Title
Elucidating the regulation of adenylyl cyclase type 9 by dynamic membrane trafficking

Author
Lazar, Andre Mingyuen

Publication Date
2019

Peer reviewed|Thesis/dissertation

Elucidating the regulation of adenylyl cyclase type 9 by dynamic membrane trafficking

by
André M. Lazar

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

Approved:

DocuSigned by:
Mark von Zastrow
A4DF1018F0A2414...

Mark von Zastrow
Chair

DocuSigned by:
Dyche Mullins
DocuSigned by:
Jack Taunton
0FD9D71201FC43C...

Dyche Mullins

Jack Taunton

Committee Members

Dedication and Acknowledgements

I am incredibly grateful for all the people who have helped me throughout my graduate career. Naturally the person who most influenced my experience in graduate school was my PI, Mark von Zastrow. Mark is the kind of scientist who is in basic research for the right reasons. His boundless energy and enthusiasm are infectious, and his willingness to pursue the hardest questions is what separates him from his peers. As a mentor, Mark has the rare ability to find the balance of guidance and freedom that each student needs to flourish. He is able to maintain a rigorous work ethic while not taking himself too seriously, which is something I hope to achieve one day.

I will forever be thankful for the help and support I received from other members of the von Zastrow lab, both past and present. First and foremost, I must thank Roshanak Irannejad, who mentored me throughout my first few years in the lab. Roshanak is a fantastic role model who is always reading and learning. She demonstrates and expects technical excellence in experimentation, and I would not be half the scientist I am today without her. Roshanak is a true joy to learn from and collaborate, and she has a big heart and a life full of fascinating stories. It is hard to imagine anyone more deserving of success and I am incredibly proud of her. She's started her own lab at UCSF and I could not recommend her more highly to incoming students.

The next person I must thank may surprise her, but Nina Tsvetanova was someone who always believed in the validity of my data and the importance of my project. She has high expectations for herself and others, and is a great person to have in your corner.

Braden Lobingier is a devoted scientist whose detail oriented nature makes him the consummate academic. He was always willing to help me troubleshoot my experiments; as the ‘Reviewer #2’ of the lab, his creativity for experimental controls is matched only by his impressive knowledge of several fields of literature. Miriam Stoeber is an incredibly talented scientist, and the depths of her empathy and social graces make her a standout amongst the scientific community. Damien Julie is a multi-talented experimentalist with creative approaches that proved invaluable for tackling the hardest problems. Yasunori Uchida is a great friend whose steady presence and quick laugh helped me through many difficult days. Thanks also to Erica Sanchez for being a ray of sunshine blasting through the clouds of cynicism in my last months of graduate school, Lea Ripoll for her chill vibes, and Aliza Ehrlich for her infectious smile.

Thanks also to Dyche Mullins, Jack Taunton and Henry Bourne for their valuable insights as my thesis advisors and mentors. Dyche is full of good cheer, and impressed me every time we spoke with his well rounded, comprehensive perspectives and absolute rigor in experimental design. He supported and encouraged me while also holding me to a high standard, and I am grateful for that balance and glad he was on my committee. Jack is one of the smartest people I’ve ever met and constantly pushed me to be my best. I knew I needed him on my committee for two reasons: his incisive criticism and his willingness to be fully engaged with my project. Henry, our resident the professor emeritus and unofficial UCSF historian shared with me his quick wit, outrageous stories, and scientific perspectives. His energy, good humor and enthusiasm made me feel included in the UCSF community.

My fellow graduate students, both inside and outside the von Zastrow lab, were constant and true companions. Alison Leaf took me under her wing before I even joined the lab. Her unique combination of ebullience and direct, in your face approach made her a joy to be around. Kate Varandas is one of the most empathetic people I know and I thank her for her kindness. Kelsie Eichel is a brilliant scientist and her sense of humor cracked me up many times throughout the years. Grace Peng and I joined the von Zastrow lab together and she had my back the whole time. Grace is a great labmate and a better friend to those lucky enough to call her one. Ben Barse-Rhyne is always willing to share his time, his opinion, and his good vibes, and I'm grateful for all of them. Joy Li is a kind and loving person who is always thinking of others first.

Thanks also to my fellow Tetrad classmates Candace Britton, Fernando Meza Gutierrez, Johnny Rodriguez, Joel Hrit, and Kelly Crotty. Together we made the first couple years of graduate school a blast and were able to help and support each other through the good times and bad. Candace and I share the exact same sense of humor and pop culture tastes, and our friendship was born out of our passion for the skits we made in our second year. I lived with Fernando, Johnny, and Joel for a year and it was awesome to be part of the Hufflepuff House even though I'm obviously a Gryffindor. Thank you Kelly for your good cheer, patience, and expansive movie knowledge. I will always remember and be thankful for all the fun times we all shared.

None of this would be possible without the help and support of my family. My parents, Aurel and Paik-kee, have always helped me construct and pursue my goals, cheered me on, and pushed me to do my best. Papa has always been a willing sounding board;

sometimes I wondered if he was more interested in my project than I was! He is a dedicated intellectual and I've learned the joys of puzzle solving from him. Mama has always fought to make sure I am a well-rounded person who has a life outside of work and interests in the world around me. She instilled in my sister and I the value of family and taught us how to treat our fellow human beings. Thank you both for always having faith that I would figure things out, even when I wasn't so sure I could. Thanks also to my little sister Katja, from whose personal journey I have learned so much about my own. Thank you Katja for always having my back, for all the times you called me to catch up, and for unabashedly being your true self. Thanks for being a great sister and a better friend. I'm proud to be your older brother and to help you through medical school as much as you helped me through graduate school.

