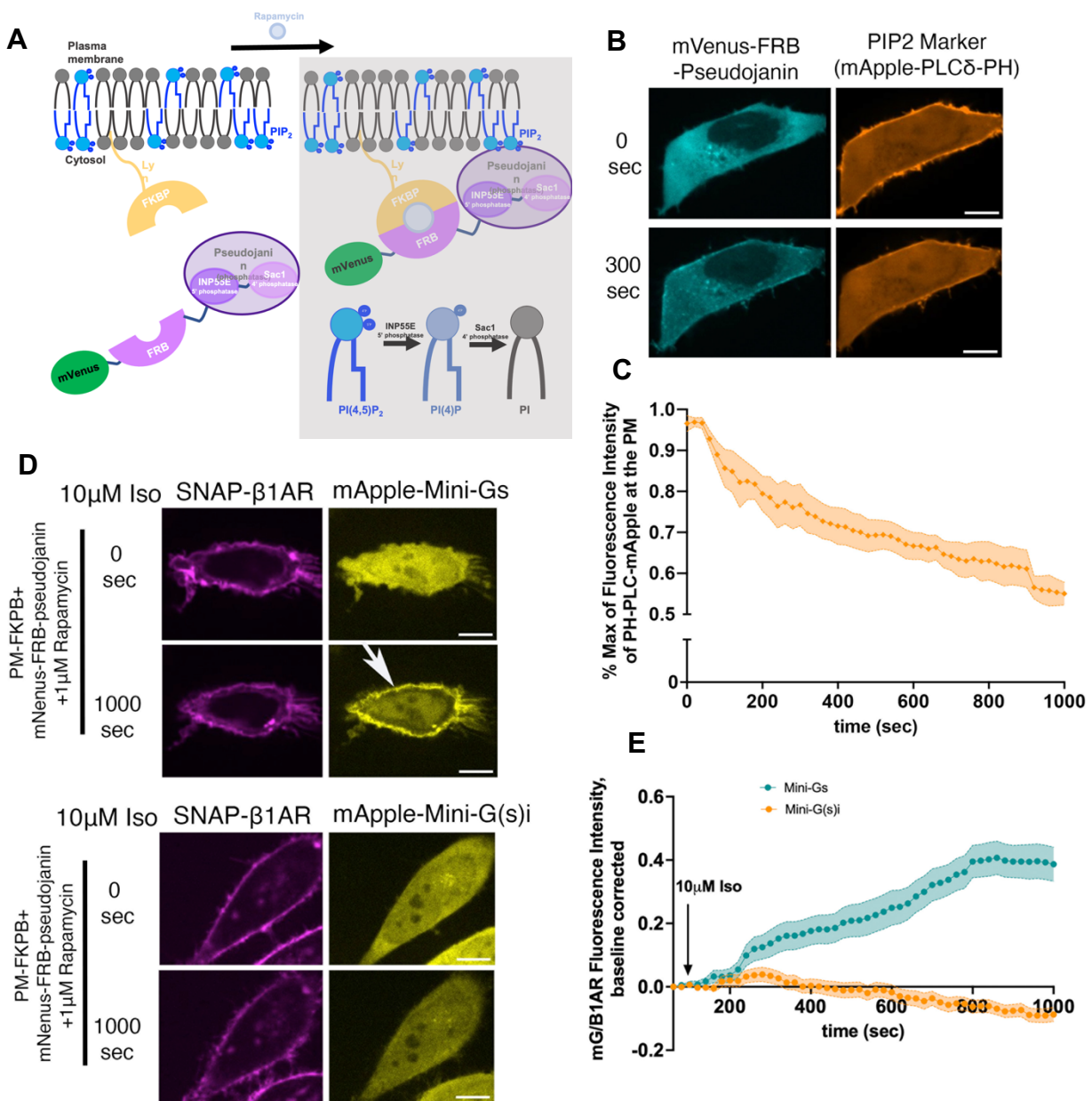


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Figure 2.3. PI(4,5)P2 depletion at the plasma membrane disrupts Mini-G(s)i recruitment to activated β 1AR. (a) Schematic of FKBP-FRB system where the FKBP fragment is targeted to the plasma membrane using a Lyn motif and the FRB fragment is fused to mVenus and a chimeric phosphatase (Pseudojanin) containing INP55E and Sac1, 5' and 4' phosphatases, respectively. Upon the addition of 1 μ M Rapamycin, the FKBP and FRB fragments dimerize allowing the chimeric phosphatase access to deplete PI(4,5)P2 at the plasma membrane (b) Representative confocal images of PI(4,5)P2 depletion using the FKBP-FRB system described. mVenus-FRB-Pseudojanin is recruited to the plasma membrane upon addition of 1 μ M Rapamycin (left panels) and the PI(4,5)P2 marker, an mApple tagged pleckstrin homology (PH) domain (mApple-PLC δ -PH, right panels), is translocated to the cytosol after 300 seconds. (c) Quantification of mApple-PLC δ -PH translocation from the plasma membrane upon PI(4,5)P2 depletion after 1 μ M Rapamycin treatment; normalized fluorescence intensity of mApple-PLC δ -PH at plasma membrane relative to maximum value. (d) Representative confocal images of SNAP-tagged β 1AR-expressing HeLa cells after PI(4,5)P2 depletion with mApple-Mini-Gs (top panels) and mApple-Mini-G(s)i (bottom panels) expression before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in the recruitment of Mini-Gs but not Mini-G(s)i to active β 1AR at the plasma membrane (Mini-Gs: n= 20 cells, 7 biological replicates; Mini-G(s)i: n= 23 cells, 4 biological replicates). Arrow indicates recruited Mini-Gs at plasma membrane; Scale bar = 10 μ m. (e) Quantification Mini-G α recruitments to activated β 1AR upon PI(4,5)P2 depletion in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 1AR. Quantifications were baseline corrected.

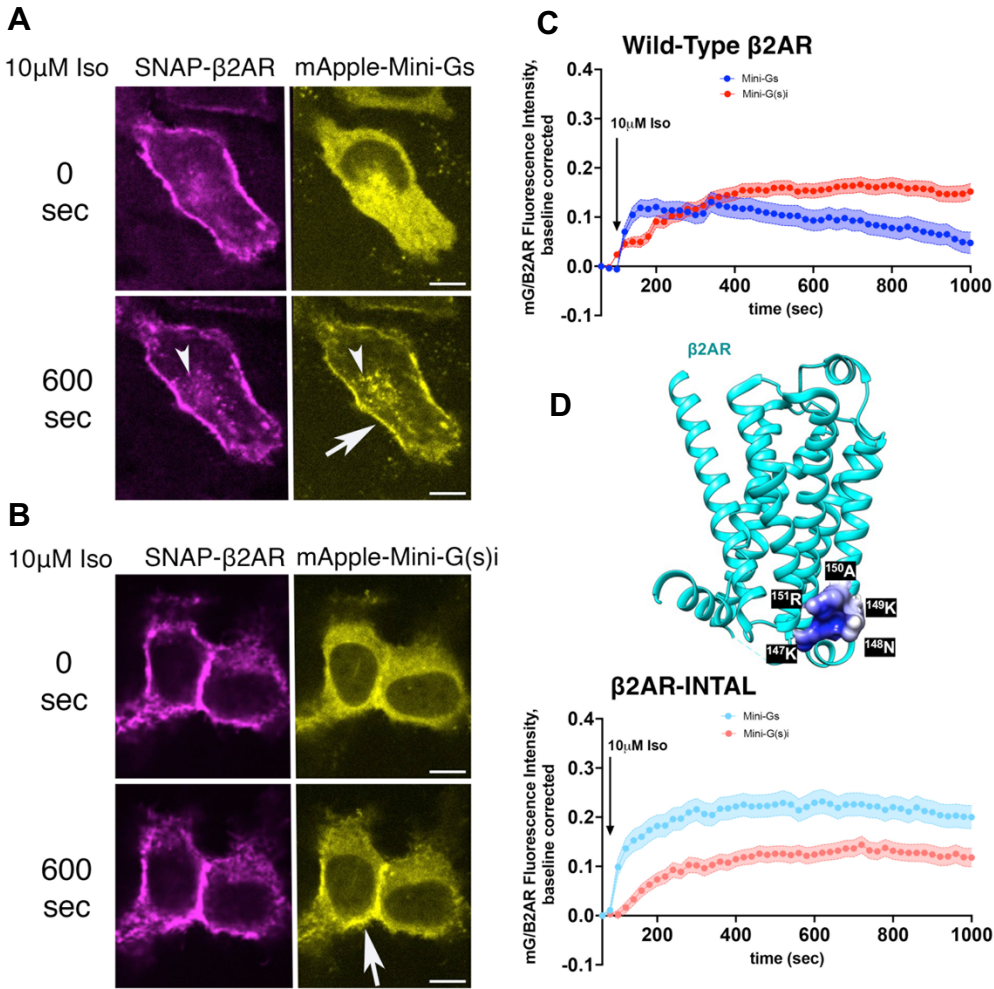
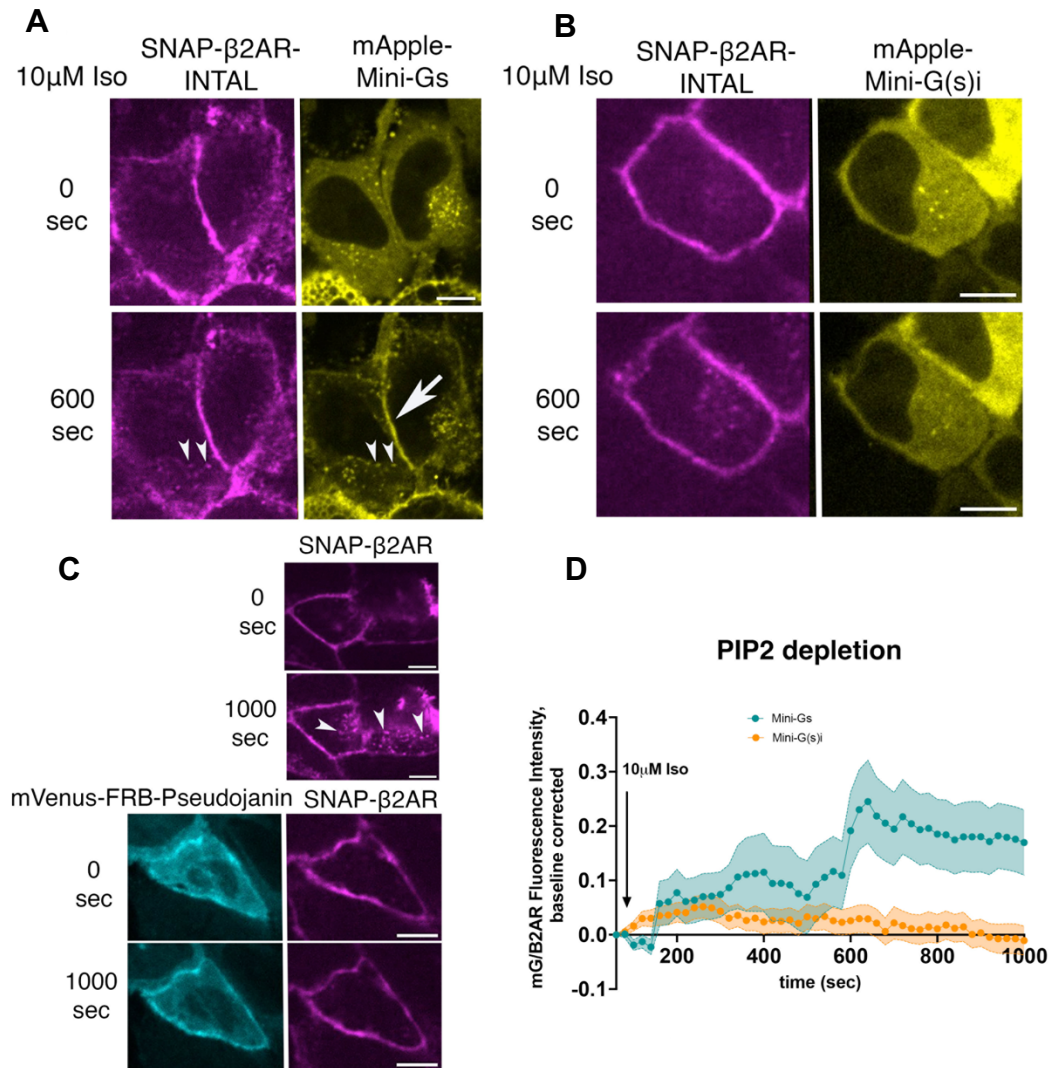


Figure 2.4. β 2AR mutant lacking PI(4,5)P2 binding sites recruits Mini-G(s)i less efficiently. (a) Confocal images of representative SNAP-tagged β 2AR-expressing HeLa cells (left panels) with mApple-Mini-Gs (right panels) expression before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in recruitment of Mini-Gs to active β 2AR at the plasma membrane ($n = 49$ cells, 4 biological replicates). Arrow indicates mApple-Mini-Gs localization at plasma membrane; Scale bar = 10 μ m. (b) Confocal images of representative SNAP-tagged β 2AR-expressing HeLa cells (left panels) with mApple-Mini-G(s)i (right panels) expression before and after 10 μ M Iso addition. Stimulation with 10 μ M Iso results in the recruitment of Mini-G(s)i to active β 2AR at the plasma membrane ($n = 34$ cells, 4 biological replicates). Arrow indicates recruited mApple-Mini-G(s)i at plasma membrane; Scale bar = 10 μ m. (c) Quantification of Mini-G recruitments to activated β 2AR at the plasma membrane in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 2AR. Quantifications were baseline corrected. (d) Top: β 2AR crystal structure (PDB: 4LDO) with predicted PI(4,5)P2 binding motif at residues 147-151 (KNKAR) showing positive electrostatic charge. Bottom: quantification of Mini-G α recruitments to activated β 2AR INTAL at the plasma membrane in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 2AR. Quantifications were baseline corrected.



Supplementary figure 2.8. β 2AR PI(4,5)P2 binding mutant modestly recruits Mini-G(s)i. (a) Confocal images of representative SNAP-tagged β 2AR INTAL expressing HeLa cells (left panels) with mApple-Mini-Gs (right panels) before and after 10 μ M Iso addition. (b) Confocal images of representative SNAP-tagged mutant β 2AR INTAL expressing HeLa cells (left panels) with mApple-Mini-G(s)i (right panels) before and after 10 μ M Iso addition. (Mini-Gs: n= 64 cells, 4 biological replicates; Mini-G(s)i: n= 54 cells, 4 biological replicates). Arrow indicates Mini-Gs recruitment at plasma membrane; arrowheads indicate internalized mutant β 2AR INTAL along with Mini-Gs; Scale bar = 10 μ m. (c) Top: confocal images of WT SNAP-tagged- β 2AR. Arrowheads indicate internalized WT β 2AR at similar levels as mutant β 2AR INTAL. Bottom: confocal images of PI(4,5)P2 depletion using the FKBP-FRB system with WT SNAP-tagged- β 2AR (right panels). mVenus-FRB-Pseudojanin is recruited to the plasma membrane upon addition of 1 μ M Rapamycin (left panels). Internalization of WT β 2AR is not observed when PI(4,5)P2 is depleted. (d) Quantification of Mini-G recruitments to activated β 2AR at the plasma membrane upon PI(4,5)P2 depletion in HeLa cells after 10 μ M Iso addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 2AR. Quantifications were baseline corrected.

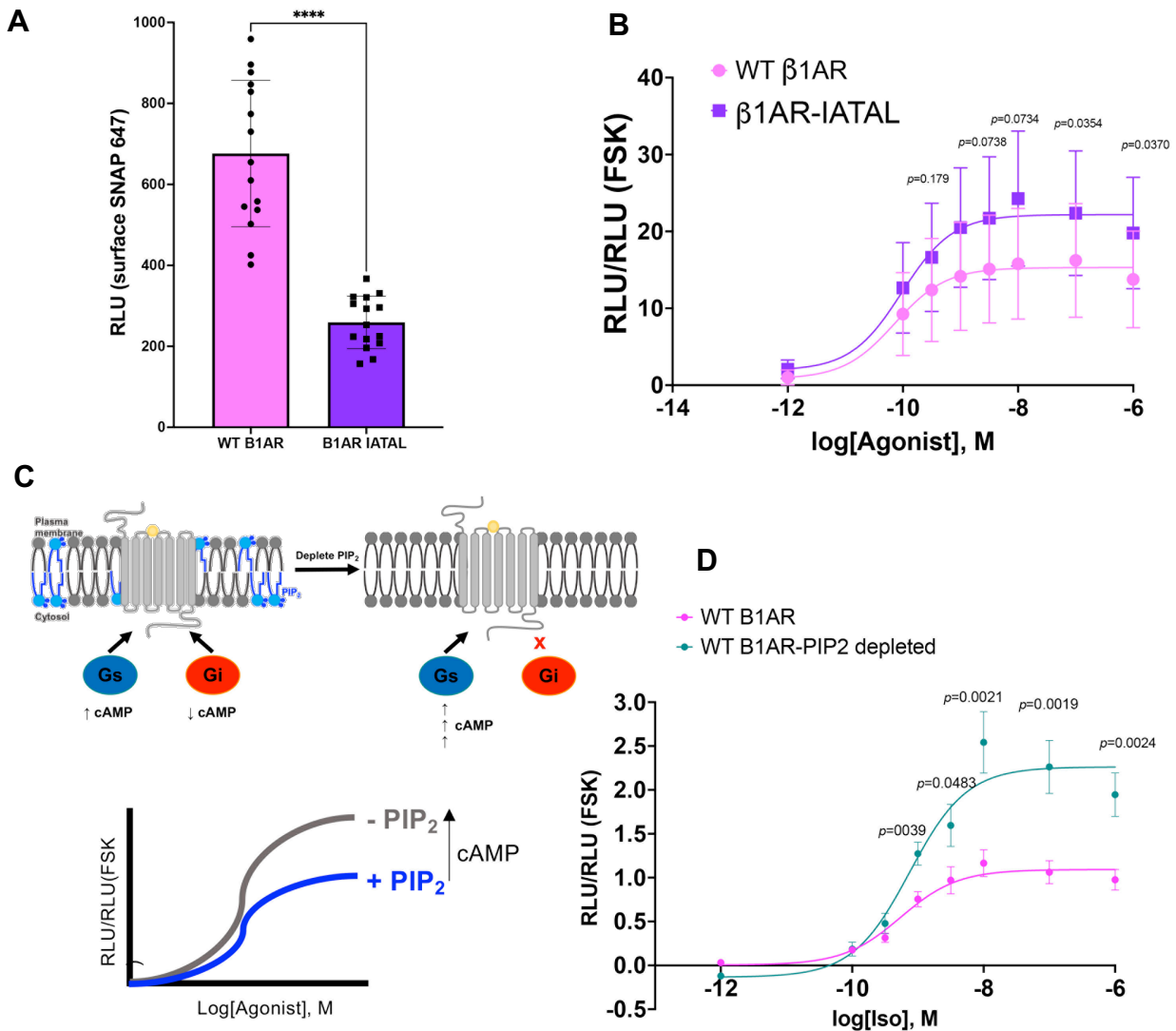
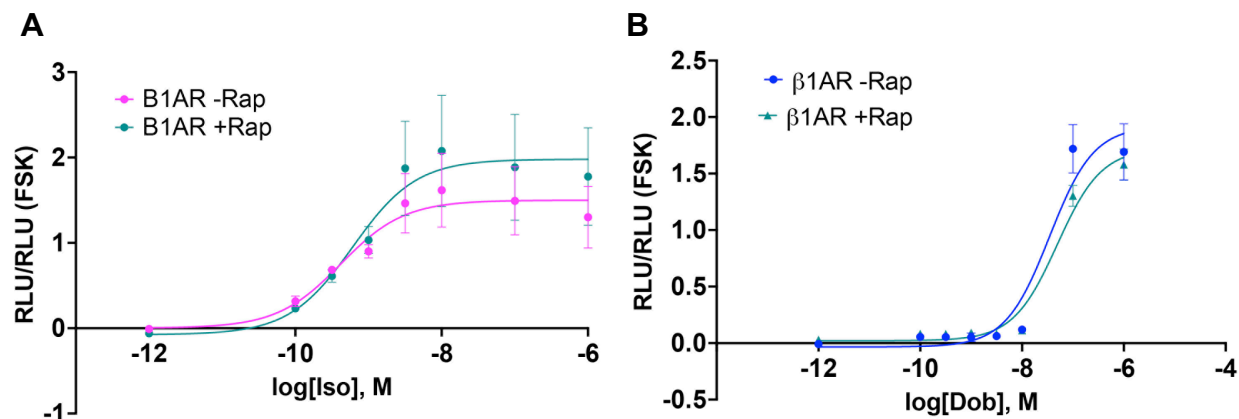


Figure 2.5. PI(4,5)P₂ modulates GPCR-mediated cAMP signaling. (a) Surface expression level of HeLa cells expressing wild type β 1AR and mutant β 1AR IATL detected by membrane-impermeant SNAP-647 dye (mean $\pm$ SEM, $n = 4$ biological replicates) (b) Forskolin-normalized wild type β 1AR and mutant β 1AR IATL-mediated cAMP response with Iso treatment at indicated concentrations in HeLa cells (mean $\pm$ SEM, $n = 15$ cells, 4 biological replicates). (c) Schematic of signaling outputs upon PI(4,5)P₂ depletion. Blue colored phospholipids represent PI(4,5)P₂ while grey phospholipids represent neutral phospholipids. (d) Forskolin-normalized β 1AR-mediated cAMP response before and after PI(4,5)P₂ depletion upon rapamycin pretreatment (1 μ M, 10 min) and at various iso concentrations in HeLa-expressing FKBP-FRB system (mean $\pm$ SEM, $n = 4$ biological replicates).