While my immediate family was in New York, my aunt Paik-Swan, my uncle Steve, and my cousins Audrey and Lauren were here in San Francisco. They were the reason I chose to come to UCSF and I'm really glad I did. My aunt and uncle are some of the warmest, most generous people I've met and I genuinely looked forward to going to their house for dinner every week or so. They were always around when I needed them, and were my home away from home for seven years. I watched Audrey grow up through middle and high school, tackling tons of challenges along the way. I couldn't be more proud of the elegant young lady she has become, and I am glad to have been a part of her life during these important years. Lauren has always been as much a sister as a cousin, and growing up with her has been a pleasure. Her distinctive laughter is infectious, and her moral clarity and raw intellect are reassuring in a political environment which seems devoid of such necessary and valuable traits.

While I have family on both the east and west coasts on whom I can rely, I also gained a new family. I met Haley Rose Smith in the summer of my second year of graduate school and quickly fell head over heels for her. Haley has the biggest heart of anyone I've ever met, and truly and deeply cares for others. She has taught me so much about the rapidly changing world outside of academia, and I am forever grateful to have been so quickly welcomed into her family, including Dave, Denise, Campbell and the whole Smith and White clans. I've always told her that I'll never quite have the words to fully describe how much I love her, but here are a few: Haley, you are a shining light in a dark world, my best friend, and an astoundingly loving and devoted partner. If I am the rock that holds you steady, you are the wind that lifts me up and carries me to new heights. It is to you that I dedicate this thesis, as without you I never would have come this far.

Abstract

Elucidating the regulation of adenylyl cyclase type 9 by dynamic membrane trafficking

by

André M. Lazar

This dissertation delves into the regulatory mechanisms that govern the dynamic redistribution of adenylyl cyclase type 9 (AC9) from the plasma membrane to the early endosome. Adenylyl cyclases (ACs) are 12-transmembrane proteins that catalyze ATP to the second messenger molecule, cyclic AMP. ACs are critical effectors of G protein activation, and AC9 is the first AC isoform described at the endosome. The introduction provides a general overview of the relationship between G protein coupled receptor (GPCR) mediated signaling and membrane trafficking. It also describes the evidence supporting GPCR-mediated signaling from the endosome, the importance of local cAMP signaling, and regulatory mechanisms that govern adenylyl cyclase isoforms. The second chapter explores the regulated endocytosis of AC9 and its contribution to a B2AR-mediated cAMP response from the endosome. We show that AC9 internalization is a result of Gs-coupled receptor activation, but more specifically that Gs activation is necessary and sufficient for this process. The third chapter describes the troubleshooting of the cell culture and fixation protocol, and ultimately determines that AC9 trafficking is sensitive to acute exposure to a negative CO2 gradient. The fourth chapter is a discussion of these findings, future directions and implications on the field.

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 1:

Introduction

1.1 Overview

This thesis focuses on the Adenylyl Cyclase type 9 (AC9), one of the nine isoforms of transmembrane adenylyl cyclases that are expressed in mammals. Adenylyl cyclases are critical effector proteins that amplify a signal that is initially detected by a G protein Coupled Receptor (GPCR). As such, the regulation of adenylyl cyclase activity is often a pivotal step in several cellular responses. The introduction chapter provides a general overview of the current model of the GPCR, G protein, and adenylyl cyclase signaling cascade, and how this relates to membrane trafficking within endocytic network. It aims to provide the proper context for the present understanding of receptor mediated signaling from various locations in the cell, as well as to present the outstanding questions in the field regarding the action of effector molecules from these locations.

1.2 Early exploration of GPCR signaling and membrane trafficking

G-protein coupled receptors constitute the largest family of signaling receptors and integral membrane proteins in mammals (Holst and Schwartz 2002). GPCRs are a major focus of pharmaceutical drug targets, with an estimated one-third of all drugs in clinical use targeting GPCRs (Hauser et al. 2017). These receptors are seven-transmembrane proteins that are inserted into the endoplasmic reticulum membrane during translation. They undergo post-translational modifications at the endoplasmic reticulum and Golgi apparatus including glycosylation, which is necessary for structural integrity and downstream signaling. GPCRs are so-named because many of their downstream signaling effects are transduced by heterotrimeric GTP-binding proteins (G proteins) to effector proteins (Weis and Kobilka 2018). Ligand binding results in stabilization of the receptor in a conformation that promotes allosteric coupling to the G protein (Weis and Kobilka 2018). This receptor-G protein coupling results in nucleotide exchange from GDP to GTP by the G protein, commonly termed as activating the G protein, allowing it to engage downstream effectors, thus propagating the signal (Hepler and Gilman 1992; Hilger, Masureel, and Kobilka 2018).

Early evidence that GPCR signaling undergoes regulation by membrane trafficking was discovered using the beta-2 adrenergic receptor (B2AR) as a model. These receptors recognize epinephrine in the bloodstream and are pivotal in mammalian adrenal response. B2ARs couple to the stimulatory G protein (Gs), which promotes adenylyl cyclase (AC) activity (Busto et al. 2000). The B2AR-Gs-AC signaling cascade was used

to demonstrate a desensitization effect in which larger doses were required to stimulate the same scale response, as measured by elevated cyclic adenosine monophosphate (cAMP) levels in the cell (Su, Harden, and Perkins 1979). Short term agonist exposure resulted in reversible reduction in cAMP production without a commensurate reduction in total number of receptors detected in whole cell lysates (Su, Harden, and Perkins 1979). Prolonged agonist exposure resulted in downregulation of receptor number that required new protein synthesis to recover (Doss, Perkins, and Harden 1981).

A nondestructive process of receptor ‘sequestration’ was detected by subcellular fractionation and radioligand-receptor binding assays (Alho et al. 1988; Chuang and Costa 1979; Staehelin and Simons 1982). This was initially described as a cycle in which agonist induced receptor redistribution from the plasma membrane fraction to the ‘soluble’ (later defined as endosome) fraction (von Zastrow and Kobilka 1992; Waldo et al. 1983). This sequestration in the soluble fraction eventually reversed, and this iteration between the plasma membrane and endosomes was found to correlate with receptor phosphorylation by GPCR kinases (GRKs) (Benovic et al. 1989). Receptor phosphorylation was also shown to attenuate receptor-G protein coupling *in vitro (Sibley et al. 1985)*, and as a result, a model in which ligand binding resulted in receptor phosphorylation and sequestration in the endosome limiting membrane was proposed (Gagnon, Kallal, and Benovic 1998).

Receptor phosphorylation was also found to promote interaction with arrestin (or beta-arrestin) proteins (Gagnon, Kallal, and Benovic 1998; Goodman et al. 1996). Arrestin was so-named because receptor engagement with arrestin correlated with a G protein-dependent signal arrest as detected by cellular cAMP levels (Ferguson et al. 1996; M. J. Lohse et al. 1990). Arrestin was demonstrated to be an endocytic adaptor protein that also bound to the clathrin lattice structure and PIP2, promoting the accumulation of B2ARs in clathrin coated pits (CCPs) (Gaidarov et al. 1999). Accumulation of receptors in CCPs was shown to be critical for endocytosis to the early endosome as well as for proteolytic downregulation in lysosomes.

1.3 An evolving view of the endosome

B2ARs are a common model both in the field and in the von Zastrow lab. Due to the historical exploration of B2AR membrane trafficking and its G protein dependent signaling, much of this system is well defined, and numerous tools are available, making it an excellent model for basic research. These tools include pharmaceutical drugs and mutants that have a wide range of effects on both signaling and trafficking of the receptor, including both agonist and antagonist effects. These tools have helped define a general model for GPCR signaling and regulation by membrane trafficking.

Central to the 'traditional model' of GPCR signaling and trafficking that was developed are these key points: 1. Ligand-induced activation of GPCRs and G proteins is initiated at the plasma membrane and 2. GPCR endocytosis is associated with events that reduce or terminate G protein activation. The commonly held belief for many years was thus that the plasma membrane was the locus of receptor-mediated signaling in the cell, and the endosome represented a pool of inactive receptors that were sequestered from further stimulation (desensitization).

The endosome has since been described beyond a sequestration mechanism. Published works from the von Zastrow lab as well as several others have explored the role of the endosome as the unit of the endocytic network. Indeed, the endosome is now understood to represent a mobile site of cargo sorting, from which receptors can be delivered to the plasma membrane (recycling) (von Zastrow and Kobilka 1992),

lysosome (degradation) (Henry et al. 2011), and the endoplasmic reticulum or Golgi apparatus (Varandas, Irannejad, and von Zastrow 2016). The endosome has also been shown to be the site of rapid receptor dephosphorylation, and G protein independent signaling from the endosome. The early endosome is thus the locus of the dynamic regulation of active and inactive receptors that can be scaffolded into signaling complexes and sorted to new locations in the cell.

In particular the discovery of the retromer complex and retromer tubule, where much of the cargo sorting decisions are made, has greatly expanded the understanding of receptor regulation by membrane trafficking (Lauffer et al. 2010; Temkin et al. 2011). Retromer binding motifs on GPCR tails allow them to gain entry into the retromer tubule (Lauffer et al. 2010), a cilia-like protuberance from the endosome that is defined by the retromer complex ring at its base and along its shaft that warps the lipid bilayer as well as selects cargo (Varandas, Irannejad, and von Zastrow 2016). This tubule will eventually undergo scission from the early endosome and can be targeted to specific locations in the cell, allowing for directed delivery based on cargo identity (Temkin et al. 2017; Choy et al. 2014).

In addition to cargo sorting, the endosome has now been described as the site for receptor-mediated signaling. As the early model described the arrest of G protein dependent signaling by phosphorylation, arrestin binding, and endocytosis, the initial focus on receptor signaling from the endosome was largely G protein independent.

Arrestin mediated signaling from the plasma membrane and endosome has been well described by several studies (Varandas, Irannejad, and von Zastrow 2016; Eichel, Jullié, and von Zastrow 2016; DeFea et al. 2000). Certain GPCRs have been characterized as remaining engaged with arrestins at the endosome well into the maturation of the endosome (Shenoy and Lefkowitz 2011). Indeed, certain Class B GPCRs have been described by the Lefkowitz lab as forming a Super Complex with arrestin and G protein (Thomsen et al. 2016). Whether this ‘megaplex’ is necessary for all ligand-induced, receptor-mediated G protein dependent signaling, remains unclear at this time.

1.4 Evidence supporting GPCR-mediated signaling at the endosome

There is now substantial evidence that endosomes are also the site of receptor-mediated G protein dependent signaling. Several different approaches have been supportive of the idea that GPCRs can activate G protein dependent signal cascades at the endosome, but none have been definitive. The three general categories of approach can be defined by their targets: 1. Disruption of receptor-ligand binding (either by agonist washout or competition with permeable or impermeable antagonists), 2. Inhibition of receptor trafficking (either by mutation or by inhibitor treatments), or 3. Detection of active-conformation receptor and G protein by conformation-specific single-chain antibodies (nanobodies).

Disruption of ligand binding by washout revealed a persistent cellular cAMP response (Calebiro et al. 2009; Ferrandon et al. 2009; Thomsen et al. 2016; Stoeber et al. 2018), and the comparison of membrane permeant and impermeant antagonists showed an incomplete effect by the impermeant antagonist (which is unable to rapidly access the endosome lumen) relative to the permeant antagonist (Thomsen et al. 2016; Stoeber et al. 2018). These suggested that GPCRS could mediate a cellular response post-endocytosis. In chapter 2, we use the comparison of permeant and impermeant antagonists as an approach to demonstrate B2AR-mediated signaling from the endosome.