Supplementary figure 2.9. PI(4,5)P2 depletion had no effect on dobutamine-mediated cAMP signaling in β 1AR expressing HeLa cells. (a) Forskolin-normalized β 1AR-mediated cAMP response with and without Rapamycin pretreatment (1 μ M, 10 min) in HeLa cells not expressing FKBP-FRB system (mean $\pm$ SEM, n = 2 biological replicates). (b) Forskolin-normalized β 1AR-mediated cAMP response before and after PI(4,5)P2 depletion upon rapamycin pretreatment (1 μ M, 10 min) and at various Dob concentrations in HeLa-expressing FKBP-FRB system (mean $\pm$ SEM, n = 2 biological replicates).

2.6 Materials and Methods

cDNA constructs/plasmids

βAR constructs

Signal Sequence-SNAP-tagged β1AR was created by amplifying β1AR from Flag-β1AR 5'- GCTG GGT CTT GGA TCC AAC GAT GAT GAC GCC GGC -3'; 5'- AT AGA ATA GGG CCC TCT AGA GCC CTA CAC CTT GGA TTC CG -3' primers and inserted into the SNAP vector using BamHI and XbaI. Mutant β1AR IATL and mutant β2AR INTAL were created by site directed mutagenesis using 5'- CG ATT GCT ACA GCT CTG GGC CTC GTG TGC ACC GT -3'; 5'- GCC CAG AGC TGT AGC AAT CGT CAG CAG GCT CTG GTA GCG -3' primers and 5'-CC ATT AAT ACA GCA TTA GTG ATC ATT CTG ATG GTG TGG ATT G -3'; 5'- AC TAA TGC TGT ATT AAT GGT CAG CAG GCT CTG GTA C -3' primers, respectively. Flag-β1AR-NanoLuc was a gift from the Lambert lab.

Mini-G probes

pmVenus-Mini-Gs, pmVenus-Mini-G(s)I constructs were gifts from Dr. Nevin Lambert. pmVenus-Mini-Gi EDGE and pmApple-Mini-Gi EDGE were created from pmVenus-Mini-G(s)i and pmApple-Mini-G(s)i by site directed mutagenesis using 5'- A AAA CAG CTC CAG AAA GAC GGG CAA GTG TAC AGG G -3'; 5'- T CTA TGA GTG GCC CTG TAC ACT TGC CCG TCT TTC TGG -3' primers for the first round of mutation (KDKQ → KDGQ), and 5'- GAA AAA CAG CTC CAG GAA GAC GGG GAA GTG -3'; 5'- AGT GGC CCT GTA CAC TTC CCC GTC TTC CTG -3' primers for the second round of mutation (KDGQ → EDGE). C-terminal domain swapped Mini-G probes were created using site directed mutagenesis using 5'- G AAT CTT CGG CAA TAC GGG CTT TTC TAA AAG CTT CGA A -3'; 5'- CG AAG CTT TTA GAA AAG CCC GTA TTG CCG AAG

ATT CA -3 for the first round of mutation (DCGLF → QYGLF) and 5'- CTT CGG CAA TAC GAG CTT CTC TAA AAG CTT CGA AT -3'; 5'- TT TTA GAG AAG CTC GTA TTG CCG AAG ATT CAT TTT GA -3 for the second round of mutation (QYGLF → QYELL) of the Gs C-terminal domain swap, and using 5'- G CAT CTC AGG GAC TGT GAG CTG CTC TAA AAG CTT CG -3'; 5'- C GAA GCT TTT AGA GCA GCT CAC AGT CCC TGA GAT GCA -3 for the first round of mutation (QYELL → DCELL) and 5'- TC AGG GAC TGT GGG CTG TTC TAA AAG CTT C -3'; 5'- AGC TTT TAG AAC AGC CCA CAG TCC CTG AGA TGC -3 for the second round of mutation (DCELL → DCGLF) of the Gi C-terminal domain swap.

Rapamycin heterodimerization constructs

Lyn-2xFKBP-Myc was used in a previous study (Irannejad et al., 2017). pmVenus-FRB-Pseudojanin (PJ) construct was created from mRFP1-1xFKBP-PJ (Hammond et al., 2012; Addgene) by amplifying PJ using 5'- GT GCT GGT GGA GGA TCC AGT GCT GGT GGT AGT GCT -3'; 5'- TTA TCT AGA TCC GGT GGA TCC TCA AGA AAC GGA GGC GAT G -3' primers and inserted into the pmVenus-FRB-C1 vector using BamHI.

Cell culture and transfections

HeLa cells (purchased from ATCC as authenticated lines CCL-2) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. Cell cultures were free of mycoplasma contamination. Transfections were performed using Transit-LT1 transfection reagent (Mirus Bio) at a 1:3 DNA to transfection reagent ratio. SNAP-tagged human β 1AR and β 2AR constructs were labeled with SNAP-cell 647 SiR (New England Biolabs, S9102S) at 1:1000 concentration for 20 min at 37°C in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4,

washed with the same media (thrice for one minute, room temperature), and imaged in the same media.

Live-cell confocal imaging

Live-cell imaging was carried out using a Nikon spinning disk confocal microscope with a $\times 60$, 1.4 numerical aperture, oil objective and a CO₂ and 37°C temperature-controlled incubator. A 488, 568 nm and 640 Voltran was used as light sources for imaging mVenus/GFP, mApple, and SNAP-647 signals, respectively. Cells expressing both SNAP-tagged receptor (1 μ g for both wildtype β 1AR and β 2AR, 4 μ g for mutant β 1AR IATAL and 2 μ g mutant β 2AR INTAL) and the indicated Mini-G probe (100 ng) were plated onto glass coverslips. Receptors were surface labeled by addition of SNAP-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Indicated agonists (isoproterenol, epinephrine, or dobutamine all from Sigma) were added and cells were imaged every 20 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. rapamycin (Sigma) was incubated for 10 min before cells were stimulated with 10 μ M isoproterenol. Time-lapse images were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS Elements).

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (<http://rsb.info.nih.gov/ij>). For measuring kinetics of Mini-G probe recruitment at the plasma and Golgi membranes over time in confocal images, analyses were performed on unprocessed TIFF images using a previously published scripts written in MATLAB, available through open access on zenob (Bakr et al., 2021; Jullie et al., 2020; Jullie et al., 2022). Briefly, the plasma

membrane and Golgi regions were selected and a mask of labeled receptor (using SNAP label) or Golgi marker were generated by thresholding the receptor or the Golgi marker signal within the selected region. The average fluorescence intensity of Mini-G was measured within the masked region and outside of the masked region, before and after addition of agonists. Values were normalized by calculating the percent relative to the maximum value, then baseline corrected using GraphPad Prism v.9.5.1 (GraphPad) software so that the first value of each condition was set to 0.

Luminescence-based cAMP assay

HeLa cells were transfected with either wildtype β 1AR, wildtype, β 2AR, mutant β 1AR IATAL, or mutant β 2AR INTAL and a plasmid encoding a cyclic-permuted luciferase reporter construct (pGloSensor-20F, Promega) and luminescence values were measured, as described previously (Irannejad et al., 2013). Briefly, cells were plated in 96-well dishes (~100,000 cells per well) in DMEM without phenol red/no serum and equilibrated to 37°C in the SpectraMax plate reader and luminescence was measured every 1.5 min for 20 min. Software was used to calculate integrated luminescence intensity and background subtraction. In rapamycin heterodimerization experiments, cells were pre-incubated with 1 μ M rapamycin (Sigma) for 10 min. 10 μ M forskolin (Sigma) was used as a reference value in each multi-well plate and for each experimental condition. The average luminescence value (measured across duplicate wells) was normalized to the maximum luminescence value measured in the presence of 10 μ M forskolin. For rapamycin-treated cells, the average luminescence value was normalized to the maximum luminescence value measured in the presence of 10 μ M forskolin and 1

μ M rapamycin. Concentration–response curves were fitted to a three-parameter binding logistic model using GraphPad Prism v.9.5.1 (GraphPad).

Western blotting

HeLa cells were lysed in extraction buffer (0.2% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM Tris at pH 7.4 and cOmplete EDTA-free Protease Inhibitor Cocktail [Roche]). After agitation at 4°C for 30 min, supernatants of samples were collected after centrifuging at 15,000 $\times$ rpm for 10 min at 4°C. Supernatants were mixed with SDS sample buffer for the protein denaturation. The proteins were resolved by SDS-PAGE and transferred to PVDF membrane and blotted for anti-SNAP (1:1000) or GAPDH (1:10,000) antibodies to detect wildtype and mutant SNAP-labeled β 1AR and β 2AR and GAPDH expression by horseradish-peroxidase-conjugated rabbit IgG, sheep anti-mouse and rabbit IgG (1:10,000 Amersham Biosciences), and SuperSignal extended duration detection reagent (Pierce).

BRET, luminescence measurements

HeLa cells were transiently transfected with Flag- β 1AR-NanoLuc, pmVenus-Mini-Gs, pmVenus-Mini-G(s)i, pmApple-Mini-Gs, pmApple-Mini-G(s)i at a 1:4 donor: acceptor ratio (receptor: Mini-G). After a 24-h incubation, cells were washed thrice with 1 $\times$ HBSS, harvested by trypsinization, and transferred to opaque black 96-well plates. Kinetic BRET and luminescence time course measurements were made using a SpectraMax plate reader. Furimazine (NanoGlo; 1:1000, Promega) was added to all wells immediately prior to making measurements with Nanoluc. Raw BRET signals were calculated as the emission intensity at 530 nm divided by the emission intensity at 480 nm. Net BRET signals were calculated as the raw BRET signal measured from cells expressing Flag-

β 1AR-NanoLuc and mVenus-Mini-G minus the raw BRET signal measured from cells expressing Flag- β 1AR-NanoLuc and mApple-Mini-G. Heatmaps represent Δ BRET_{max} (Figure 1e).

Sequence alignments and receptor structures

All sequence alignments in Supplementary information (Supp figs. 2 and 4) were performed with Clustal Omega Multiple Sequence Alignment (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Sequences were extracted from UniProt (<https://www.uniprot.org/>). Structures in Figures 2 and 4 correspond to a previous publication (Xu et al., 2021), and can be found in Protein Data Bank (PDB: 7BTS and 4LDO, β 1AR and β 2AR, respectively). Figures were generated using UCSF Chimera v.1.17.3.

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2.8 Acknowledgements

We thank Drs. Aashish Manglik, Mark von Zastrow, and Nevan Lambert for reagents and helpful discussion. I thank Dr. Quynh Mai for mentorship and guidance throughout this project, I would have given up on it long ago if it were not for her encouragement. She instructed me on the confocal microscope and signaling assays. I thank Dr. Ting-Yu Lin for advice on techniques such as cloning, expression, and western blotting.

Chapter 3:
**Characterizing the molecular determinants of β AR subcellular
localization**

Tracy Knight and Evelyn Hernandez

3.1 Abstract

Although highly homologous in sequence, β 1AR and β 2AR contain different localizations in cardiomyocytes. β 1AR localizes at the plasmalemma of cardiomyocyte and in perinuclear/Golgi apparatus regions, while β 2AR localizes at the T-tubules and in endosomes after receptor internalization. The two sections of β 1AR and β 2AR that diverge the most in amino acid sequence are in the intracellular loop 3 (ICL3) and C-terminal tail (CTT). Here we swap the ICL3 regions of β 1AR and β 2AR to generate chimeras that allow us to test whether this region contains a Golgi retention signal for β 1AR that are lacking in β 2AR. Indeed, the presence of the β 1AR- β 2ICL3 chimera is significantly reduced at the Golgi compared to wildtype β 1AR. However, we do not observe significant Golgi exit for the β 2AR- β 1ICL3 chimera compared to wildtype β 2AR. This suggests that although β 1AR ICL3 may contain a Golgi retention signal that allows β 1AR to retain at the Golgi, it is not sufficient to cause β 2AR to retain at the Golgi. This may indicate that β 2AR contains additional Golgi exit motifs, or stronger plasma membrane targeting motifs than β 1AR that promotes its exit from the Golgi to the plasma membrane.

3.2 Introduction

Cardiomyocytes express two β AR isoforms, β 1AR and β 2AR, that regulate cardiac function through activation of G protein-mediated cAMP production (Rohrer et al., 1996; Chruscinski et al., 1996; Rohrer et al., 1998; Devic et al., 2001; Boivin et al., 2006). β 1AR comprises ~80% of β ARs expressed in cardiomyocytes, and β 2AR the remaining ~20% (Rohrer et al., 1999). Despite having similar effectors, β 1AR and β 2AR elicit significantly different effects on cardiac functions. For example, β 1AR plays a dominant role in

chronotropy (heart rate), inotropy (force of contraction) and lusitropy (relaxation) responses in healthy cardiomyocytes, whereas β 2AR produces modest chronotropic effect and no lusitropic responses (Rohrer et al., 1999). Interestingly, β 1AR and β 2AR have very distinct membrane localizations in cardiomyocytes. β 1AR is localized in the plasma membrane and perinuclear/Golgi regions, whereas β 2AR is localized in the T-tubules of adult cardiomyocytes and does not retain at the Golgi (Boivin et al., 2006). More importantly, our lab has shown that the plasma membrane - and Golgi-localized β 1ARs activate G protein-mediated cAMP responses and regulate distinct PKA effectors at each location, and β 2ARs activate G protein-mediated signaling from the plasma membrane and endosomes (Irannejad et al., 2013; Irannejad et al., 2017; Lin and Mai et al., 2023).

Cardiomyocytes have intricate membrane structures, such as the transverse T-tubules and complex arrangement of organelles like the sarcoplasmic reticulum, which provide physical barriers that limit β AR-stimulated cAMP production to local membrane vicinities (Xiang et al., 2011). Membrane-associated phosphodiesterases (PDEs), which degrade cAMP, put an additional limit as to how far into the cell cAMP can diffuse and activate protein kinase A (PKA) for the phosphorylation of downstream effectors (Xiang et al., 2011). Due to this tight regulation of localized cAMP production and degradation and the membrane localizations of both β ARs, cAMP may be produced and regulated locally at different compartments. I hypothesize that this distinct membrane localization and signaling function of β 1AR and β 2AR could provide an explanation for their distinct roles in regulating cardiac function. What determines β ARs subcellular localization is not

known. This chapter aims to characterize the molecular determinants of β ARs subcellular localization and function.

Once synthesized and properly folded at the ER, transmembrane proteins, including GPCRs, exit the ER and Golgi to be localized at the plasma membrane (Dong et al., 2007). The specific motifs and machineries regulating β AR retention at the Golgi is not known. Three mechanisms of Golgi retention for other transmembrane cargos that could potentially be applied to β ARs are Golph3-mediated Golgi retention, Golgin-160-mediated trafficking, and arginine-rich motifs (Tu et al., 2008; Hicks et al., 2006; Gilbert et al., 2014; Banfield et al., 2011; Shiwerski et al., 2019). Glycosyltransferases are Golgi-localized transmembrane proteins that modify glycoproteins. These glycosyltransferases are type II integral membrane proteins, meaning that they contain a single transmembrane region, a short N-terminal tail that faces the cytoplasm, and the catalytic C-terminal domain that exists in the Golgi lumen. It has been shown that glycosyltransferases are retained at the Golgi through the interaction of their (F/L)(L/I/V)XX(R/K) motif on the N-terminal tail with a cytoplasmic peripheral Golgi membrane protein named Golph3 (Vps74p in yeast) (Tu et al., 2008). Golph3 deletion caused several glycosyltransferases to be mis-localized to parts of the cell that appeared as a vesicle-like haze or, in the case of yeast, the vacuole lumen. Mutating the (F/L)(L/I/V)XX(R/K) motif in the glycosyltransferases caused comparable mis-localization as the Golph3 deletion (Tu et al., 2008). Interestingly, β 1ARs also have a (F/L)(L/I/V)XX(R/K) motif in its intracellular loop 3 (ICL3) region (LVALR) which faces the cytoplasm, but β 2AR does not have the proposed Golph3 interacting motif. This predicted

interaction of $\beta 1AR$ but not $\beta 2AR$ to Golph3 could explain the lack of Golgi $\beta 2AR$ localization at the endogenous level.