Endocytic blockade of the B2AR was achieved in two main ways: genetic or chemical. Genetic manipulation included the mutation of key tail residues that are phosphorylated and thus necessary for arrestin binding, expression of a dominant-negative form of dynamin (necessary for scission of a clathrin coated pit from the plasma membrane), and knockdown of clathrin. Chemical manipulation included treatment with small molecule inhibitors that inhibit the activity of key proteins necessary for endocytosis (DYNGO-4a is used to block dynamin activity in this study), or use of agonists for the B2AR that induce a cAMP response via Gs but do not promote B2AR endocytosis. Both genetic and chemical blockade of receptor endocytosis showed that restriction of the receptor to the plasma membrane reduced the overall response rather than increasing it (Calebiro et al. 2009; Ferrandon et al. 2009; Lyga et al. 2016; Irannejad et al. 2013). This was consistent with the idea that there is a cellular response originating at the endosome as well as the plasma membrane.

Finally, the most recent evidence that GPCRs can mediate a signal that is transduced by G protein at the endosome used nanobody biosensors. These single-chain antibodies, cloned into an EGFP vector, were used to demonstrate that B2ARs could respond to a membrane permeant agonist isoproterenol both at the plasma membrane and the endosome (Irannejad et al. 2013). Typically dispersed throughout the cytoplasm, agonist stimulation of the B2AR resulted in the rapid recruitment of Nb80-EGFP, which recognizes the ‘active conformation’ of the receptor, to the plasma membrane, and then to the endosome (Irannejad et al. 2013). Further, Nb37-EGFP,

which preferentially binds to the nucleotide-free state of the Gs protein, was recruited to the endosome after treatment with isoproterenol (Irannejad et al. 2013). These data are consistent with the idea that there is ligand-receptor binding at the endosome and that this results in G protein activation. Similar strategies were used in the context of the Mu-Opioid Receptor (Stoeber et al. 2018) and Thyroid Stimulating Hormone Receptor (A et al. 2018)

Proponents of endosome signaling favor a model in which the endosome functions to trap ligand within the lumen, raising or at least preserving the effective agonist concentration (Bowman, Shiwarski, and Puthenveedu 2016). This promotes ligand rebinding after dissociation from the receptor, consistent with the sustained response model. Additionally, GPCR-G protein activation at the endosome can preferentially promote a GPCR-dependent transcriptional response that is absent from cells in which GPCR-G protein activation occurs only at the plasma membrane (Tsvetanova and von Zastrow 2014; Calebiro et al. 2009; Jean-Alphonse et al. 2014).

Detractors of endosome signaling point to the rapid diffusion that the small molecule cAMP exhibits in the cell. The source of the cAMP does not affect the structure of the molecule, so it can be difficult to see why an effector at another cellular location might differentiate between cAMP that originates from the plasma membrane or from the endosome. It remains unclear whether the rise in overall cAMP concentration in the cell is relevant to a cellular response, or if local generation of high-concentrations cAMP

(acute cAMP gradients) is of more importance. Scaffolding of signaling molecules is a common phenomenon in signal cascades, and it has been shown that enforced proximity enhances local signaling by cAMP (Agarwal, Clancy, and Harvey 2016; C. Lohse et al. 2017; Esseltine and Scott 2013). Local phosphodiesterase (PDE) activity can also create a low local cAMP concentration, potentially buffering against a rise in global cAMP levels nearby relevant effectors.

1.5 AKAPs confer spatial and effector specificity on cAMP signaling

Spatial compartmentalization of cAMP-dependent signaling is accomplished in part by A-Kinase Anchoring Proteins (AKAPs) (Dessauer 2009). AKAPs perform a scaffolding function, coordinating several critical signaling molecules into close proximity (Gros et al. 2006). This typically includes Protein Kinase A, a primary effector of cAMP in many cell types, and phosphodiesterases (PDEs) which degrade cAMP, reducing local concentration (Davare 2001). AKAPs can also be involved in the scaffolding of GPCRs, G proteins, and ACs, and several effectors including calcium channels in neurons (Davare 2001). AKAPs can thus link individual signaling pathways through forced proximity, with clinical relevance. Of great importance is the fact that each AKAP only binds certain AC isoforms, and exhibit differential subcellular localization patterns.

There is substantial evidence that the location of cAMP production is relevant for its downstream effects. Initiation of a cAMP response from the plasma membrane and a response from endosomes or cytoplasm have been demonstrated to have differential results (Tsvetanova and von Zastrow 2014). It is now clear that AKAPs and their preferential binding of different ACs and effectors is a mechanism for conferring specificity of outcome upon a universal signal in cAMP (Esseltine and Scott 2013). Local production of cAMP by ACs is necessarily more relevant to AKAP-bound effectors than not. Furthermore, PDE activity (coordinated by AKAPs) may function to either filter out ‘noise’ of a rise in cytoplasmic cAMP or even inhibit the spread of cAMP from AKAP-bound ACs (Smith et al. 2006). Coordination of signaling cascades by AKAPs at

various locations in the cell represents a more dynamic model of GPCR-mediated signaling. Although there is a massive diversity in GPCRs, G proteins, downstream effectors, and even nine AC isoforms, cAMP remains unchanged throughout a wide variety of signaling pathways. AKAPs are necessary for the recovery of input-output specificity after a receptor-mediated signal is transduced through a molecule that retains no inherent information.

To our knowledge, only AKAP79/150 has been described in EEA1+ early endosomes so far. Palmitoylation of two conserved cysteine residues (C36 and C129) on the N-terminal tail of AKAP79/150 promotes association with lipid rafts and Rab11-positive endosomes in Cos7 cells and hippocampal neurons (Keith et al. 2012). This palmitoylation was also found to promote targeting of PKA and calcineurin to endosomes in these cells (Delint-Ramirez et al. 2015). Further, AKAP79/150 palmitoylation is required for synaptic homeostasis and long term potentiation in hippocampal neurons (Keith et al. 2012). It is clear that targeting of this scaffolding protein to the endosome has large functional relevance, and strongly implicates the coordination of a cAMP response with local effectors at the endosome.

1.6 Adenylyl Cyclases

The GPCR-G protein-Adenylyl Cyclase-cAMP signal cascade one of the most highly studied signal transduction pathways in mammals. Despite a great diversity of receptors and eventual downstream effects, cAMP responses always involve receptor and G protein coupling, and G protein regulation of adenylyl cyclase activity (Sunahara, Dessauer, and Gilman 1996). This basic structure has been described in a vast number of biological processes including (but not limited to): oogenesis, embryogenesis, larval development, hormone secretion, glycogen breakdown, smooth muscle relaxation, cardiac constriction, olfaction, and learning & memory (Sadana and Dessauer 2009). Insights into the structure and function of adenylyl cyclases have come from several fields, and basic research progress has an intimate connection with pharmaceutical drug development.

Adenylyl cyclases are ATP-pyrophosphate lyase which converts ATP to cyclic AMP and pyrophosphate (Gilman 1989). Nine mammalian isoforms of transmembrane adenylyl cyclases have been defined, and a tenth ‘soluble’ form (sAC) has been recognized (Sadana and Dessauer 2009). All transmembrane ACs share a similar topology: two repeats of a 6-transmembrane anchor domain followed by a catalytic domain in the cytoplasm (Krupinski et al. 1989). The cytoplasmic domains (C1 and C2) are over 40% identical in both primary sequence and tertiary structure. C1 and C2 are highly conserved across isoforms, and interact with each other to create a pseudosymmetric site that can be regulated by different factors. C2 differs from C1 in that it binds the

stimulatory G protein (Gs), which promotes catalytic activity, while C1 can (depending on the isoform) bind the inhibitory G protein (Gi) (Taussig, Iniguez-Lluhi, and Gilman 1993).

All transmembrane AC isoforms are sensitive to Gs. There are four classes of AC isoforms that have been defined based on the regulatory mechanisms that they are sensitive to. Group 1 includes AC1, AC3, and AC8. These ACs are also stimulated as a result of elevated levels of Ca^{2+} through calmodulin (Choi, Xia, and Storm 1992). Group 2 includes AC2, AC4, and AC6, all of which are stimulated by $G_{\beta\gamma}$ but not by Gi (Tang and Gilman 1991). Group 3 constitutes AC5 and AC6, which are sensitive to both $G_{\beta\gamma}$ and Gi, and are inhibited by an increase in free Ca^{2+} (Tang and Gilman 1991). Last but not least, Group 4 includes AC9 which is stimulated by Gs but not Gi, inhibited by calcineurin and Protein Kinase C, and is unique amongst AC isoforms in that it is relatively insensitive to forskolin (FSK), a diterpene molecule that promotes AC activity in a G protein independent manner in ACs 1-8 .

AC isoforms are differentially expressed through mammals. AC1 is expressed in brain cells and in the adrenal medulla, and has been demonstrated to be involved in learning, memory, synaptic plasticity, and opiate withdrawal (Xia et al. 1993; Tzavara et al. 1996; Wu et al. 1995). AC2 is expressed in brain, lung, skeletal muscle, and heart tissues. AC3 is most associated with cilia and olfaction, and is found in the olfactory epithelium, pancreas, brain, heart, lung, and testes tissues (Z. Wang et al. 2006; Zou et al. 2007).

AC5 and AC6 are largely expressed in similar tissues, including heart, kidney, lunch, testis, and adrenal medulla (Espinasse et al. 1995). AC8 is expressed in brain, lung, pancreas, testis and adrenal medulla (H. Wang et al. 2003). AC4, AC7, and AC9 display widespread expression throughout cell types, and AC9 has been found to be involved in B2AR-mediated signaling in lung tissue and smooth muscle airway tissue more generally (Small et al. 2003).

There are several studies that have suggested the involvement of specific AC isoforms in a cAMP response originating from endosome-localized GPCRs. Parathyroid Hormone Receptor (PTHR) has been shown to mediate a sustained signal from the endosome and has functional coupling with the B2AR (Ferrandon et al. 2009). PTHR appears to specifically activate AC2 post endocytosis, as demonstrated by selective inhibition of AC2, and overexpression of AC2 (Ferrandon et al. 2009). Soluble AC (sAC) has been implicated in the propagation of a cAMP signal that is endocytosis dependent. However, studies that identify subcellular localization of AC isoforms within the same cell type and across cell types are few and far between. This is partially hindered by the lack of highly specific antibodies that can differentiate between AC isoforms, and that endogenous expression of ACs is typically fairly low (limiting CRISPR-based tagging). As such, the subcellular localization of AC isoforms, and more specifically, whether they are present at the endosome, is poorly described.

1.7 Adenylyl Cyclase Type 9

Adenylyl Cyclase Type 9 is widely expressed throughout cell types, including the central nervous system and heart (Berndt et al. 2013; Paterson et al. 1995). Knockdown studies have provided clear evidence that AC9 plays a role in neutrophil chemotaxis and cardiac function (Li et al. 2017). A polymorphism of the ADCY9 gene that encodes AC9 disrupts B2AR mediated signaling and is associated with risks for asthma, mood and body weight disorders (Small et al. 2003; Berndt et al. 2013). AC9 associates with the AKAP Yotiao and is coupled to I_{Ks} channel activity and cardiac repolarization (Li et al. 2012). Despite its clinical relevance, AC9 is perhaps the least studied of the AC isoforms and conflicting reports of its regulation have hindered progress in building a model for its function.

AC9 is a Gs-regulated effector with several unique properties. AC9 is the most phylogenetically distant isoform and has been described to have an autoinhibitory domain in the C2b catalytic unit that is not shared amongst AC isoforms (Pálvölgyi et al. 2018). Despite this autoinhibition, AC9 has also been described to have the highest basal activity amongst AC isoforms (Baldwin et al. 2019). AC9 was initially classified as the only Group 4 AC isoform, having been described as sensitive to inhibition by calmodulin and PKC, but not Gi or $G_{\beta\gamma}$ (Dessauer et al. 2017).