The Golgin family of proteins are described by their Golgi localization and lengthy coiled coil motifs, which have been implicated in Golgi structure and aiding the bulk flow of proteins through the compartment (Hicks et al., 2006). Golgin-160 interacts with $\beta 1AR$ through three basic amino acids, KRR (i.e. tribasic motif) upstream of the LVALR motif in its ICL3. Deletion of Golgin-160 in HeLa cells has been shown to decrease plasma membrane localization of $\beta 1AR$ (Hicks et al., 2006). Furthermore, mutating -KRR- to -AAA- in $\beta 1AR$ abrogates its interaction with Golgin-160 and decreases its transport from the Golgi to the plasma membrane (Gilbert et al., 2014). Interestingly, $\beta 2AR$ contains a leucine in place of the lysine, making it an LRR motif, which could explain its lack of Golgi retention at the endogenous level.

Lastly, the overrepresentation of arginine, particularly in -RRR- and -RXR- motifs, have been implicated in actively precluding the exit of proteins from the ER and Golgi (Banfield et al., 2011). One clear example of such mechanism in GPCRs is the delta opioid receptor (DOR), which has been shown to be retained at the Golgi via two -RXR- motifs in its C-terminal tail (Shiwarski et al., 2019). Similar to the $\beta 1AR$ and $\beta 2AR$ localization conundrum, DOR is localized at Golgi and plasma membrane, but the highly homologous μ -opioid receptor (MOR) only localizes to the plasma membrane. Furthermore, MOR contains only one -RXR- motif, suggesting that perhaps two -RXR- motifs are necessary at the C-terminal tail for efficient Golgi retention. $\beta 1AR$ does contain two -RXR- motifs in its C-terminal tail, whereas, $\beta 2AR$ does not contain any.

3.3 Results

β 1AR and β 2AR have 66% homology. Sequence alignment show most divergence in their ICL3 and C-terminus tail. There are three potential Golgi retention motifs in β 1AR that are not present in β 2AR: LVALR and KRR motifs in the ICL3 that interact with Golph3 and Golgin-160, respectively, and the arginine (-RXR-) motifs in the C-terminal tail. Given these distinctions, I mutated the -RXR- motifs in β 1AR to test whether this also may be causing Golgi retention (Figure 3.1). I also swap the ICL3 regions of β 1AR and β 2AR to attempt to disrupt β 1AR Golgi retention, increase β 2AR Golgi retention, cause receptor re-localization, and ultimately affect β AR function in cardiomyocytes (Figure 3.1).

To test whether the -RXR- motifs dictate β 1AR Golgi localization, I mutated the -RXR- motifs to -AAA- in SNAP-tagged β 1AR constructs. Immunofluorescence microscopy was performed using this mutant and a Golgi marker, GM130 in fixed HeLa cells. Substantial amounts of β 1AR in the Golgi was still observed when comparing the -RXR- β 1AR to wildtype β 1AR (Figure 3.2, not quantified). Therefore, we concluded that -RXR- mutations are not sufficient to interfere with β 1AR Golgi retention. They may be modestly important for Golgi retention but are not necessary or sufficient for Golgi exit to occur.

To test whether the ICL3 dictates β AR Golgi localization, I made SNAP-tagged β 1AR and β 2AR constructs with their ICL3 regions swapped. Immunofluorescence microscopy was performed using SNAP tagged- β ARs and a Golgi marker, GalT-GFP in live HeLa cells (Figure 3.3). We found that β 1AR- β 2ICL3 (β 1AR with β 2AR ICL3 chimera) trafficked more to the plasma membrane than wildtype β 1AR. This suggested that β 1AR ICL3 contained a Golgi retention signal that once removed, promoted β 1AR Golgi exit.

However, $\beta 2AR$ - $\beta 1ICL3$ ($\beta 2AR$ with $\beta 1AR$ ICL3 chimera) did not retain more at the Golgi as expected, suggesting that there are other regions in $\beta 2AR$ that allow for Golgi exit.

3.4 Discussion

Even though $\beta 1AR$ and $\beta 2AR$ are highly homologous GPCRs, are stimulated by epinephrine and norepinephrine, and are regulating cardiac function, these receptors have distinct subcellular localizations and elicit unique cardiac outputs. The regions where these receptors diverge the most are in the ICL3 and C-terminal tails regions. When key Golgi-retention motifs in the C-terminal tail of $\beta 1AR$ that have been shown to retain other GPCRs at the Golgi were mutated, its Golgi retention was not affected, suggesting that for $\beta 1AR$, these motifs are not necessary or sufficient to promote Golgi exit.

Since the C-terminal tail mutations in $\beta 1AR$ did not promote Golgi exit, the next step was to swap the ICL3 regions of $\beta 1AR$ and $\beta 2AR$ and test whether this influenced βAR Golgi retention. When $\beta 1AR$ contained $\beta 2AR$ ICL3, significantly less $\beta 1AR$ was retained at the Golgi compared to wildtype $\beta 1AR$, indicating that $\beta 1AR$ ICL3 does contain some sort of Golgi retention signal that allows for $\beta 1AR$ Golgi residence. However, when $\beta 2AR$ contained $\beta 1AR$ ICL3, no significant difference was observed in Golgi retention. This suggests that there are other factors in $\beta 2AR$ sequence that promote its Golgi exit. One region could be the $\beta 2AR$ C-terminal tail, which is also quite different than $\beta 1AR$ C-terminal tail. Perhaps adding in the $\beta 1AR$ -RXR- motifs could further promote $\beta 2AR$ Golgi retention. Additionally, N-linked glycosylation has previously been attributed to being critical for plasma membrane localization (Yan et al., 2002). Another factor that could be influencing $\beta 2AR$ Golgi exit and more efficient plasma membrane targeting is that $\beta 2AR$ is further glycosylated at its N-terminal domain than $\beta 1AR$, with $\beta 2AR$ containing three

glycosylation sites (Li et al., 2017) and β 1AR containing only one (He et al., 2002). While previous studies have determined that mutating glycosylation sites on β ARs did not change their activities and plasma membrane localization (Rands et al., 1990), perhaps a combined effect of ICL3 sequence, -RXR- motifs, and altering glycosylation post-translational modifications cause a synergistic effect in β AR subcellular localization.

Future directions include transducing the β 1AR- β 2ICL3 chimera into neonatal cardiomyocytes derived from β AR knockout (KO) mice to provide functional and more physiological significance. Chimeric β 1AR- β 2ICL3 can be transduced into β AR-double KO cardiomyocytes and its localization can be validated in this physiologically relevant system using immunofluorescence microscopy. Our lab has demonstrated that activation of Golgi-localized β 1AR leads to the activation of Golgi-localized PKA which in turn phosphorylates PLB, a key downstream effector of relaxation response in cardiomyocytes (Lin and Mai et al., 2023). In addition, we have shown that plasma membrane-localized β 1AR signaling regulates cTnI and ryanodine receptor 2 (RyR2), additional PKA effectors in cardiomyocytes that are markers of the contraction response (Lin and Mai et al., 2023). It would be interesting to test whether β 1AR- β 2ICL3 chimera only allows for cTnI and RyR2 phosphorylation and no PLB phosphorylation. To do this, isolating β AR-KO cardiomyocytes and comparing the phosphorylation levels of P-PLB and P-cTnI using phospho-specific antibodies by western blotting when transducing wildtype β 1AR and β 1AR- β 2ICL3 chimera. This information would provide further insight on what determines β AR subcellular localizations and could elucidate mechanisms that cause Golgi retention in other GPCRs.

3.5 Figures

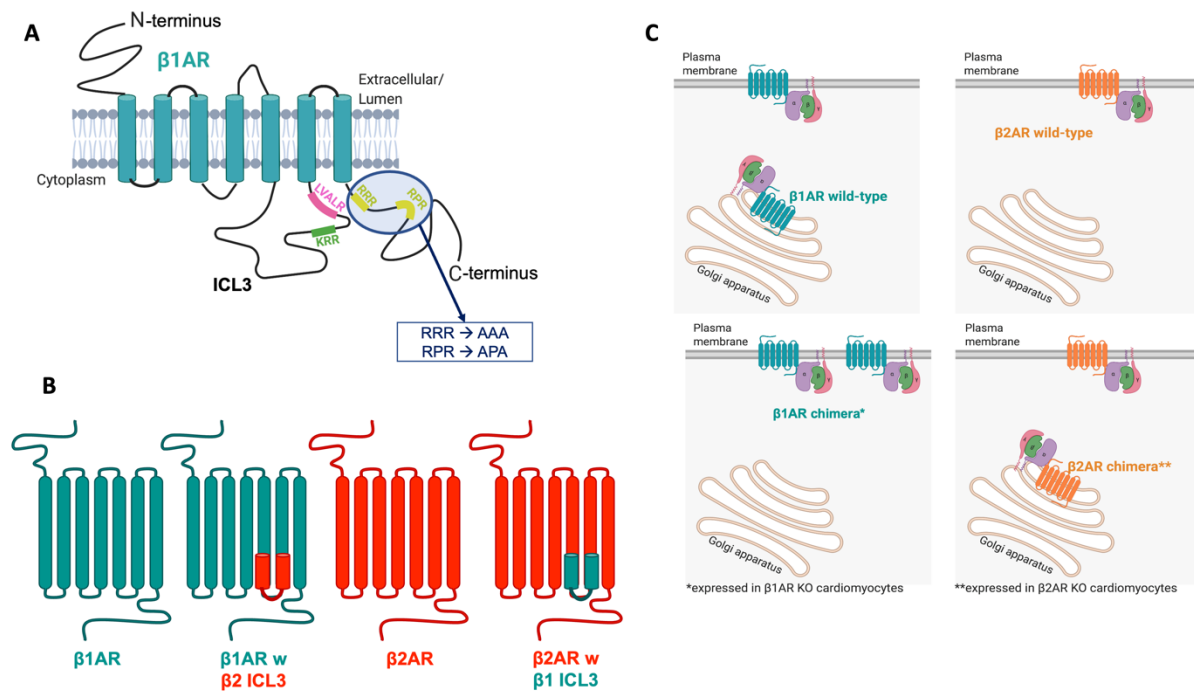


Figure 3.1 Swapping key differing features of β ARs may promote opposing cellular localizations. A) Proposed Golgi retention motifs for β 1AR. LVALR and KRR are in the intracellular loop 3 (ICL3) and the RRR motifs are in the C-terminal tail of β 1AR. B) ICL3 of β 1AR and β 2AR are swapped to make chimeras. C) Comparison of predicted localizations of wildtype (WT) versus chimeric β ARs.

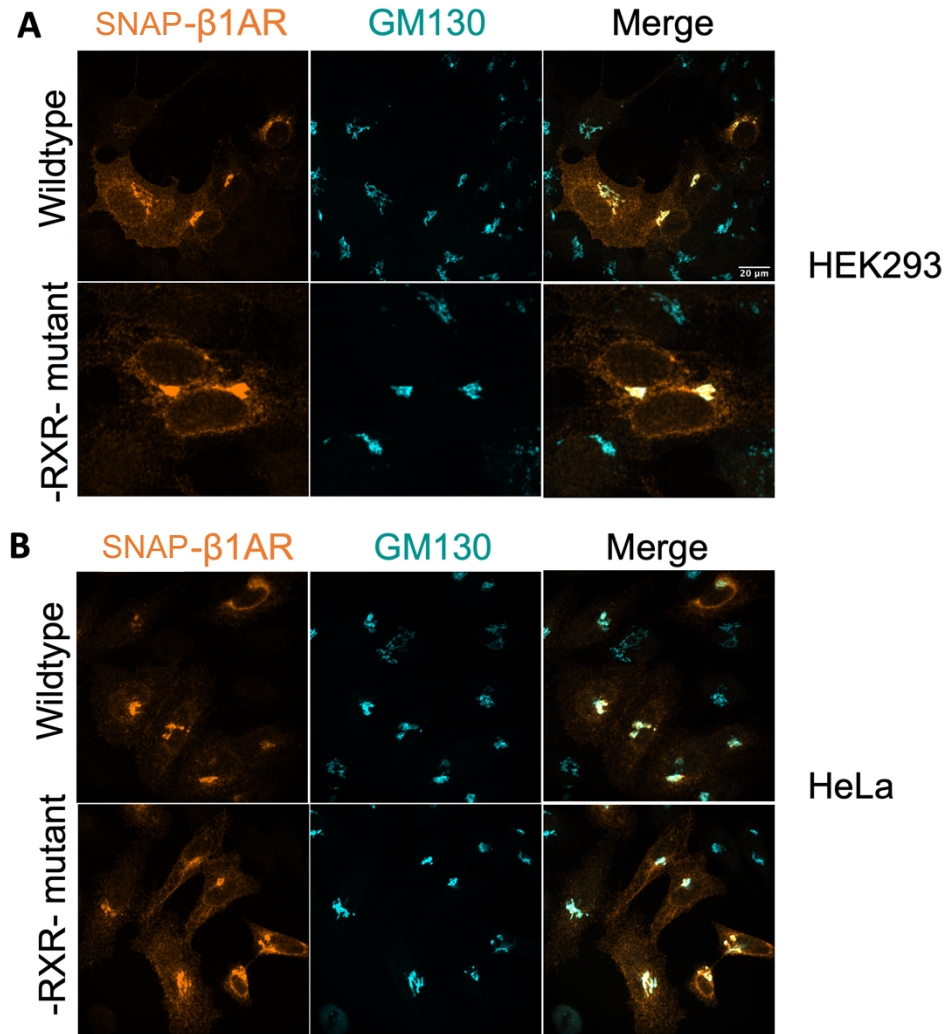


Figure 3.2 C-terminal -RXR- mutations are not sufficient to promote Golgi exit for β 1AR. A) HEK293 fixed cells expressing WT β 1AR (top) and -RXR- mutant β 1AR (bottom). SNAP-labeled β 1AR panels on the left, Golgi marker GM130 in the middle, and merge on the right. B) HeLa fixed cells expressing WT β 1AR (top) and -RXR- mutant β 1AR (bottom). SNAP-labeled β 1AR panels on the left, Golgi marker GM130 in the middle, and merge on the right.

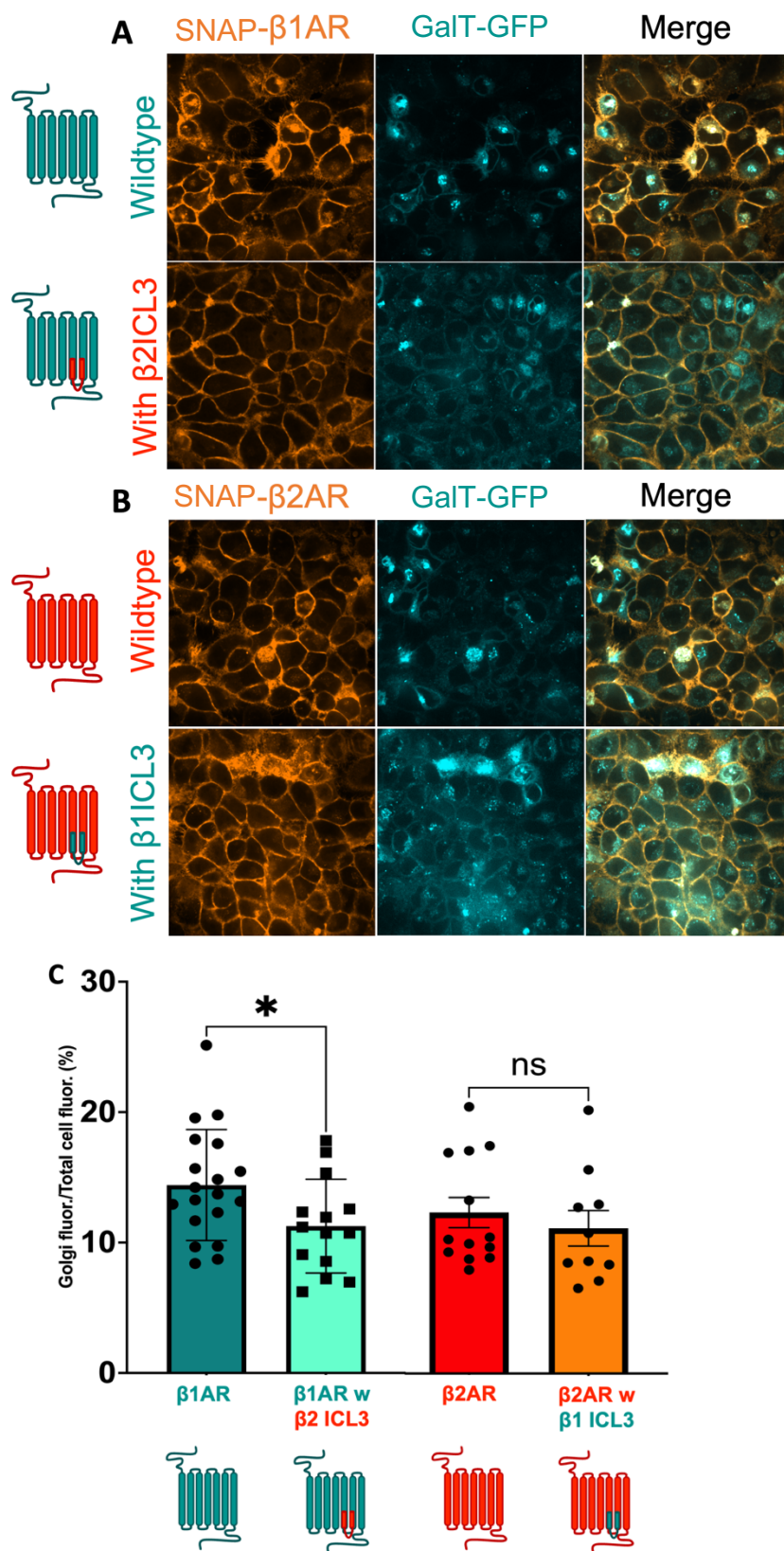


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Figure 3.3 ICL3 swapping promotes Golgi exit for β 1AR but does not promote Golgi retention in β 2AR. A) HeLa live cells expressing WT β 1AR (top) and ICL3 β 1AR chimera (bottom). SNAP-labeled β 1AR panels on the left, Golgi marker GM130 in the middle, and merge on the right. β 1AR w β 2ICL3 chimera appears to be retained less at the Golgi than WT β 1AR. B) HeLa live cells expressing WT β 2AR (top) and ICL3 β 2AR chimera (bottom). SNAP-labeled β 2AR panels on the left, Golgi marker GM130 in the middle, and merge on the right. β 2AR w β 1ICL3 chimera does not appear to be retained more at the Golgi than WT β 2AR. C) Quantification of A and B. Fluorescence of SNAP- β ARs at Golgi regions marked by GM130 were divided by total cell SNAP- β AR fluorescence. WT β 1AR is significantly retained more at the Golgi than β 1AR w β 2ICL3. No significance in Golgi retention is found for WT β 2AR and β 2AR w β 1ICL3.

3.6 Materials and Methods

cDNA constructs/plasmids

SNAP- β 1AR -RXR- mutant was generated by site directed mutagenesis on SNAP- β 1AR construct (see chapter 2 or 4 materials and methods) using 5'- CC GCG GCC GCG CAC GCG ACC CAC GGA GAC G -3' and 5'- C GTG CGC GGC CGC GGC AGC CCT GCG CGC -3' primers (-RRR- to -AAA-), and 5'- AC GCT CCG GCG GCC TCG GGC TGT CTG GCC -3' and 5'- A GGC CGC CGG AGC GTC TCC GTG GGT CGC GT -3' primers (-RPR- to -APA-). SNAP- β 1AR and β 2AR ICL3 swapped constructs were ordered from Twist Biosciences. Amino acids containing SNAP-receptor (β 1AR or β 2AR)-stop codon (*) sequences were input into Twist website and CMV-F backbone was selected to be optimized for mammalian cell expression. GalT-GFP was utilized previously by the lab (Puri et al., 2022).

Cell culture and transfections

HEK293 and HeLa cells (purchased from ATCC as authenticated lines CRL-1573 and CCL-2, respectively) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. Cell cultures were free of mycoplasma contamination. Transfections were performed using Transit-LT1 transfection reagent (Mirus Bio) at a 1:3 DNA to transfection reagent ratio. SNAP-tagged β AR constructs was labeled with SNAP-cell 546 SiR (New England Biolabs, S9102S) at 1:1000 concentration for 20 min at 37°C in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4, washed with the same media (thrice for one minute, room temperature), and imaged in the same media.

Fixed confocal cell imaging

Cells were permeabilized with saponin to reduce the cytoplasmic background, as described previously (*Lobert and Stenmark, 2012*). Briefly, HEK293 and HeLa cells were permeabilized with 0.05% saponin (Sigma) in PEM buffer (80 mM K-Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl₂) for 5 min on ice. Cells were then fixed with 3% paraformaldehyde in PBS for 10 min and then quenched with 50 mM NH₄Cl in PBS for 15 min. Primary antibodies anti- SNAP antibody (NEB P9310S) (1:1000) and GM130 (BD Biosciences 610822) (1:1000) were diluted in PBS supplemented with 0.05% saponin. Confocal images were taken using Nikon spinning disk confocal microscope with a 60 × 1.4 numerical aperture, oil objective.

Live-cell confocal imaging

Live-cell imaging was carried out using a Nikon spinning disk confocal microscope with a ×60, 1.4 numerical aperture, oil objective and a CO₂ and 37°C temperature-controlled incubator. A 488nm and 568 nm Voltran was used as light sources for imaging 488 secondary antibody (GM130), GFP and SNAP-546 signals, respectively. Cells expressing SNAP-tagged receptors were plated onto glass coverslips. Receptors were surface labeled by addition of SNAP-Cell 546 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Cells were imaged in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. Images were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS Elements).

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (<http://rsb.info.nih.gov/ij>). Briefly,

Golgi- β 1AR and total cell β 1AR fluorescence was measured (using SNAP label). Golgi- β 1AR was identified by colocalization with Golgi marker GalT-GFP. Golgi- β 1AR fluorescence values were divided by total cell β 1AR fluorescence values and represented as a percentage using GraphPad Prism v.9.5.1 (GraphPad) software.

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3.8 Acknowledgements

I thank Tracy Knight (future PhD) for advancing this project further during her rotation in Spring 2022. Mentoring her was an amazing experience as she is a brilliant and clever scientist. I also thank Drs. Shaeri Mukherjee, Natalia Jura, Abigail Buchwalter, and Jeremy Reiter for useful insight in this project during the qualification exam process.

Chapter 4:

**Full agonists promote dual Gs- and Gi-protein coupling to β 1AR while
partial and potent agonists bias β 1AR to Gs coupling**

Evelyn Hernandez

4.1 Abstract

While most GPCRs are categorized by the pathway they activate, which derives from coupling to a single cognate G protein, some GPCRs can couple to more than one G protein type and thus initiate multiple types of signaling cascades. This dual coupling can arise either through receptor activation by the same drug or biasing the receptor towards one G protein or another using different drugs. In chapter 2, I highlighted the importance of anionic phospholipids in stabilizing β AR conformations to be able to accommodate G_i coupling as well as its cognate G_s . Here, I tested four different drugs: isoproterenol, epinephrine, dobutamine, and BI167107. Isoproterenol and epinephrine are both full β 1AR agonists that promote dual G protein coupling at the plasma membrane (Chapter 2). Isoproterenol is membrane impermeant, thus it cannot be used to study activation of the Golgi-localized β 1AR. Epinephrine enters the cell via organic cation transporters, activating Golgi-localized β 1AR and only promotes G_s recruitment (Chapter 2). Dobutamine is a selective partial agonist for β 1AR and BI167107 is a highly potent agonist for β ARs. Both drugs only activate G_s at the plasma membrane and Golgi. Given that these drugs have differing recruitment patterns, to further determine whether the behaviors of Mini-G protein biosensors change when the C-terminal domains are switched, I created Mini-G chimeras to test their recruitment at the plasma membrane and Golgi. I concluded that the G_s C-terminal domain is a robust determinant for β 1AR- G protein coupling selectivity, however, it is not the only factor because Mini-G probes with a non-cognate C-terminal domain was still able to bind strongly to β 1AR. This is consistent with what was shown in chapter 2, where I showed that PIP2 facilitates the dual coupling of G_s and G_i .

4.2 Introduction

As mentioned before, the C-terminal domains (CTD) of each G protein has been classically defined to provide selectivity to receptor coupling. The CTD sequences from different G proteins are distinct, especially in the last five to ten amino acids. This is the region that embeds itself into the core of the receptor when interacting. Gs and Gi coupled receptor structures show different modes of G protein binding, where the CTD of Gs seems to require a more open receptor core, while the Gi CTD engages a more closed structure (Van Eps et al., 2018). A more recent study showed that receptors tend to be flexible, and their G protein binding core region cannot differ between different types of G protein engagement since several GPCRs were shown to bind to non-cognate G proteins (Jang et al., 2023). Another paper solved several turkey β 1AR structures with full, partial, and weak partial agonists. Two of those drugs were isoproterenol and dobutamine, one full agonist with one aromatic ring, and one partial agonist with 2 aromatic rings, which I use in this chapter. Their studies found no significant differences between these drugs in making overall structural changes (Warne et al., 2019). However, looking at the atomic contacts that isoproterenol and dobutamine make with β 1AR, dobutamine makes more hydrophobic contacts and hydrogen bonds in the orthostatic pocket and transmembrane 7, potentially allowing β 1AR to be in a more open conformation. This could be rendering β 1AR more accommodating for Gs coupling but not allowing Gi to make the tighter contacts with the receptor. Perhaps the lack of significant changes in the overall conformation with these drugs is due to the fact that receptor was stabilized in the active conformation using a nanobody and not Mini-Gs and Mini-Gi proteins, that would provide more physiologically relevant structures for comparison.

Another study employed nuclear magnetic resonance (NMR) to elucidate the dynamics of β 2AR engaging Gs and Gi. Less spectral changes for β 2AR-Gi than β 2AR-Gs were observed, suggesting that there are less drastic transmembrane domain movements when β 2AR binds to Gi versus Gs (Ma et al., 2020). The use of NMR is beneficial here because, although resolution is not as high as when using crystallography or cryo-electron microscopy, protein dynamics and movements can be better studied.

Serotonin receptor 4 (5HT4) is the only serotonin receptor with the ability to couple to both Gs and Gi proteins (Huang et al., 2022). It was shown that what gave the receptor the flexibility to accept two opposing G proteins was the movement of its transmembrane 5 and 6 alpha helices. When 5HT4 engaged Gs, its TM5 appeared longer while its TM6 was shorter, and the opposite was true when 5HT4 engaged Gi (Huang et al., 2022). When TM6 was artificially lengthened in Gi-coupled serotonin receptors such as 5HT1A or 5HT1B, the receptor was now able to couple to Gs more efficiently, and the opposite was true for Gs-coupled serotonin receptors such as 5HT6 or 5HT7 and lengthening TM5 (Huang et al., 2022). Although structural studies provide great clues that zoom in on the specific amino acids that can be driving dual G protein coupling mechanisms, these remain snapshots of what is occurring in a living cell.

As was shown in chapter 2, anionic phospholipids appear to be stabilizing β AR conformations to couple to both Gs and Gi coupling. In this chapter, I test the ability of four drugs, isoproterenol, epinephrine, dobutamine, and BI167107, to promote G protein coupling in β 1AR. Isoproterenol and epinephrine both are full β 1AR agonists that allow for dual G protein coupling at the plasma membrane as I showed in chapter 2 using Mini-G biosensors. Dobutamine is a partial agonist selective for β 1AR and BI167107 is a highly

potent agonist for β ARs, primarily used in structural studies (Rasmussen et al., 2011; Ma et al., 2020; Xu et al., 2021). These latter two drugs only activate Gs at the plasma membrane, as shown by Mini-Gs recruitment to the plasma membranes upon stimulation with these drugs but not Mini-G(s)i or Mini-GiEDGE (our more Gi-like biosensor, see chapter 2 for details on Mini-GiEDGE generation). Furthermore, epinephrine, dobutamine, and BI167107 access the internal pools of β 1AR by either OCT3 transport (epinephrine) or permeability due to drug hydrophobicity (dobutamine and BI167107). These three drugs are shown to recruit only Gs at the Golgi apparatus, and not Gi. To decipher if the behaviors of our biosensors change when the G protein CTD are switched, we created Mini-G chimeras to test their recruitment at the plasma membrane and Golgi.

4.3 Results

When stimulated with 10 μ M isoproterenol or 10 μ M epinephrine in HeLa cells expressing SNAP- β 1AR and Mini-Gs, Mini-G(s)i, or Mini-GiEDGE, all Mini-G probes show recruitment to the plasma membrane with Mini-Gs coupling first, followed by slightly weaker Mini-G(s)i. and subtly Mini-GiEDGE at later time points (Figure 4.1). When cells were stimulated with 10 μ M dobutamine or 10nM BI167107, only Mini-Gs was recruited to the plasma membrane, and not Mini-G(s)i or Mini-GiEDGE (Figure 4.1). Epi gains access to the internal Golgi pool of β 1AR by organic cation transporters (OCT3) and dobutamine and BI167107 access the Golgi by diffusing across HeLa cell plasma membranes due to their high hydrophobicity, therefore, we can compare these drugs' activities at the Golgi apparatus. All three of these drugs promote only Mini-Gs coupling at the Golgi (Figure 2.1 and Supplemental figures 4.3 and 4.4).

To test the effect of CTD on the Mini-G probes, the last five amino acids of Mini-Gs (QYELL), Mini-G(s)i (DCGLF), and Mini-GiEDGE (DCGLF) were swapped to create chimeras which we named Mini-Gs Gi CTD (DCGLF), Mini-G(s)i (QYELL) and Mini-GiEDGE (QYELL) (Figure 4.1 and 4.2). The last five amino acids were selected because this is where G protein CTD sequences diverge the most (Supplemental figure 2.4). When stimulated with 10 μ M isoproterenol or 10 μ M epinephrine in HeLa cells expressing SNAP- β 1AR and Mini-Gs Gi CTD, Mini-G(s)i Gs CTD, or Mini-GiEDGE Gs CTD, all Mini-G probes show recruitment to the plasma membrane with Mini-Gs Gi CTD coupling first, followed by Mini-G(s)i Gs CTD and Mini-GiEDGE Gs CTD at later time points (Figure 4.2). The only drastic difference is that the efficiency for Mini-GiEDGE Gs CTD to couple to the plasma membrane is improved significantly. When stimulated with 10 μ M dobutamine or 10 nM BI167107, HeLa cells expressing SNAP- β 1AR and Mini-Gs Gi CTD and Mini-G(s)i Gs CTD show recruitment to the plasma membrane with Mini-G(s)i Gs CTD coupling first, followed by Mini-Gs Gi CTD (Figure 4.2). At the plasma membrane, Mini-GiEDGE Gs CTD is still not recruited to the plasma membrane upon 10 μ M dobutamine and 10 nM BI167107 stimulation. This data suggests that the CTD is indeed important for determining coupling selectivity and recruitment patterns, corroborating previous studies that point to the CTD as a main determinant of G protein selectivity. However, given that Mini-GiEDGE Gs CTD chimera was not able to couple to β 1AR at the plasma membrane using dobutamine and BI167107 and that Mini-Gs Gi CTD does not lose its coupling efficiency when stimulated with all drugs, this suggests that there are other sections in the G protein other than the CTD that allow it to strongly couple to β 1AR.

At the Golgi we do not see recruitment of Mini-Gs Gi CTD nor Mini-G(s)i Gs CTD when stimulated with epinephrine, dobutamine or BI167107, but we do see Golgi recruitment of Mini-GiEDGE Gs CTD (Supplemental figures 4.6, 4.7, and 4.8). This suggests that the Gs CTD is what may be conferring Golgi β 1AR selectivity. However, at the plasma membrane, it appears that Mini-G needs more than the Gs CTD for coupling using Gs-biased drugs. This is because we see a gain in Golgi recruitment using Mini-GiEDGE Gs CTD, while we still do not see its recruitment at the plasma membrane using these drugs. This could suggest that indeed anionic phospholipids like PIP2 are playing an important role at the plasma membrane in providing structural stability for β 1AR to couple to Gs and Gi.

4.4 Discussion

While the G-protein CTD has been classically defined as what determines its selectivity, the N-terminal domain has been more recently been shown to also be crucial. In this chapter, we observed altered kinetics and coupling patterns when Mini-G proteins CTD were swapped. β 1AR has differing G-protein coupling patterns at different compartments when activated by full agonists such as isoproterenol and epinephrine, with Gs and Gi coupling at the plasma membrane but only Gs at the Golgi. When partial or potent agonists are used such as dobutamine and BI167107, only Gs can couple at both plasma membrane and Golgi. Isoproterenol and epinephrine have similar chemical structures, each containing one aromatic ring (Figure 4.1), while dobutamine and BI167107 share more chemical commonalities, each containing two aromatic rings which gives them their hydrophobic nature and thus renders them permeable to the plasma membrane (Figure 4.1). It could be that dobutamine and BI167107 hold the receptor too

open to accommodate Gi coupling, given that it has been shown that a larger drug like dobutamine contains more hydrophobic contacts in the orthostatic pocket than a smaller drug like isoproterenol (Warne et al., 2019). Another possibility that explains the inability of Gi to couple to β 1AR could be that these drugs are so hydrophobic that they rush into the cell's interior so fast that it does not allow for Gi to couple, since it takes slightly longer to bind to β 1AR (note delay in kinetic curve when stimulated with isoproterenol and epinephrine, Figure 4.1).

What became clear from these studies is that the Gs CTD is a strong determinant for β 1AR-G protein coupling selectivity. However, it also became clear that the G protein CTD is not the only determinant for β 1AR-G protein coupling selectivity because Mini-Gs Gi CTD was still able to bind strongly to β 1AR at the plasma membrane when stimulated with isoproterenol and epinephrine. Therefore, it seems like for plasma membrane β 1AR, there is more interplay occurring at different regions of the G protein (NTD and CTD) with other factors at that organelle, such as PIP2, as was shown in chapter 2, that allow for the dual coupling of Gs and Gi.

For Golgi β 1AR however, it appears that the Gs CTD is what renders the receptor capable of coupling to any G protein. This is because when β 1AR is stimulated with epinephrine, only wildtype Mini-Gs couples to Golgi β 1AR, and wildtype Mini-G(s)i, which is a Gs and Gi chimera containing a more Gs-like NTD core but a Gi CTD, cannot couple to Golgi β 1AR. Wildtype Mini-GiEDGE also does not couple to Golgi β 1AR when stimulated with epinephrine. However, Mini-GiEDGE Gs CTD can couple to Golgi β 1AR when stimulated with epinephrine, and with dobutamine and BI167107I, it couples to the Golgi only, and not the plasma membrane. There is still some slight recruitment of Mini-

Gs Gi CTD at the Golgi and lack of recruitment with Mini-Gi Gs CTD at the Golgi, but this could be because only the last five amino acids of the G protein CTD were swapped and not the full CTD sequence where G proteins diverge, which is in their last ten amino acids. Perhaps if more residues are accounted for, then full recapitulation of each G protein might have been observed in these type of recruitment studies.

Determining which pathways are activated when certain drugs are utilized becomes important not just for our overall understanding of GPCR biology but for heart health as well. Although BI167107 is not used in any type of clinical setting because it is of its high potency, epinephrine is the endogenous ligand that stimulates β 1AR and β 2AR to produce proper heart function and isoproterenol and dobutamine have been utilized short-term in hospital or clinic settings to deal with cardiac decompensation due to debilitated heart muscle (Mraz and Rorabaugh, 2007). Isoproterenol has been substituted as a therapeutic drug by other compounds that are more specific for β AR subtypes such as dobutamine, which is β 1AR-specific (Abel and Rorabaugh, 2007). Dobutamine is usually administered when other cardiac treatments have been unsuccessful (Mraz and Rorabaugh, 2007). Therefore, it is important to understand the compartmentalized consequences of β AR stimulation and which pathways are being activated when using certain drugs. Furthermore, β 1AR has primarily been classified and studied mostly as a Gs-coupled receptor, but according to these studies, it can also engage Gi when using certain drugs. It is important to follow up on this finding in cardiomyocytes to better understand how β 1AR functions in healthy settings versus disease settings, and this can inform future drug design and can aid physicians in administering and prescribing the most appropriate medication.

Future directions include testing the recruitment patterns of wildtype and CTD swapped Mini-G probes using confocal microscopy in a more physiologically relevant system by transducing wildtype and CTD swapped Mini-G probes in isolated mouse cardiomyocytes and corroborating the results we observed in HeLa cells. It would also be useful to detect proteins via western blot in isolated mouse cardiomyocytes after treating with these drugs to identify which drug promotes more efficient activation of the necessary downstream pathways.