However, there are several conflicting reports of AC9 regulation, which may be explained by the discovery that AC9 can both homodimerize and heterodimerize with

AC5 and AC6 (Baldwin et al. 2019). The most recent study of AC9 regulation done *in vitro* found that AC9 is insensitive to direct inhibition by calmodulin, PKC, Gi and $G_{\beta\gamma}$ (Baldwin et al. 2019). It remains possible that AC9 activity can be indirectly affected by these mechanisms *in vivo* through heterodimerization with other AC isoforms, likely with cell type specificity. All other AC isoforms are either stimulated (AC2, 4, 5, 6, 7) or inhibited (AC 1, 3, 8) by $G_{\beta\gamma}$ through either direct or indirect means (sequestration of Gs), and it has been shown that $G_{\beta\gamma}$ indirectly activates AC9 in neutrophils. PKC and calmodulin activity may need to be scaffolded (possibly by an AKAP) to facilitate phosphorylation of AC9, or may act on another protein which can regulate AC9 in cells.

AC9 is also unique amongst AC isoforms in its relative insensitivity to the diterpene forskolin. Forskolin binds to all AC isoforms and stimulates cAMP production in a G protein independent manner (Qi et al. 2019). AC9 has been shown to bind FSK and even exhibit enhanced catalytic activity as a result, but this is contingent on engagement with Gs (Qi et al. 2019; Baldwin et al. 2019). Furthermore, AC9 has high basal activity relative to other ACs and requires a higher concentration of activated Gs to increase its catalytic activity (Baldwin et al. 2019). This high basal activity appears to be important for its physiological roles, particularly in cardiac function, as AC9 knockout in mice resulted in bradycardia and diastolic dysfunction (Li et al. 2017).

AC isoforms have typically been assumed to be present at the plasma membrane for the bulk of their functionally relevant activity. Initial observations in other cells types

showed AC9 localization to the plasma membrane generally. AC9 is most typically associated with the AKAP Yotiao (encoded by the AKAP9 gene), which has been described as localized to the plasma membrane thus far (Li et al. 2012). However, AC9 has been described at the Golgi apparatus in Cos7 cells, and was found to be necessary for trafficking of a GPCR-like receptor (KDELR) between the ER and Golgi (Cancino et al. 2014). In addition, a 2013 mass spectrometry study by the Cullen lab in which retromer components SNX27 and VPS35 were knockdown showed surface depletion of AC9 (amongst many other hits, but no other AC isoforms) (Steinberg et al. 2013). This suggests that AC9 can both be present at the endosome and be sorted into recycling tubules by the retromer complex.

In summary, the landscape of receptor-mediated cAMP signaling has evolved from a static model in which the plasma membrane is the locus of a massive cAMP gradient that rapidly diffuses to secondary targets in the cell. We now have a dynamic model which membrane trafficking can regulate receptor-mediated signaling from various locations in the cell with differential outcomes. A critical outstanding question is whether Gs regulated effectors are present in the endocytic network, and how they are coordinated into proximity with GPCR-G protein activation. This thesis focuses largely on adenylyl cyclase type 9 (AC9). We show that agonist engagement of the B2AR results in the redistribution of AC9 from the plasma membrane to the early endosome, and characterize this process as well as describe AC9 as a major contributor to the B2AR-mediated cAMP response from the endosome in HEK293 cells.

1.8 References

Agarwal, Shailesh R., Colleen E. Clancy, and Robert D. Harvey. 2016. “Mechanisms Restricting Diffusion of Intracellular cAMP.” *Scientific Reports*. https://doi.org/10.1038/srep19577.

A, Godbole, A. Godbole, S. Lyga, M. J. Lohse, and D. Calebiro. 2018. “Internalized TSH Receptors En Route to the TGN Induce Local Gs-Protein Signaling and Gene Transcription.” *Yearbook of Paediatric Endocrinology*. https://doi.org/10.1530/ey.15.3.3.

Alho, H., O. Dillion-Carter, C. P. Moxham, C. C. Malbon, and D. M. Chuang. 1988. “Changes in Immunohistochemical Properties of Beta-Adrenergic Receptors in Frog Erythrocytes by Isoproterenol-Induced Desensitization.” *Life Sciences* 42 (3): 321–28.

Baldwin, Tanya A., Yong Li, Cameron S. Brand, Val J. Watts, and Carmen W. Dessauer. 2019. “Insights into the Regulatory Properties of Human Adenylyl Cyclase Type 9.” *Molecular Pharmacology*. https://doi.org/10.1124/mol.118.114595.

Benovic, J. L., A. DeBlasi, W. C. Stone, M. G. Caron, and R. J. Lefkowitz. 1989. “Beta-Adrenergic Receptor Kinase: Primary Structure Delineates a Multigene Family.” *Science* 246 (4927): 235–40.

Berndt, Sonja I., Stefan Gustafsson, Reedik Mägi, Andrea Ganna, Eleanor Wheeler, Mary F. Feitosa, Anne E. Justice, et al. 2013. “Genome-Wide Meta-Analysis Identifies 11 New Loci for Anthropometric Traits and Provides Insights into Genetic

Architecture.” *Nature Genetics* 45 (5): 501–12.

Bowman, Shanna Lynn, Daniel John Shiwarski, and Manojkumar A. Puthenveedu. 2016. “Distinct G Protein–coupled Receptor Recycling Pathways Allow Spatial Control of Downstream G Protein Signaling.” *The Journal of Cell Biology* 214 (7): 797–806.

Busto, R., I. Carrero, P. Zapata, B. Colás, and J. C. Prieto. 2000. “Multiple Regulation of Adenylyl Cyclase Activity by G-Protein Coupled Receptors in Human Foetal Lung Fibroblasts.” *Regulatory Peptides* 95 (1-3): 53–58.

Calebiro, Davide, Viacheslav O. Nikolaev, Maria Cristina Gagliani, Tiziana de Filippis, Christian Dees, Carlo Tacchetti, Luca Persani, and Martin J. Lohse. 2009. “Persistent cAMP-Signals Triggered by Internalized G-Protein-Coupled Receptors.” *PLoS Biology* 7 (8): e1000172.

Cancino, Jorge, Anita Capalbo, Antonella Di Campli, Monica Giannotta, Riccardo Rizzo, Juan E. Jung, Rosaria Di Martino, et al. 2014. “Control Systems of Membrane Transport at the Interface between the Endoplasmic Reticulum and the Golgi.” *Developmental Cell* 30 (3): 280–94.

Choi, E. J., Z. Xia, and D. R. Storm. 1992. “Stimulation of the Type III Olfactory Adenylyl Cyclase by Calcium and Calmodulin.” *Biochemistry* 31 (28): 6492–98.

Choy, Regina Wai-Yan, Minjong Park, Paul Temkin, Bruce E. Herring, Aaron Marley, Roger A. Nicoll, and Mark von Zastrow. 2014. “Retromer Mediates a Discrete Route of Local Membrane Delivery to Dendrites.” *Neuron*. https://doi.org/10.1016/j.neuron.2014.02.018.

Chuang, D. M., and E. Costa. 1979. “Evidence for Internalization of the Recognition Site of Beta-Adrenergic Receptors during Receptor Subsensitivity Induced by (-)-Isoproterenol.” *Proceedings of the National Academy of Sciences of the United States of America* 76 (6): 3024–28.

Davare, M. A. 2001. “A Beta 2 Adrenergic Receptor Signaling Complex Assembled with the Ca2 Channel Cav1.2.” *Science*. https://doi.org/10.1126/science.293.5527.98.

DeFea, K. A., J. Zalevsky, M. S. Thoma, O. Déry, R. D. Mullins, and N. W. Bunnett. 2000. “Beta-Arrestin-Dependent Endocytosis of Proteinase-Activated Receptor 2 Is Required for Intracellular Targeting of Activated ERK1/2.” *The Journal of Cell Biology* 148 (6): 1267–81.

Delint-Ramirez, Ilse, Debbie Willoughby, Gerald R. V. Hammond, Laura J. Ayling, and Dermot M. F. Cooper. 2015. “Palmitoylation Targets AKAP79 Protein to Lipid Rafts and Promotes Its Regulation of Calcium-Sensitive Adenylyl Cyclase Type 8.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.a111.243899.

Dessauer, Carmen W. 2009. “Adenylyl Cyclase–A-Kinase Anchoring Protein Complexes: The Next Dimension in cAMP Signaling.” *Molecular Pharmacology* 76 (5): 935–41.

Dessauer, Carmen W., Val J. Watts, Rennolds S. Ostrom, Marco Conti, Stefan Dove, and Roland Seifert. 2017. “International Union of Basic and Clinical Pharmacology. CI. Structures and Small Molecule Modulators of Mammalian Adenylyl Cyclases.” *Pharmacological Reviews* 69 (2): 93–139.

Doss, R. C., J. P. Perkins, and T. K. Harden. 1981. “Recovery of Beta-Adrenergic

Receptors Following Long Term Exposure of Astrocytoma Cells to Catecholamine. Role of Protein Synthesis." *The Journal of Biological Chemistry* 256 (23): 12281–86.

Eichel, K., D. Jullié, and M. von Zastrow. 2016. "β-Arrestin Drives MAP Kinase Signalling from Clathrin-Coated Structures after GPCR Dissociation." *Nature Cell Biology* 18 (3): 303–10.

Espinasse, I., V. Iourgenko, N. Defer, F. Samson, J. Hanoune, and J. J. Mercadier. 1995. "Type V, but Not Type VI, Adenylyl Cyclase mRNA Accumulates in the Rat Heart during Ontogenic Development. Correlation with Increased Global Adenylyl Cyclase Activity." *Journal of Molecular and Cellular Cardiology* 27 (9): 1789–95.

Esseltine, Jessica L., and John D. Scott. 2013. "AKAP Signaling Complexes: Pointing towards the next Generation of Therapeutic Targets?" *Trends in Pharmacological Sciences* 34 (12): 648–55.

Ferguson, S. S., W. E. Downey 3rd, A. M. Colapietro, L. S. Barak, L. Ménard, and M. G. Caron. 1996. "Role of Beta-Arrestin in Mediating Agonist-Promoted G Protein-Coupled Receptor Internalization." *Science* 271 (5247): 363–66.

Ferrandon, Sébastien, Timothy N. Feinstein, Marian Castro, Bin Wang, Richard Bouley, John T. Potts, Thomas J. Gardella, and Jean-Pierre Vilardaga. 2009. "Sustained Cyclic AMP Production by Parathyroid Hormone Receptor Endocytosis." *Nature Chemical Biology* 5 (10): 734–42.

Gagnon, A. W., L. Kallal, and J. L. Benovic. 1998. "Role of Clathrin-Mediated Endocytosis in Agonist-Induced down-Regulation of the beta2-Adrenergic

Receptor." *The Journal of Biological Chemistry* 273 (12): 6976–81.

Gaidarov, Ibragim, Jason G. Krupnick, John R. Falck, Jeffrey L. Benovic, and James H. Keen. 1999. "Arrestin Function in G Protein-Coupled Receptor Endocytosis Requires Phosphoinositide Binding." *The EMBO Journal*. https://doi.org/10.1093/emboj/18.4.871.

Gilman, A. G. 1989. "Transmembrane Signaling, G Proteins, and Adenylyl Cyclase." *Harvey Lectures* 85: 153–72.

Goodman, O. B., Jr, J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic. 1996. "Beta-Arrestin Acts as a Clathrin Adaptor in Endocytosis of the beta2-Adrenergic Receptor." *Nature* 383 (6599): 447–50.