4.5 Figures

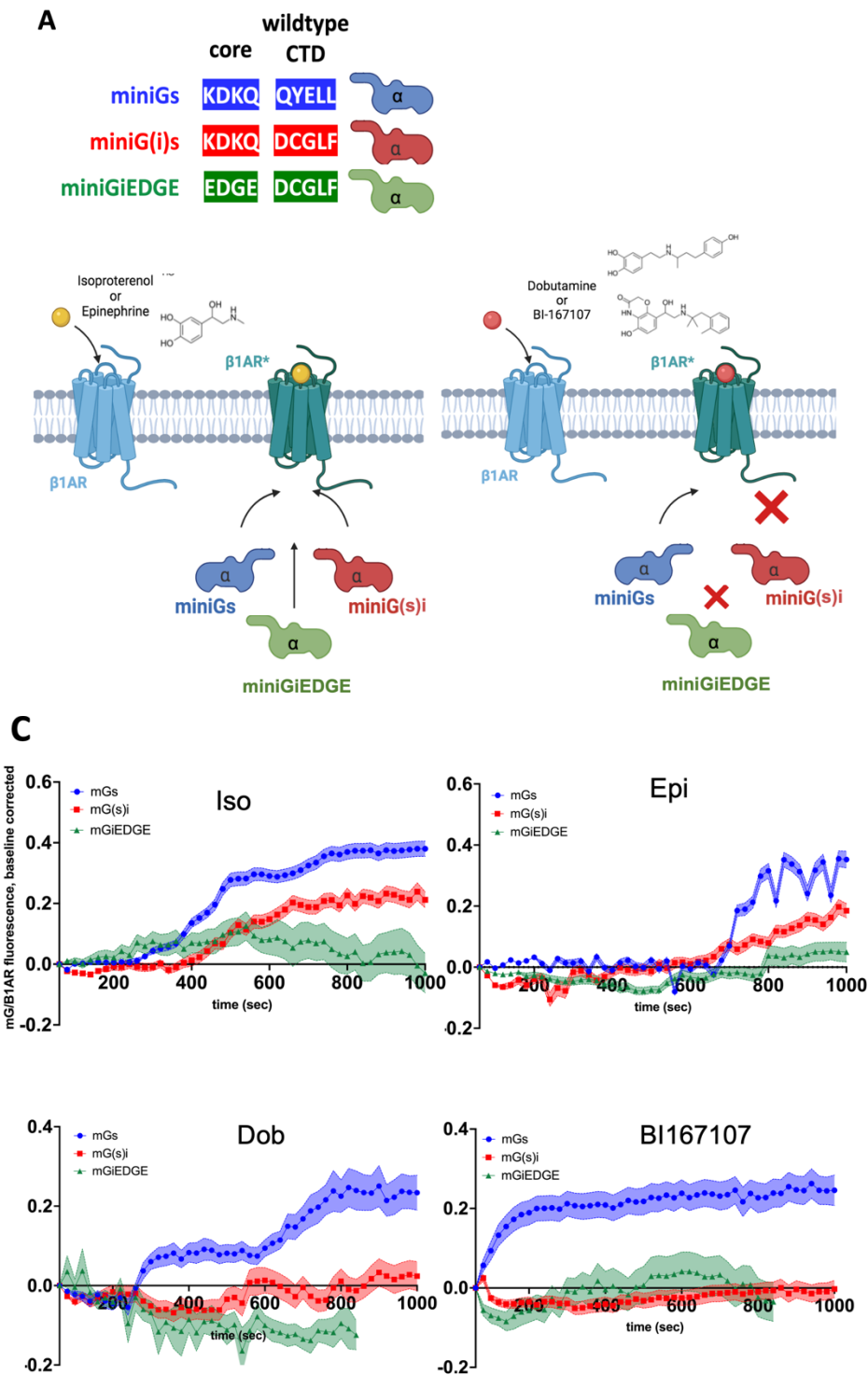
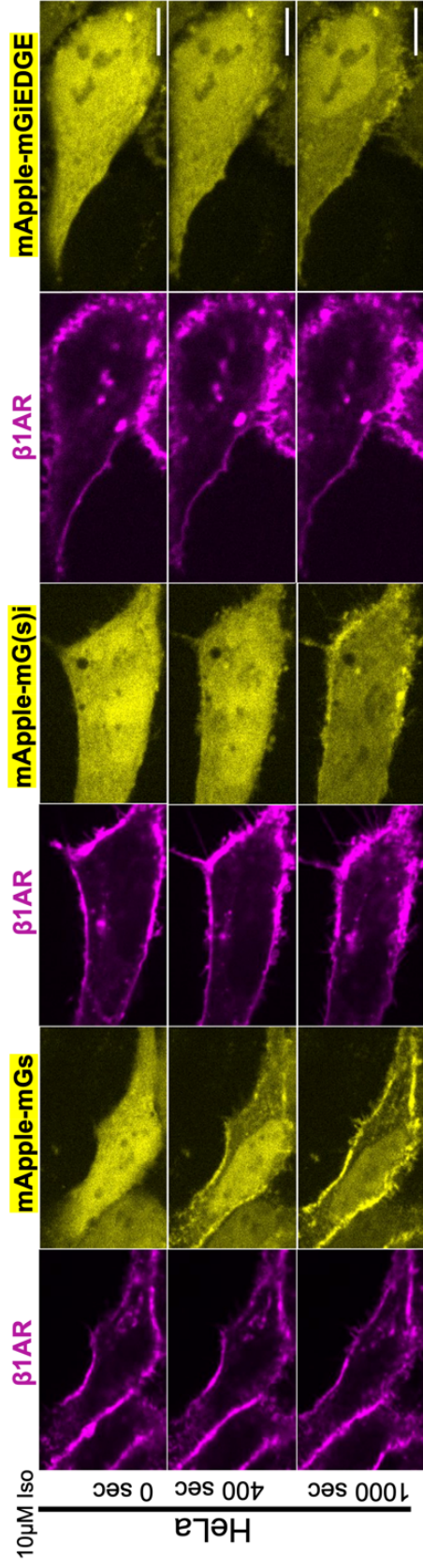
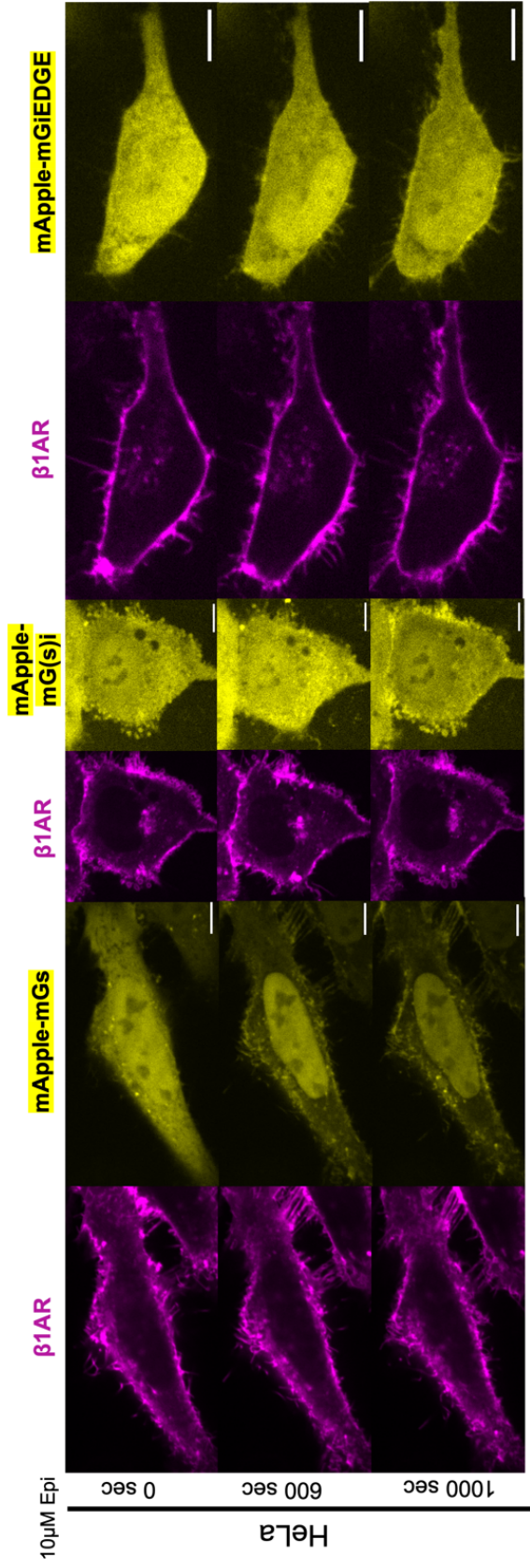


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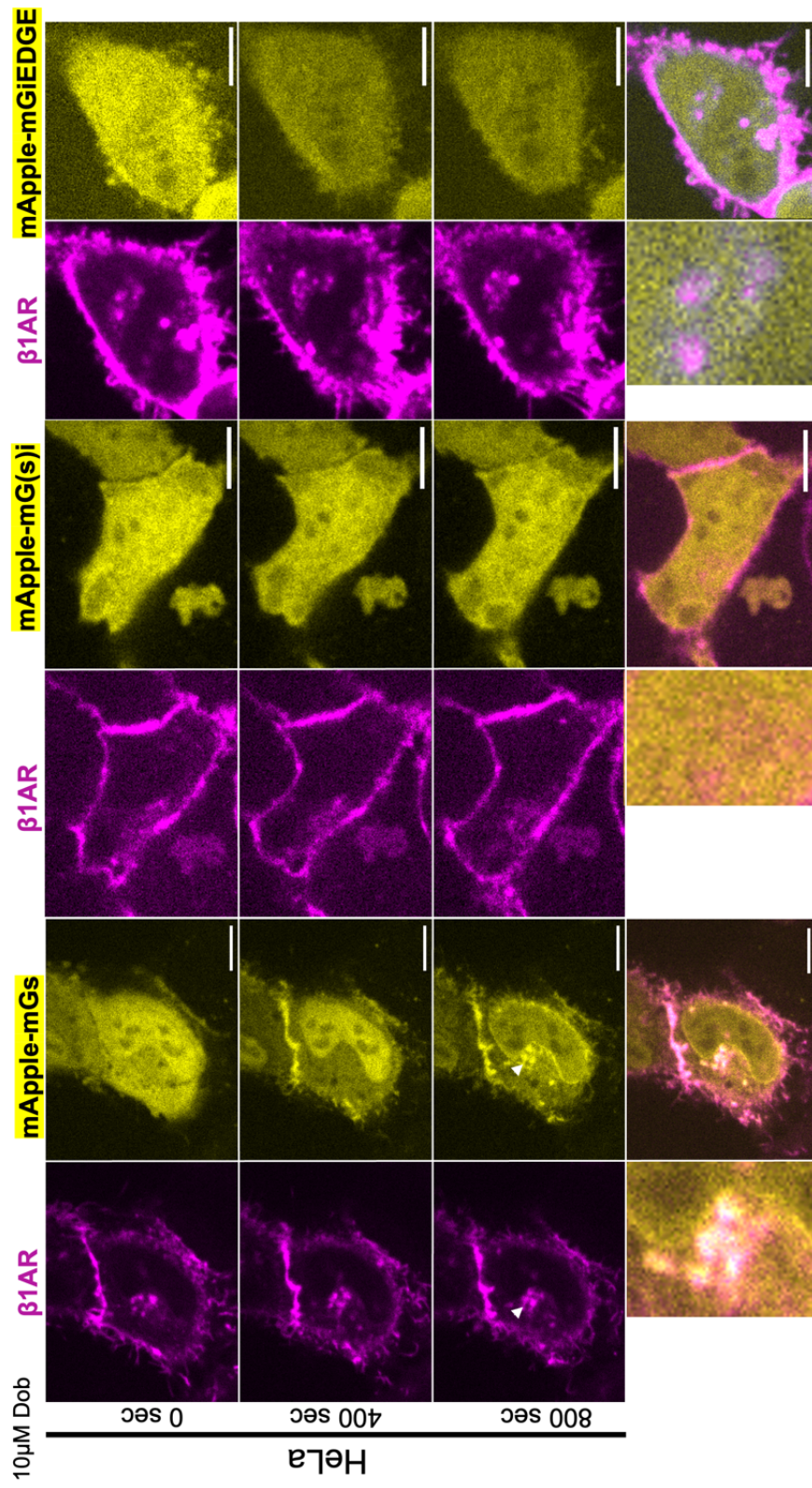
Figure 4.1 Conformational biosensors for G α s and G α i proteins detect activated β 1AR at the plasma membrane upon Isoproterenol (Iso) and epinephrine (Epi) stimulation but is biased to G α s with dobutamine (Dob) and potent agonist BI167107. A) The N-terminal core region of Mini-G(s)i was mutated from its positive sequence (KDKQ) derived from G α s sequence to its original negative sequence (EDGE) to create a more G α i-like biosensor which we named MiniGi-EDGE. Mini-Gs contains the CTD of G α s (QYELL) and Mini-G(s)i and MiniGi-EDGE contain the CTD of G α i (DCGLF). B) Iso and Epi stimulation promote recruitment of all three biosensors, while Dob and potent agonist BI167107 only promote recruitment of Mini-Gs. Note the similarities in chemical structure of drugs Iso and Epi (one aromatic ring) and Dob and BI167107 (very hydrophobic, two aromatic rings, cell permeable). C) Quantifications of β 1AR activation at the plasma membrane in HeLa cells after 10 μ M Iso, 10 μ M Epi, 10 μ M Dob, or 10nM BI167107 addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 1AR. Quantifications were baseline corrected after addition of drugs. Recruitment is defined as all error bars clearing the x-axis (i.e. all error bars above 0).



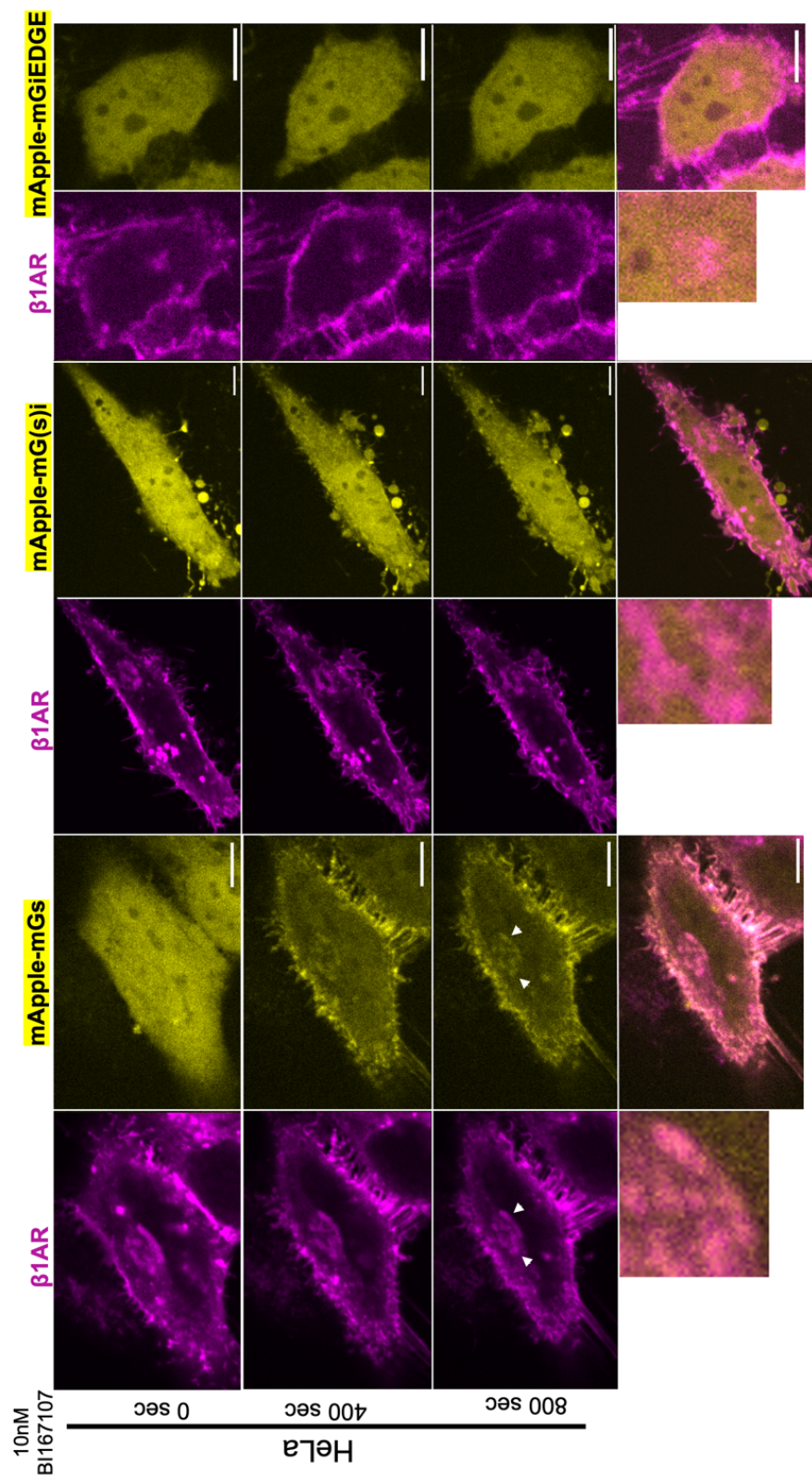
Supplementary figure 4.1 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs (yellow left panels), mApple-Mini-G(s)i (yellow middle panels), and mApple-MiniGi-EDGE (yellow right panels) expression before and after 10 μM Iso addition. Stimulation with 10 μM Iso results in recruitment of Mini-Gs, Mini-G(s)i, and MiniGi-EDGE to active $\beta 1AR$ at the plasma membrane in HeLa cells. Scale bar = 10 μm .



Supplementary figure 4.2 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs (yellow left panels), mApple-Mini-G(s)i (yellow middle panels), and mApple-Mini-Gi-EDGE (yellow right panels) expression before and after 10 μM Epi addition. Stimulation with 10 μM Epi results in recruitment of Mini-Gs, Mini-G(s)i, and Mini-Gi-EDGE to active $\beta 1AR$ at the plasma membrane in HeLa cells. Scale bar = 10 μm .



Supplementary figure 4.3 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs (yellow left panels), mApple-Mini-G(s)i (yellow middle panels), and mApple-MiniGi-EDGE (yellow right panels) expression before and after $10\mu M$ Dob addition. Stimulation with $10\mu M$ Dob results in recruitment of only Mini-Gs (three biological replicates), but not Mini-G(s)i (n= 26 cells, five biological replicates) or MiniGi-EDGE (n=14 cells, three biological replicates) to active $\beta 1AR$ at the plasma membrane and Golgi in HeLa cells. Arrows indicate recruited Mini-Gs at the Golgi; Scale bar = $10\mu m$.



Supplementary figure 4.4 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs (yellow left panels), mApple-Mini-G(s)i (yellow middle panels), and mApple-MiniGi-EDGE (yellow right panels) expression before and after 10 nM B1167107 addition. Stimulation with 10 nM B1167107 results in recruitment of only Mini-Gs ($n = 38$ cells, three biological replicates), but not Mini-G(s)i ($n = 26$ cells, five biological replicates) or MiniGi-EDGE ($n = 14$ cells, three biological replicates) to active $\beta 1AR$ at the plasma membrane and Golgi in HeLa cells. Arrows indicate recruited Mini-Gs at the Golgi; Scale bar = 10 μm

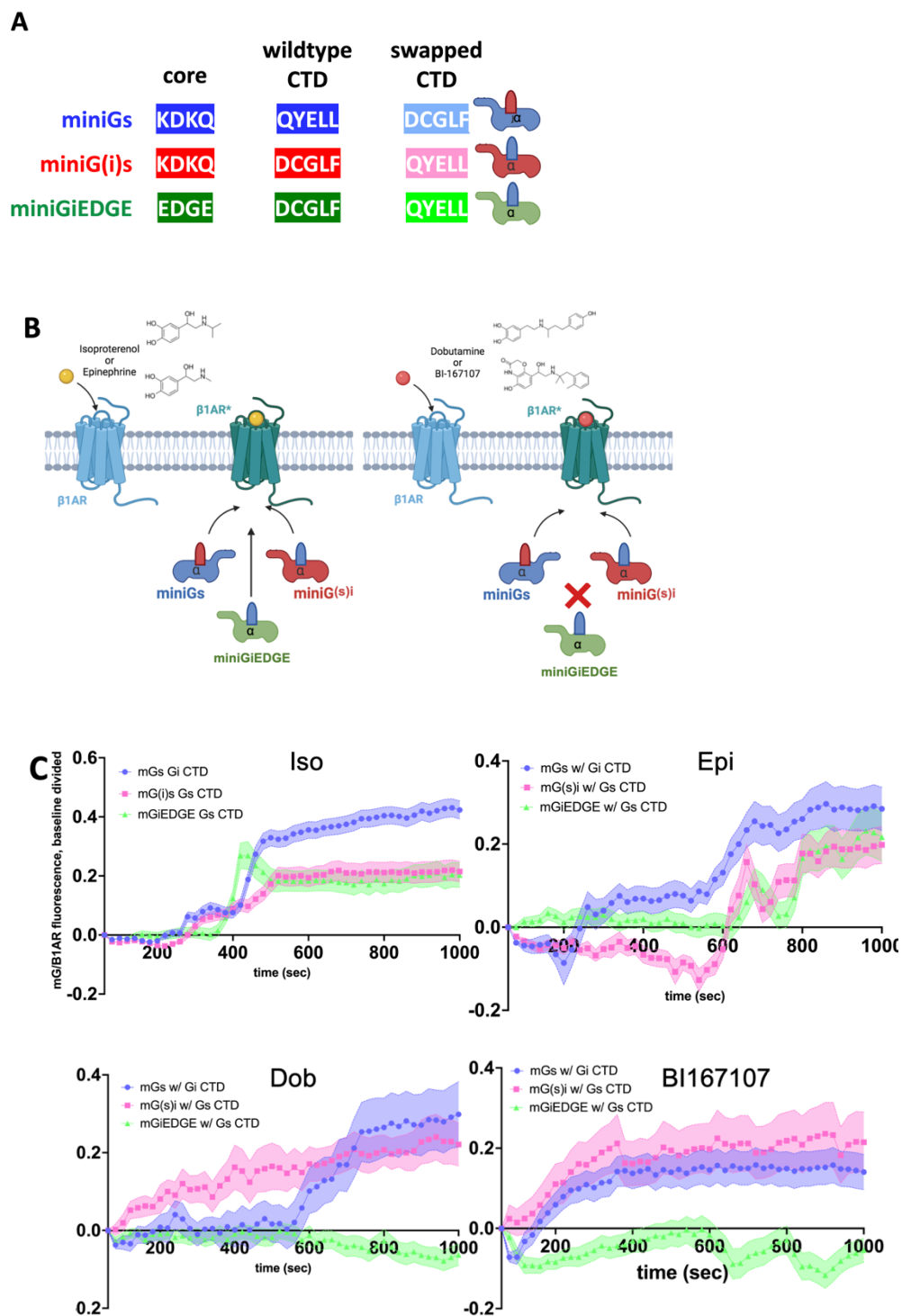
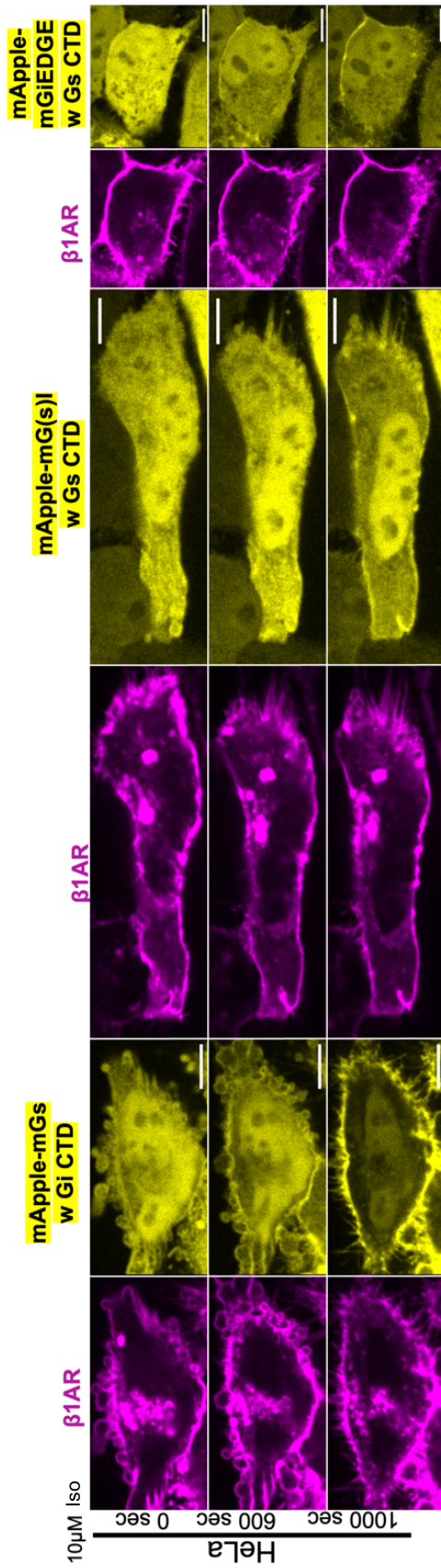
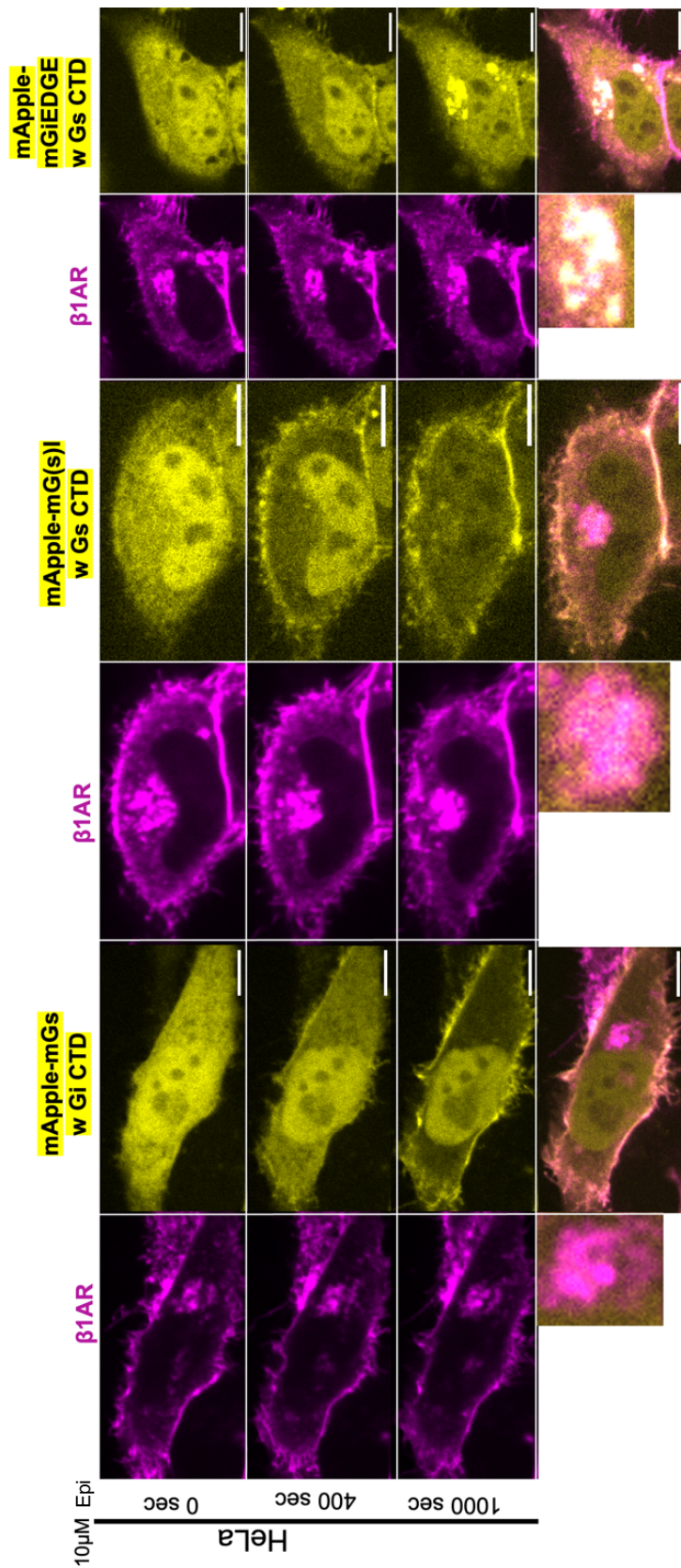


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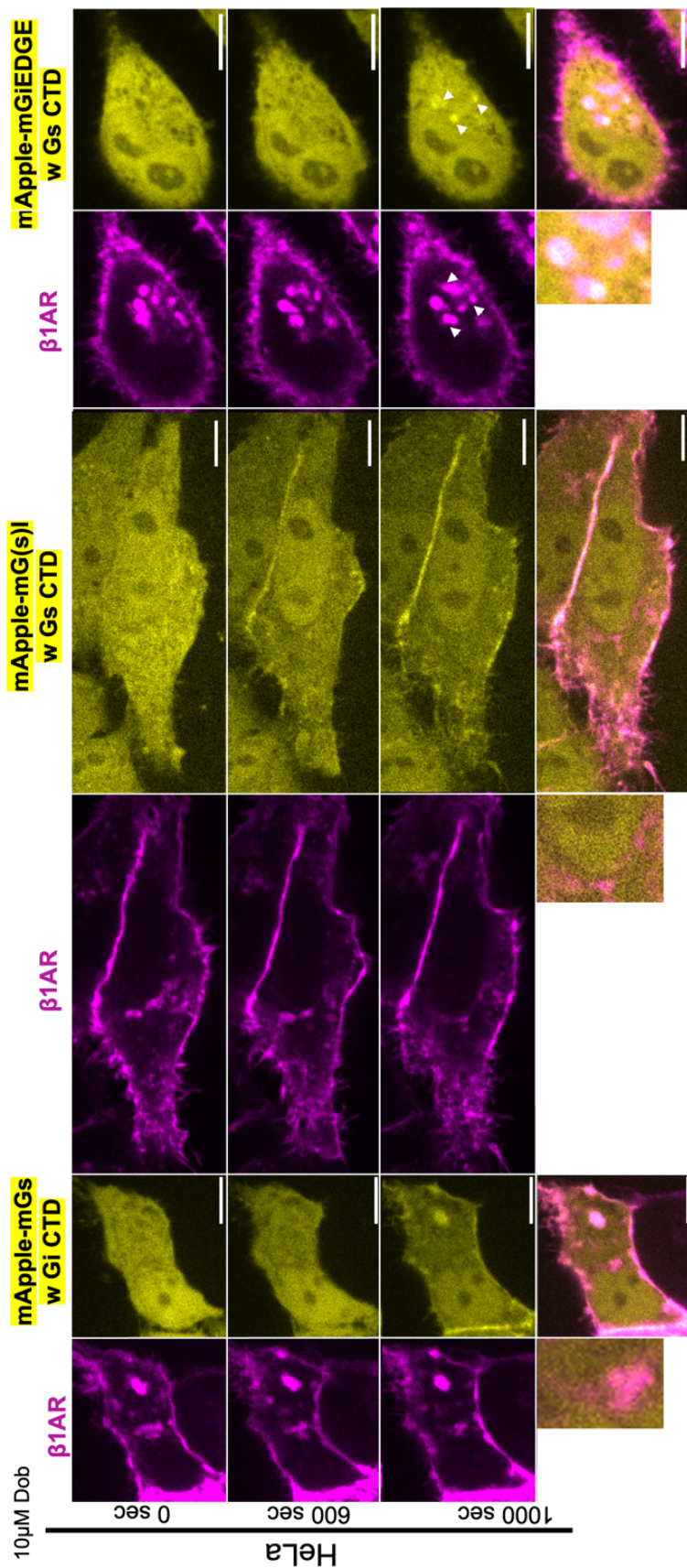
Figure 4.2 Swapping the C-terminal domains (CTD) of Mini-G probes does not alter their recruitment patterns to activated β 1AR at the plasma membrane. A) The CTD of all Mini-G probes were swapped. Mini-Gs contains the CTD of G α i (DCGLF) and Mini-G(s)i and MiniGi-EDGE contain the CTD of G α s (QYELL). B) Isoproterenol (Iso) and epinephrine (Epi) stimulation promote recruitment of all three biosensors, while dobutamine (Dob) and potent agonist BI167107 only promote recruitment of Mini-Gs with Gi CTD and Mini-Gi with Gs CTD (the latter is essentially Mini-G(s)i mutated back Mini-Gs). C) Quantifications of β 1AR activation at the plasma membrane in HeLa cells after 10 μ M Iso, 10 μ M Epi, 10 μ M Dob, or 10nM BI167107 addition; normalized fluorescence intensity of Mini-G probes at plasma membrane relative to SNAP-tagged- β 1AR. Quantifications were baseline corrected after addition of drugs. Recruitment is defined as all error bars clearing the x-axis (i.e. all error bars above 0).



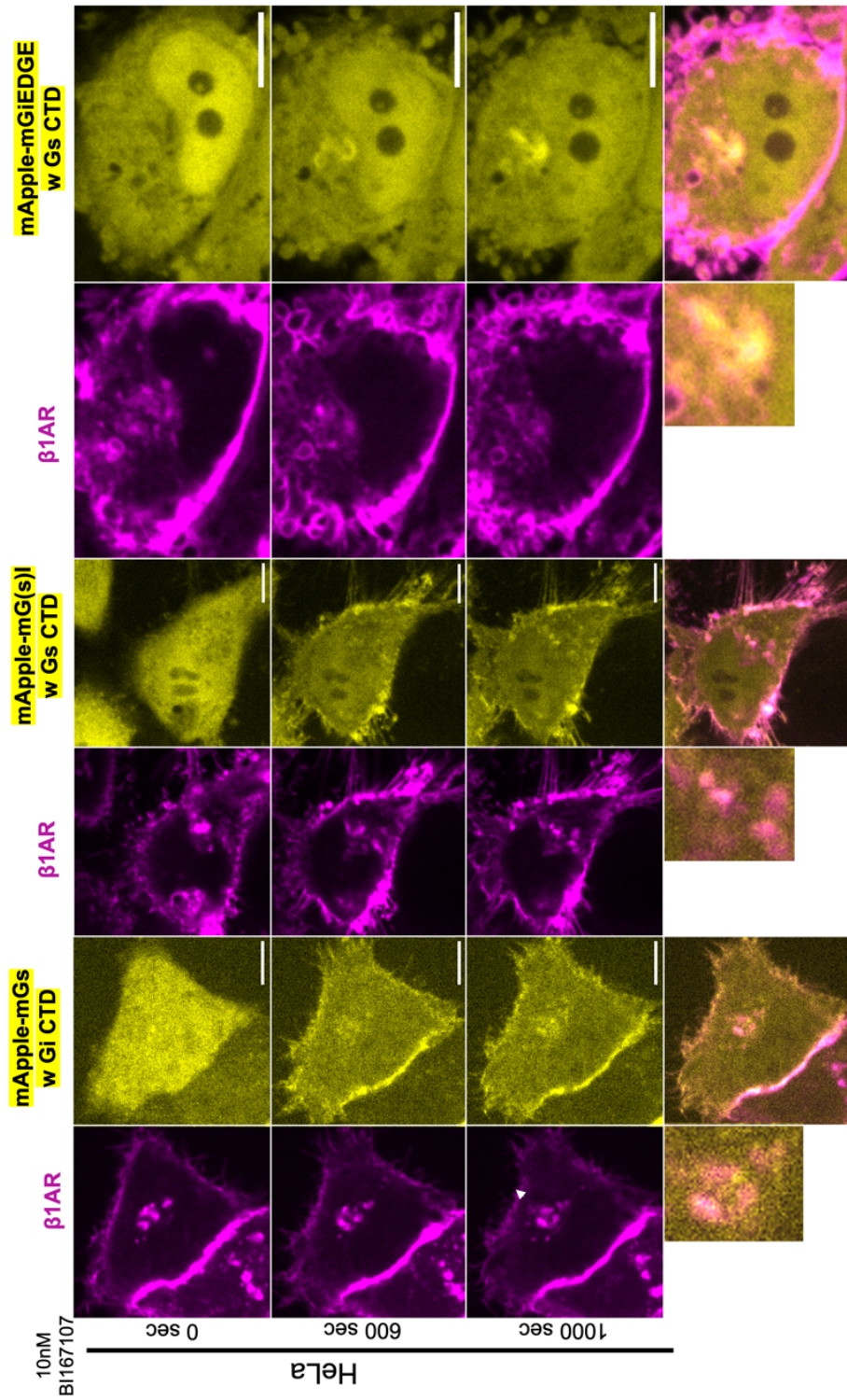
Supplementary figure 4.5 Confocal images of representative SNAP-tagged β1AR-expressing HeLa cells (magenta panels) with mApple-Mini-Gs w Gi CTD (yellow left panels), mApple-Mini-G(s)j w Gs CTD (yellow middle panels), and mApple-MiniGi-EDGE w Gs CTD (yellow right panels) expression before and after 10 μM Iso addition. Stimulation with 10 μM Iso results in recruitment of Mini-Gs, Mini-G(s)j, and MiniGi-EDGE to active β1AR at the plasma membrane in HeLa cells but only MiniGi-EDGE w Gs CTD at the Golgi. Scale bar = 10 μm.



Supplementary figure 4.6 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs w Gi CTD (yellow left panels), mApple-Mini-G(s)j w Gs CTD (yellow middle panels), and mApple-MiniGi-EDGE w Gs CTD (yellow right panels) expression before and after 10 μM Epi addition. Stimulation with 10 μM Epi results in recruitment of Mini-Gs, Mini-G(s)j, and MiniGi-EDGE to active $\beta 1AR$ at the plasma membrane in HeLa cells. Scale bar = 10 μm .



Supplementary figure 4.7 Confocal images of representative SNAP-tagged β1AR-expressing HeLa cells (magenta panels) with mApple-Mini-Gs w Gi CTD (yellow left panels), mApple-Mini-G(s)i w Gs CTD (yellow middle panels), and mApple-MiniGi-EDGE w Gs CTD (yellow right panels) expression before and after 10 μM Dob addition. Stimulation with 10 μM Dob results in recruitment of Mini-Gs and Mini-G(s)i to active β1AR at the plasma membrane in HeLa cells but only MiniGi-EDGE w Gs CTD at the Golgi. Scale bar = 10 μm.



Supplementary figure 4.8 Confocal images of representative SNAP-tagged $\beta 1AR$ -expressing HeLa cells (magenta panels) with mApple-Mini-Gs w Gi CTD (yellow left panels), mApple-Mini-G(s)j w Gs CTD (yellow middle panels), and mApple-MiniGi-EDGE w Gs CTD (yellow right panels) expression before and after 10 nM BI167107 addition. Stimulation with 10 nM BI167107 results in recruitment of Mini-Gs and Mini-G(s)j to active $\beta 1AR$ at the plasma membrane in HeLa cells and all three at the Golgi. Scale bar = 10 μm .

4.6 Materials and Methods

βAR constructs

Signal Sequence- SNAP-tagged β1AR was created by amplifying β1AR from Flag-β1AR 5'- GCTG GGT CTT GGA TCC AAC GAT GAT GAC GCC GGC -3'; 5'- AT AGA ATA GGG CCC TCT AGA GCC CTA CAC CTT GGA TTC CG -3' primers and inserted into the SNAP vector using BamHI and XbaI.

Mini-G probes

pmVenus-Mini-Gs, pmVenus-Mini-G(s)I constructs were gifts from Dr. Nevin Lambert. pmVenus-Mini-Gi EDGE and pmApple-Mini-Gi EDGE were created from pmVenus-Mini-G(s)i and pmApple-Mini-G(s)i by site directed mutagenesis using 5'- A AAA CAG CTC CAG AAA GAC GGG CAA GTG TAC AGG G -3'; 5'- T CTA TGA GTG GCC CTG TAC ACT TGC CCG TCT TTC TGG -3' primers for the first round of mutation (KDKQ → KDGQ), and 5'- GAA AAA CAG CTC CAG GAA GAC GGG GAA GTG -3'; 5'- AGT GGC CCT GTA CAC TTC CCC GTC TTC CTG -3' primers for the second round of mutation (KDGQ → EDGE). C-terminal domain swapped Mini-G probes were created using site directed mutagenesis using 5'- G AAT CTT CGG CAA TAC GGG CTT TTC TAA AAG CTT CGA A -3'; 5'- CG AAG CTT TTA GAA AAG CCC GTA TTG CCG AAG ATT CA -3 for the first round of mutation (DCGLF → QYGLF) and 5'- CTT CGG CAA TAC GAG CTT CTC TAA AAG CTT CGA AT -3'; 5'- TT TTA GAG AAG CTC GTA TTG CCG AAG ATT CAT TTT GA -3 for the second round of mutation (QYGLF → QYELL) of the Gs C-terminal domain swap, and using 5'- G CAT CTC AGG GAC TGT GAG CTG CTC TAA AAG CTT CG -3'; 5'- C GAA GCT TTT AGA GCA GCT CAC AGT CCC TGA GAT GCA -3 for the first round of mutation (QYELL → DCELL) and 5'- TC AGG GAC TGT

GGG CTG TTC_TAA AAG CTT C -3'; 5'- AGC TTT TAG AAC AGC CCA CAG TCC CTG AGA TGC -3 for the second round of mutation (DCELL → DCGLF) of the Gi C-terminal domain swap.

Cell culture and transfections

HeLa cells (purchased from ATCC as authenticated lines CCL-2) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. Cell cultures were free of mycoplasma contamination. Transfections were performed using Transit-LT1 transfection reagent (Mirus Bio) at a 1:3 DNA to transfection reagent ratio. SNAP-tagged human β 1AR construct was labeled with SNAP-cell 647 SiR (New England Biolabs, S9102S) at 1:1000 concentration for 20 min at 37°C in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4, washed with the same media (thrice for one minute, room temperature), and imaged in the same media.

Live-cell confocal imaging

Live-cell imaging was carried out using a Nikon spinning disk confocal microscope with a $\times 60$, 1.4 numerical aperture, oil objective and a CO2 and 37°C temperature-controlled incubator. A 568 nm and 640 Voltran was used as light sources for imaging mApple and SNAP-647 signals, respectively. Cells expressing SNAP-tagged receptor (1 μ g for wildtype β 1AR) and the indicated Mini-G probe (100 ng) were plated onto glass coverslips. Receptors were surface labeled by addition of SNAP-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Indicated agonists (isoproterenol, epinephrine, or dobutamine all from Sigma; BI167107 was a gift from Dr. Aashish Manglik) were added and cells were imaged every 20 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. Time-lapse images

were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS Elements).

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (<http://rsb.info.nih.gov/ij>). For measuring kinetics of Mini-G probe recruitment at the plasma and Golgi membranes over time in confocal images, analyses were performed on unprocessed TIFF images using a previously published scripts written in MATLAB, available through open access on zenodo (Jullie D et al., 2020; Jullie D et al., 2022). Briefly, the plasma membrane and Golgi regions were selected and a mask of labeled receptor (using SNAP label) or Golgi marker were generated by thresholding the receptor or the Golgi marker signal within the selected region. The average fluorescence intensity of Mini-G was measured within the masked region and outside of the masked region, before and after addition of agonists. Values were normalized by calculating the percent relative to the maximum value, then baseline corrected using GraphPad Prism v.9.5.1 (GraphPad) software so that the first value of each condition was set to 0.

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4.8 Acknowledgments

I thank Dr. Aashish Manglik for supplying BI167107 and for helpful discussion.

Chapter 5:

Determining interactomes of β 1AR at the plasma membrane and Golgi apparatus using APEX-MS

Evelyn Hernandez, Qiongyu Li, Ruth Huttenhaim, Nevan Krogan, and Roshanak Irannejad

5.1 Abstract

GPCRs are the largest family of membrane receptors in the human genome and are responsible for sensing a wide range of signals between cells and tissues. GPCRs were previously thought to only function at the plasma membrane. Although it has been widely accepted that GPCRs have residences at other organelles other than the plasma membrane such as at endosomes after receptor internalization and the endoplasmic reticulum (ER) and Golgi apparatus as proteins are being synthesized, processed, and trafficked to the plasma membrane. It was assumed that GPCRs were dormant at these subcellular locations and could only be activated at the plasma membrane due to hormone and drug access. Recent evidence has shown that certain GPCRs, such as β 1AR, β 2AR, D1DR, MOR and DOR, can be activated at these subcellular compartments. To identify compartment specific GPCR signaling interactomes, we are using a proximity labeling approach, based on an engineered ascorbic acid peroxidase, to identify location specific protein partners of an exemplary GPCR, β 1AR, and comparing its interactomes upon activation at the plasma membrane versus Golgi apparatus. This has been previously performed for β 2AR to determine its different interactomes when it is activated at the plasma membrane versus the endosomes.

5.2 Introduction

Activation of β 1AR leads to G protein-mediated cAMP production and PKA-mediated phosphorylation of key subcellular Ca^{2+} regulatory proteins such as phospholamban (PLB) and Troponin I (cTnI). Our lab has shown that phosphorylated PLB (P-PLB), a perinuclear localized protein regulating Ca^{2+} ATPase pumps to mediate the relaxation response, colocalizes with the Golgi, whereas phosphorylated cTnI (P-cTnI), a

marker of the contraction response, does not (Lin and Mai et al., 2023). Furthermore, using β 1AR antagonists with different membrane permeabilities, we showed that Golgi-localized β 1ARs regulate relaxation responses via PLB phosphorylation, whereas plasma membrane-localized β 1ARs regulate contraction responses via cTnI phosphorylation. Moreover, previous studies on β 1AR-, β 2AR-, and double β 1AR/ β 2AR-knockout (KO) mice, showed that β AR stimulation of contraction and relaxation are mediated almost exclusively by β 1AR, but heart rate is controlled by both β ARs (Rohrer et al., 1999). More recently, it has been shown that β AR-mediated L-type Ca^{2+} channel (LTCC) activation is dependent on Rad phosphorylation by PKA at the t-tubules, a Ras-like GTPase known for its capacity to inhibit all high-voltage-activated Ca^{2+} channels in an unphosphorylated form (Liu et al., 2020). These studies suggest that although there is a high degree of relatedness between both β 1AR and β 2AR in terms of sequence and structure (66% homology), and similar effectors (e.g. adenylyl cyclase), the roles of β 1AR and β 2AR are not redundant, even in tissues where they are co-expressed like in cardiomyocytes (Rohrer et al., 1999).

This chapter aims to test the following: Given the differing β 1AR and β 2AR subcellular localizations, and their unique roles in regulating cardiac output, I hypothesize that β AR signaling from their distinct, specific compartments dictates their roles in cellular and physiological function due to the local recruitment of unique set of effectors/proteins. Several interacting partners are known for plasma membrane-localized β 1AR, such as the heterotrimeric Gs protein, PDZ binding proteins, and phosphodiesterases (e.g. PDE4D8), to name a few (Xiang et al., 2002; Richter et al., 2008; Xiang 2011; Yang et al., 2015). However, information on the interacting partners for Golgi-localized β 1AR is

lacking. More importantly, how β 1AR signaling at the Golgi is regulated is not known. In this chapter, I aim to unveil some of these key players in the compartmentalized β 1AR interactome via an unbiased approach described below:

To determine unknown effector proteins and regulatory machinery for β 1AR signaling from the plasma membrane and the Golgi in an unbiased approach, I used engineered ascorbic acid peroxidase (APEX) followed by mass spectrometry (MS). APEX-MS has been successfully used to decipher GPCR interactomes, such as the β 2AR, DOR, and angiotensin II type 1 receptor, at different subcellular locations (Lobingier et al., 2017; Paek et al., 2017). APEX-MS has also been used to determine the interactome LTCC, which allows Ca^{2+} entry into the cell upon β AR stimulation in cardiomyocytes (Liu et al., 2020). These studies not only unveiled new interacting proteins but also help explain novel regulatory mechanisms. One such example is the discovery of Rad GTPase as a regulatory protein inhibiting LTCC activity. It has been shown that phosphorylation of Rad by PKA releases its inhibitory effect on LTCC and allows increased Ca^{2+} entry into the cells. This inhibitory interaction between Rad-LTCC and the phosphorylation of Rad being what frees LTCC is reminiscent of the relationship between PLB and SERCA pumps at the sarcoplasmic reticulum when Golgi- β 1AR is stimulated and with this approach I plan to identify new such regulatory mechanisms (Liu et al., 2020).

5.3 Results

I successfully anchored APEX to the plasma membrane or Golgi in HEK293 cells stably expressing β 1AR and confirmed that the enzyme is activated when hydrogen peroxide was added in the media (Figure 5.1). I then labeled proteins in the vicinity of

either plasma membrane or Golgi-localized β 1AR and compared the labeled proteins from dobutamine-stimulated β 1AR to non-stimulated β 1AR. I used dobutamine and not isoproterenol because dobutamine is a membrane permeant β 1AR agonist and I wanted to ensure that Golgi- β 1AR was activated. I did not use epinephrine in this case because HEK293 cells do not express organic cation transporters (OCT3) that allow for epinephrine to enter the cell interior, thus unable to activate Golgi- β 1AR in those cells.

We identified several target proteins and decided to focus on the Golgi + and – dobutamine targets. In the volcano plot of the hits from the Golgi labeling profile (Figure 5.2), we compare the proteins that were labeled in the unstimulated samples to proteins that were labeled in the dobutamine stimulated samples. Although the p values did not appear to be significant, we found a significant fold change in some target proteins. These targets include enzymes that are important for trafficking and phosphoinositide synthesis. The two that I will be focusing on in this chapter are phospholipid phosphatase 3 (LPP3) and phosphoinositide synthase (PIS). PIS was upregulated by 1.7-fold [nearly a 2-fold change] and LPP3 was downregulated -0.58-fold [above a half-fold change]. Two other targets that stood out include enzymes involved in phospholipid synthesis or modification such as 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase η -2 (PLCH2) and phosphatidylglycerophosphate synthase 1 (PGS1). One last protein that was shown to interact with Golgi- β 1AR upon dobutamine stimulation was an A-Kinase Anchoring Protein, AKAP10. AKAPs are known to anchor PKA to specific membrane compartments.

Three of the selected target proteins are enzymes that function to regulate phosphatidic acid (PA) generation, the precursor and substrate for the phosphoinositide

synthesis pathway, where PIS is involved. PIS is the only enzyme expressed in the mammalian genome that is involved in generating phosphoinositide (PI). PIS is expressed in the Golgi and endoplasmic reticulum (ER), where it generates PI from cytidine diphosphate diacylglycerol (CDP-DAG) (Blunsom and Cockcroft, 2020). From there, PI transporting proteins shuttle it to other organelles. As highlighted in chapter 2, organelle membranes tend to contain their own unique PI identity, such as PI(4,5)P₂ on the plasma membrane and PI(4)P on the Golgi.

On the plasma membrane, PI is converted to PI(4)P by PI4 kinase. PI(4)P at the plasma membrane is quickly phosphorylated at the 5' position to make PI(4,5)P₂. At the Golgi, PI is converted to PI(4)P, and unlike at the plasma membrane, it retains its PI(4)P identity. PA that is localized at the ER is a precursor of PI. PA has two sources: de novo from glycerol-3-phosphate (G-3-P) synthesis and when it is recycled back from the plasma membrane to the ER upon DAG conversion to PA by DAG kinases (Blunsom and Cockcroft, 2020).

It has been shown that activation of Golgi- β 1AR leads to activation of an specific Golgi effector, EPAC (exchange protein activated by 3'-5'-cAMP) which can bind cAMP and activate PLC ϵ , leading to PI(4)P cleavage and producing DAG and IP₂ (Nash et al., 2019). This increased PI(4)P cleavage caused by activation of Golgi- β 1AR was shown to create hypertrophic response in rat cardiomyocytes (Nash et al., 2019). The accumulation of DAG upon activation of Golgi- β 1AR can also contribute to generation of PA.

Another protein target that we identified was LPP3, which localizes at the plasma membrane, ER, and Golgi (Uniprot). LPP3 is a phospholipid phosphatase and its mechanism of action at the ER is to convert PA into DAG, most likely from the G-3-P

pathway. At the Golgi LPP3 converts PA to DAG from a different phospholipid source (phosphocholine). LPP3 can regulate the overall increase in DAG throughout the cell due to its various cellular localizations. It has also been shown that LPP3 is important for cargo trafficking. The silencing of LPP3 causes retrograde trafficking to be affected where less tubulation is seen from the Golgi to the ER and the motility of tubules is slowed down (Gutierrez-Martinez et al., 2013). This can potentially be explained by the decrease in DAG caused by silenced LPP3 because DAG provides negative membrane curvature that is required for COPI-dependent retrograde trafficking (Bard and Malhortra, 2006). Therefore, less DAG means that cells do not get the C-shaped structures that is required to promote membrane fission. This led me wonder whether the expressions and localizations of LPP3 and PIS are affected upon β 1AR stimulation .

At the ER, PA has two options: to be converted into DAG by LPP3 or be converted to CDP-DAG by CDS enzymes and then to PI by PIS. However, the creation of CDP-DAG by CDS enzymes is the slowest, rate-limiting step of the synthesis of PI (Blunsom and Cockcroft, 2020). Therefore, upregulation of PIS will not have a significant effect in the formation of PI if the level of CDS enzymes are unchanged. Thus, LPP3 downregulation might be of significant importance as it will slow down the conversion of PA to DAG. This would also make sense in the context of PLC ϵ -mediated PI(4)P conversion to DAG (Nash et al., 2019). In this case, presumably a negative feedback loop is further slowing down LPP3 activity to reduce DAG generation and avoid overflowing cells with enhanced DAG production. Similarly, the enhanced PIS activity upon β 1AR activation at the Golgi is driven by PI(4)P hydrolysis at Golgi. This will allow cells to replenish PI by increasing PIS activity and recruitment at the Golgi. As discussed above,

PIS is the only enzyme expressed in the mammalian genome that catalyzes the conversion of CDP-DAG to PI. Therefore, it would make sense for PIS to compensate for the loss of PI upon activation of Golgi- β 1AR. However, this can only occur along with LPP3 loss of activity. Accordingly, the synthesis of poly-phosphoinositides like PI(4,5)P₂ and PI(4)P has been shown to be regulated by the supply of PI and not the activity of PI4 kinases (Sonh et al., 2018).

To test these ideas, PIS and LPP3 constructs were ordered from addgene and tagged with GFP and expressed in HeLa cells. PIS appeared to localize at the Golgi and colocalized with Golgi- β 1AR (Figure 5.3). LPP3 appeared to localize at the plasma membrane and Golgi overlapping with β 1AR at those locations (Figure 5.3). Cells were stimulated with 10 μ M isoproterenol to test whether any changes in PIS-GFP or LPP3-GFP localization can be observed. We found that PIS-GFP localization is slightly increased, whereas, LPP3-GFP localization at the Golgi is decreased (Figure 5.3). This was observed when cells were stimulated with 10 μ M, isoproterenol, a full agonist that is not efficiently delivered into the cells to activate the Golgi- β 1AR. The follow up step would be to stimulate β 1AR expressing HeLa cells with dobutamine or epinephrine, two agonists shown to efficiently activate β 1AR at the Golgi membrane (Ref). Thus I predict that the effects would be more pronounced in these conditions.

5.4 Discussion

Future directions include confirming these targets in cardiomyocytes and validating their role and importance in Golgi- β 1AR signaling. Transducing plasma membrane- and Golgi-APEX into rat or mouse cardiomyocytes would provide a much more physiologically relevant system. This can then be followed by testing for altered levels of DAG and PI(4)P

using biosensors, checking for increased cell size as a sign of hypertrophy, and probing for phosphorylation of histone deacetylase 5, an indicator of hypertrophy that has been shown to increase when DAG levels are elevated. The mRNA levels of atrial natriuretic factor can also be measured, whose increased levels are an indicator of hypertrophy (Vikstrom et al., 1998). Furthermore, comparing the effects of Golgi- β 1AR stimulation in diseased versus healthy cardiomyocytes would be interesting to observe whether the upregulation of PIS and/or the downregulation of LPP3 is affected when heart cells no longer function properly. Lastly, chapter 4 highlighted how different drugs can bias β 1AR to couple to Gs or Gi proteins. Therefore, comparing the different APEX-MS protein profiles when stimulating β 1AR with these different drugs can be important for determining which pathways are pronounced depending on the drug used.

Corroborating the importance of PIS and LPP3 would be interesting because this can be tied back to the importance of phosphoinositides in β 1AR signaling. In chapter 2 I highlighted the importance of anionic phospholipids, namely PI(4,5)P2, in stabilizing β AR structures to be able to accommodate Gs and Gi proteins at the plasma membrane. This chapter unveiled how Golgi- β 1AR can also be involved indirectly in the phosphoinositide synthesis pathway.

This chapter aimed to understand the functional consequences of receptor signaling from each membrane compartment in regulating cellular responses. Compartmentalized signaling challenges the dogma in the GPCR field and has much left to uncover. Following up on these findings will be crucial to unveiling how signaling from different compartments by β 1AR regulates heart contractility response and provide further clues as to how to specifically manipulate each compartmentalized receptor in the heart

failure. This can also provide further clues as to how other GPCRs behave subcellularly. Furthermore, understanding other downstream players of Golgi- β 1AR would be useful in understanding the pathological causes of β 1AR related cardiomyopathies.

5.5 Figures

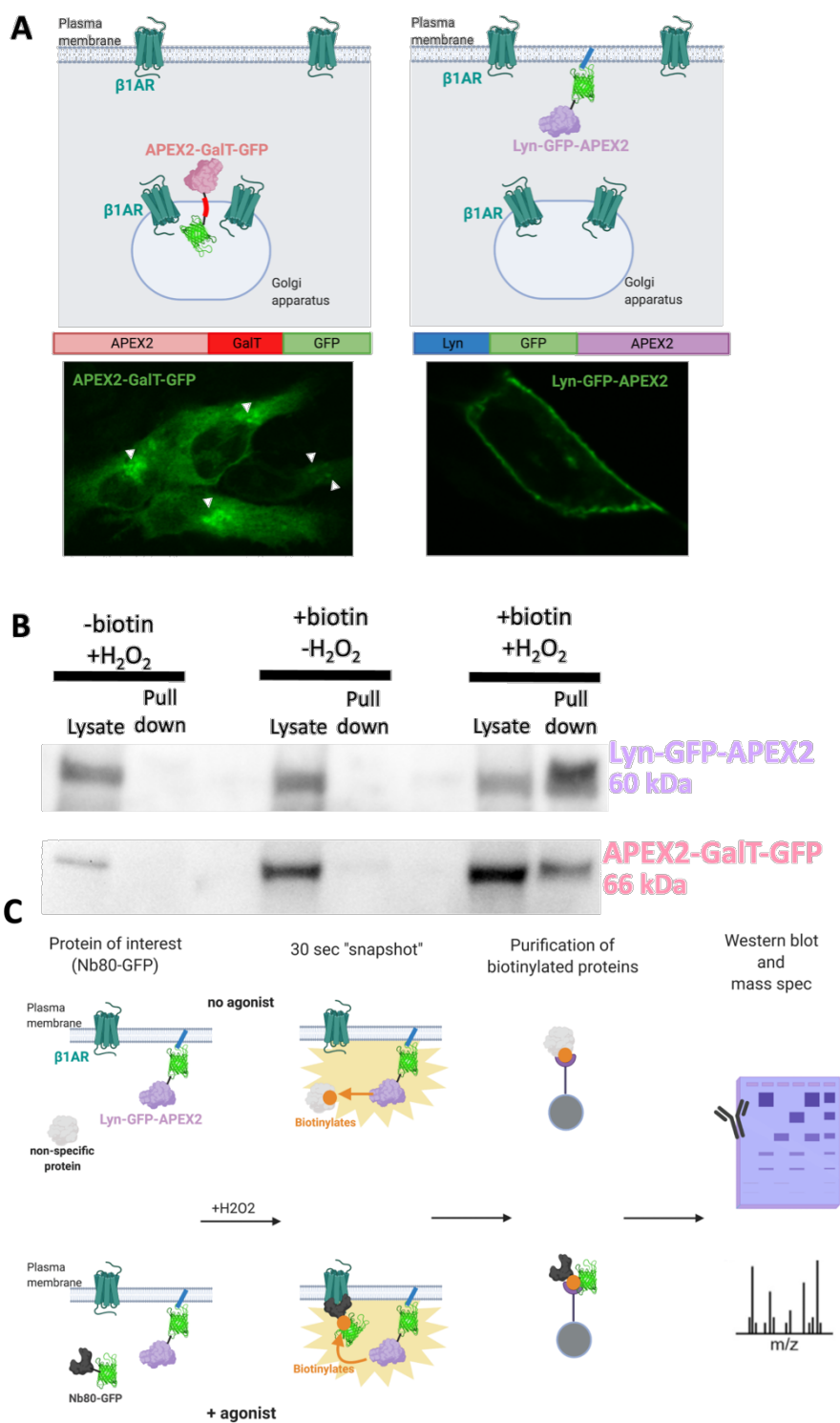


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Figure 5.1 APEX-MS schematic and validation of construct expression and function. A) Golgi (right) and plasma membrane (PM) (left) targeted APEX2 schematic (top). GalT is the Golgi targeting sequence, Lyn is the PM targeting sequence. Constructs and spinning disk confocal microscopy images of APEX2-GalT-GFP and Lyn-GFP-APEX2 in HEK293 cells (below). B) Western blot detection using anti-GFP antibody. As can be seen by the lack of bands at the pull-down lanes, labeling by APEX2 in the control lanes (-biotin, +peroxide and +biotin, -peroxide) does not occur. When biotin and peroxide are added to the cells expressing the APEX2 constructs, we see biotinylation of proteins in the pull-down lane, confirming functionality of our APEX enzyme. C) Reaction scheme for unstimulated (top) and agonist-stimulated (bottom) APEX2 labelling. Scheme for the PM (Lyn) targeted APEX2 is shown, the idea is the same for Golgi (GalT) targeted APEX2.

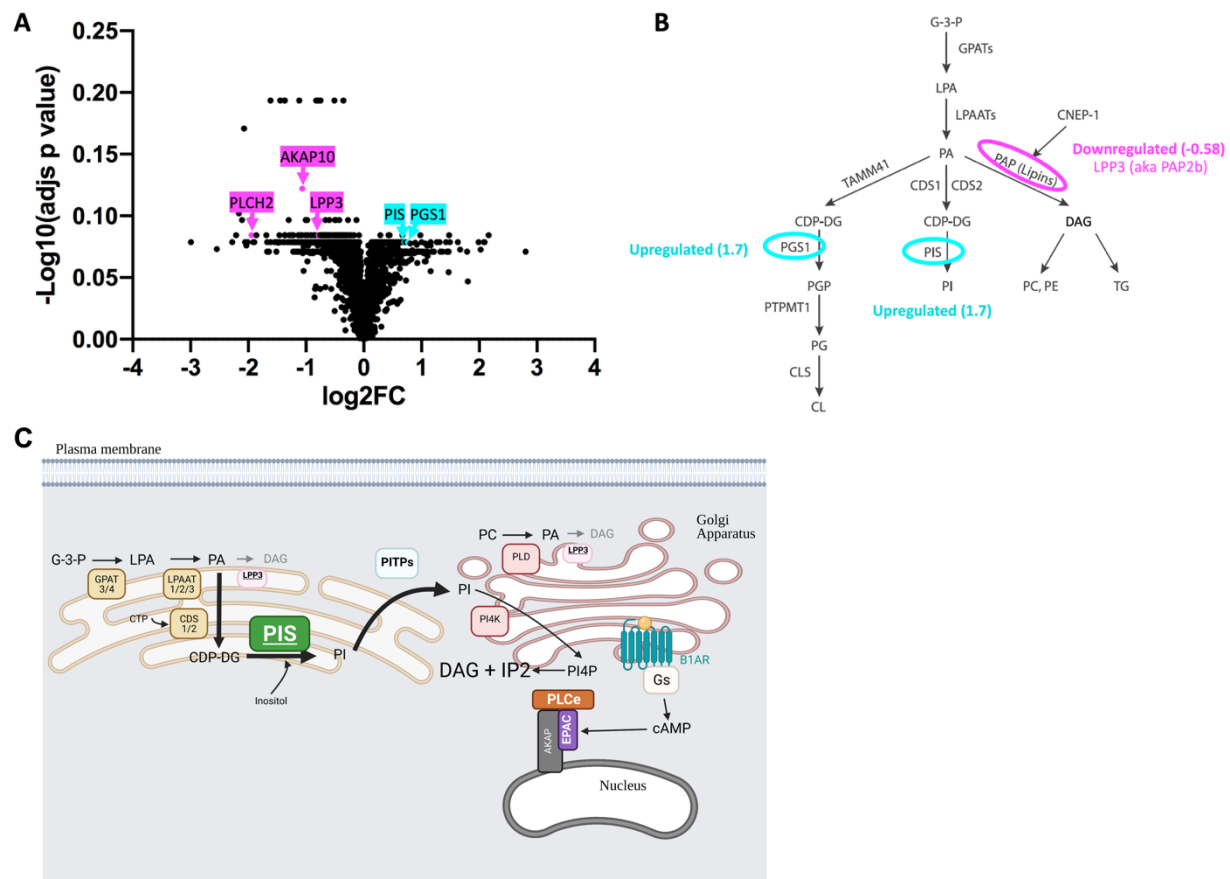


Figure 5.2 Golgi-APEX hits show changes in protein profiles involved in phospholipid and phosphoinositide pathways. A) Volcano plot of Golgi-APEX hits with log 2-fold change values on the x axis and adjusted p values on the y axis. Hits of interest included values that were either up- or downregulated by 0.5-fold change (i.e., a half-fold change). Selected hits were chosen due to their involvement in GPCR related pathways (AKAP10) and phospholipid and phosphoinositide processes (PIS, LPP3, PGS1, PLCH2). B) Three of the selected hits are after the phosphatidic acid (PA) branchpoint, which is a precursor for phosphoinositide (PI), and thus all other phosphoinositides important in membrane compositions (PI(4,5)P2, PI(4)P, and PI(3)P) and diacyl glycerol (DAG) which is increased during PI(4)P hydrolysis. C) Schematic of the potential interplay of PIS and LPP3 in cells when Golgi- β 1AR is stimulated, adding onto the findings in Nash et al., 2019 where stimulating specifically Golgi- β 1AR caused PI4P cleavage. PIS is green and bolded to represent upregulation, LPP3 is shown smaller and dim to represent downregulation, bolded black arrows represent an increase in that reaction pathway, non-bolded black arrows represent normal pathway activity, and light grey arrows represent an increase in that reaction pathway.

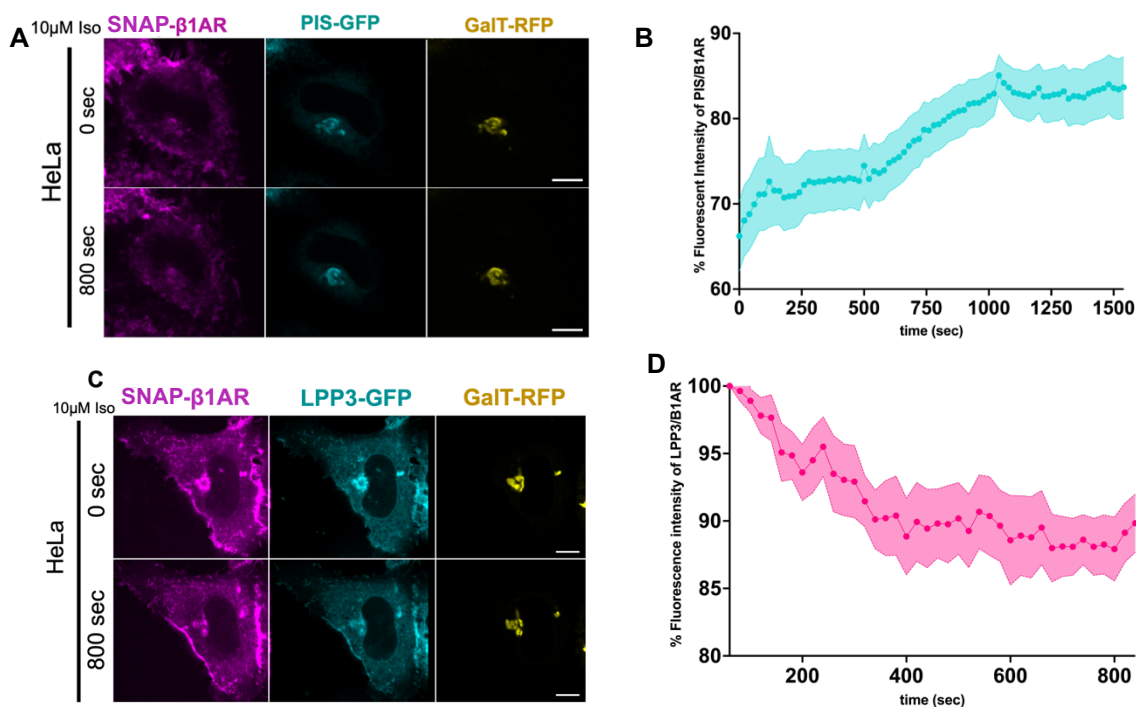


Figure 5.3 Golgi-APEX hits show changes in protein profiles involved in phospholipid and phosphoinositide pathways. A) Live cell imaging of HeLa cells expressing SNAP-β1AR (left panels), PIS fused to GFP (middle panels), and Golgi marker GalT-RFP (right panels). PIS-GFP is localized at the Golgi, as is β1AR. A slight increase in fluorescence of PIS-GFP is observed after 10 μM Iso stimulation. B) Quantification of PIS-GFP upregulation after β1AR activation in HeLa cells after 10 μM Iso addition; normalized fluorescence intensity of PIS-GFP at the Golgi relative to Golgi SNAP-tagged-β1AR (n=14 cells; one biological replicate). Fluorescence of PIS-GFP at the Golgi increases from ~70% to ~85% at the Golgi. C) Live cell imaging of HeLa cells expressing SNAP-β1AR (left panels), LPP3 fused to GFP (middle panels), and Golgi marker GalT-RFP (right panels). LPP3-GFP is localized at the Golgi and plasma membrane, as is β1AR. A slight decrease in fluorescence of LPP3-GFP is observed after 10 μM Iso stimulation. D) Quantification of LPP3-GFP downregulation after β1AR activation in HeLa cells after 10 μM Iso addition; normalized fluorescence intensity of LPP3-GFP at the Golgi relative to Golgi SNAP-tagged-β1AR (n=9 cells; one biological replicate).

5.6 Materials and Methods

cDNA constructs/plasmids

Lyn-APEX-GFP and APEX-GaIT-GFP

These constructs were gifts from the von Zastrow lab.

PIS-GFP and LPP3-GFP

PIS-GFP and LPP3-GFP were created by amplifying PIS and LPP3 from constructs bought from addgene using 5'- G CGC TAC CGG ACT CAG ATG CCA CCA TGC CAG ACG AAA -3'; 5'- T GGT GGC GAC CGG TGG ATC CTT GTC CTT CTT CTT GGC GCG -3' (PIS) and 5'- G GAC TCA GAT CTC GAG ATG CAA AAC TAC AAG TAC GAC AAA GC -3'; 5'- T GGT GGC GAC CGG TGG ATC CTT CAA CAT CAT GTT GTG GTG ATT GTT -3' (LPP3) primers and inserted into a GFP vector using XhoI and BamHI.

Cell culture and transfections

HeLa cells (purchased from ATCC as authenticated lines CCL-2) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. Cell cultures were free of mycoplasma contamination. Transfections were performed using Transit-LT1 transfection reagent (Mirus Bio) at a 1:3 DNA to transfection reagent ratio. SNAP-tagged human β 1AR construct was labeled with SNAP-cell 647 SiR (New England Biolabs, S9102S) at 1:1000 concentration for 20 min at 37°C in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4, washed with the same media (thrice for one minute, room temperature), and imaged in the same media.

Live-cell confocal imaging

Live-cell imaging was carried out using a Nikon spinning disk confocal microscope with a $\times 60$, 1.4 numerical aperture, oil objective and a CO₂ and 37°C temperature-

controlled incubator. A 488 nm, 568 nm and 640 Voltran was used as light sources for imaging mApple and SNAP-647 signals, respectively. Cells expressing SNAP-tagged receptor (1 μ g for wildtype β 1AR) and the indicated Mini-G probe (100 ng) were plated onto glass coverslips. Receptors were surface labeled by addition of SNAP-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Indicated agonists (isoproterenol, epinephrine, or dobutamine all from Sigma; BI167107 was a gift from Dr. Aashish Manglik) were added and cells were imaged every 20 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. Time-lapse images were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS Elements).

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (<http://rsb.info.nih.gov/ij>). For measuring kinetics of Mini-G probe recruitment at the plasma and Golgi membranes over time in confocal images, analyses were performed on unprocessed TIFF images using a previously published scripts written in MATLAB, available through open access on zenodo (Jullie D et al., 2020; Jullie D et al., 2022). Briefly, the plasma membrane and Golgi regions were selected and a mask of labeled receptor (using SNAP label) or Golgi marker were generated by thresholding the receptor or the Golgi marker signal within the selected region. The average fluorescence intensity of Mini-G was measured within the masked region and outside of the masked region, before and after addition of agonists. Values were normalized by calculating the percent relative to the maximum value, then

baseline corrected using GraphPad Prism v.9.5.1 (GraphPad) software so that the first value of each condition was set to 0.

Proximity labeling assay

Protocol was followed as in Lobingier et al., 2017. Briefly, β 1AR stable cell lines HEK293 cells were seeded onto 10 cm dishes and transfected with Lyn-APEX-GFP or APEX-GalT-GFP 24h after seeding. After 24h post-transfection, 10 μ L biotin phenol (500 mM stock) was added to each 10 cm dish for a final concentration of 500 μ M and incubated for 30 minutes at 37C. For Golgi-APEX samples, 10 μ M dobutamine was added 10 mins after the addition of biotin phenol to allow enough time for Golgi β 1AR stimulation (20mins). For plasma membrane-APEX samples, 10 μ M dobutamine was added 25 mins after biotin phenol addition to allow for plasma membrane- β 1AR stimulation (5 mins). After the biotin phenol and dobutamine incubations, dishes were taken out of 37C and 5 mL of media was removed from each dish. 5 mL of a 2 mM H₂O₂ media mixture was added to each dish for a final 1 mM H₂O₂ concentration. Cells were incubated with light agitation for 1 minute before H₂O₂ media was aspirated. Cells were washed 3x with 5 mL ice cold quenching buffer (TBS supplemented with 1mM CaCl₂, 10 mM sodium ascorbate, 1 mM sodium azide, and 1 mM Trolox). An additional 5 mL quenching buffer are added to the cells and left on ice for 20 mins. For western blotting, quenching buffer is removed and cells were lysed with 500 μ L RIPA for each plate. Samples are collected in Eppendorf tubes and sonicated and spun at 10,000xG for 10 min. Supernatant is added to streptavidin agarose resin and rotated overnight at 4C. Western blot was performed as described in chapter 2 materials and methods and a GFP primary antibody was utilized followed by an HRP secondary antibody. For sample that are immediately sent of for

mass spectrometry, cells are gently scraped in quenching buffer following the 20 min ice incubation, placed in 15 mL conical tubes and spun at 500xG for 5 mins. Quenching buffer is then aspirated from pellet and cells are flash frozen until further use. For more details on mass spectrometry, see materials and methods in Lobingier et al., 2017.

APEX statistics

This was performed by our collaborators in Dr. Nevan Krogan's laboratory by Dr. Qiongyu Li under the leadership of Dr. Ruth Huttenheim. For more details on APEX-MS statistical analysis, see materials and methods in Lobingier et al., 2017.

5.7 References

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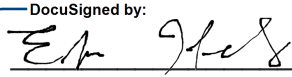
5.8 Acknowledgements

I thank Drs. Qiongyu Li, Ruth Huttenhaim, Nevan Krogan for performing the mass spectrometry and proteomic analysis. I also thank Drs. Shaeri Mukherjee and Jeremy Reiter for helpful discussion regarding this project in my first thesis committee meeting.

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