Gros, Robert, Qingming Ding, Jozef Chorazyczewski, J. Geoffrey Pickering, Lee E. Limbird, and Ross D. Feldman. 2006. "Adenylyl Cyclase Isoform–Selective Regulation of Vascular Smooth Muscle Proliferation and Cytoskeletal Reorganization." *Circulation Research*, October. https://doi.org/10.1161/01.RES.0000245189.21703.c0.

Hauser, Alexander S., Misty M. Attwood, Mathias Rask-Andersen, Helgi B. Schiöth, and David E. Gloriam. 2017. "Trends in GPCR Drug Discovery: New Agents, Targets and Indications." *Nature Reviews. Drug Discovery* 16 (12): 829–42.

Henry, Anastasia G., Ian J. White, Mark Marsh, Mark von Zastrow, and James N. Hislop. 2011. "The Role of Ubiquitination in Lysosomal Trafficking of δ-Opioid Receptors." *Traffic* 12 (2): 170–84.

Hepler, John R., and Alfred G. Gilman. 1992. “G Proteins.” *Trends in Biochemical Sciences*. https://doi.org/10.1016/0968-0004(92)90005-t.

Hilger, Daniel, Matthieu Masureel, and Brian K. Kobilka. 2018. “Structure and Dynamics of GPCR Signaling Complexes.” *Nature Structural & Molecular Biology* 25 (1): 4–12.

Holst, Birgitte, and Thue Schwartz. 2002. “Molecular Structure and Function of 7TM G-Protein- Coupled Receptors.” *Textbook of Receptor Pharmacology, Second Edition*. https://doi.org/10.1201/9781420041071.sec2.

Irannejad, Roshanak, Jin C. Tomshine, Jon R. Tomshine, Michael Chevalier, Jacob P. Mahoney, Jan Steyaert, Søren G. F. Rasmussen, et al. 2013. “Conformational Biosensors Reveal GPCR Signalling from Endosomes.” *Nature* 495 (7442): 534–38.

Jean-Alphonse, Frederic, Shanna Bowersox, Stanford Chen, Gemma Beard, Manojkumar A. Puthenveedu, and Aylin C. Hanyaloglu. 2014. “Spatially Restricted G Protein-Coupled Receptor Activity via Divergent Endocytic Compartments.” *The Journal of Biological Chemistry* 289 (7): 3960–77.

Keith, Dove J., Jennifer L. Sanderson, Emily S. Gibson, Kevin M. Woolfrey, Holly R. Robertson, Kyle Olszewski, Rujun Kang, Alaa El-Husseini, and Mark L. Dell’acqua. 2012. “Palmitoylation of A-Kinase Anchoring Protein 79/150 Regulates Dendritic Endosomal Targeting and Synaptic Plasticity Mechanisms.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 32 (21): 7119–36.

Krupinski, J., F. Coussen, H. A. Bakalyar, W. J. Tang, P. G. Feinstein, K. Orth, C. Slaughter, R. R. Reed, and A. G. Gilman. 1989. “Adenylyl Cyclase Amino Acid Sequence: Possible Channel- or Transporter-like Structure.” *Science* 244 (4912): 1558–64.

Lauffer, Benjamin E. L., Cristina Melero, Paul Temkin, Cai Lei, Wanjin Hong, Tanja Kortemme, and Mark von Zastrow. 2010. “SNX27 Mediates PDZ-Directed Sorting from Endosomes to the Plasma Membrane.” *The Journal of Cell Biology*. https://doi.org/10.1083/jcb.201004060.

Li, Yong, Tanya A. Baldwin, Yan Wang, Janani Subramaniam, Anibal Garza Carbajal, Cameron S. Brand, Shane R. Cunha, and Carmen W. Dessauer. 2017. “Loss of Type 9 Adenylyl Cyclase Triggers Reduced Phosphorylation of Hsp20 and Diastolic Dysfunction.” *Scientific Reports* 7 (1): 5522.

Li, Yong, Lei Chen, Robert S. Kass, and Carmen W. Dessauer. 2012. “The A-Kinase Anchoring Protein Yotiao Facilitates Complex Formation between Adenylyl Cyclase Type 9 and the IKs Potassium Channel in Heart.” *The Journal of Biological Chemistry* 287 (35): 29815–24.

Lohse, Christian, Andreas Bock, Isabella Maiellaro, Annette Hannawacker, Lothar R. Schad, Martin J. Lohse, and Wolfgang R. Bauer. 2017. “Experimental and Mathematical Analysis of cAMP Nanodomains.” *PloS One* 12 (4): e0174856.

Lohse, M. J., J. L. Benovic, J. Codina, M. G. Caron, and R. J. Lefkowitz. 1990. “Beta-Arrestin: A Protein That Regulates Beta-Adrenergic Receptor Function.” *Science* 248 (4962): 1547–50.

Lyga, Sandra, Silvia Volpe, Ruth C. Werthmann, Konrad Götz, Titiwat Sungkaworn, Martin J. Lohse, and Davide Calebiro. 2016. “Persistent cAMP Signaling by Internalized LH Receptors in Ovarian Follicles.” *Endocrinology* 157 (4): 1613–21.

Pálvölgyi, Adrienn, James Simpson, Ibolya Bodnár, Judit Bíró, Miklós Palkovits, Tamás Radovits, Paul Skehel, and Ferenc A. Antoni. 2018. “Auto-Inhibition of Adenylyl Cyclase 9 (AC9) by an Isoform-Specific Motif in the Carboxyl-Terminal Region.” *Cellular Signalling* 51 (November): 266–75.

Paterson, J. M., S. M. Smith, A. J. Harmar, and F. A. Antoni. 1995. “Control of a Novel Adenylyl Cyclase by Calcineurin.” *Biochemical and Biophysical Research Communications* 214 (3): 1000–1008.

Qi, Chao, Simona Sorrentino, Ohad Medalia, and Volodymyr M. Korkhov. 2019. “The Structure of a Membrane Adenylyl Cyclase Bound to an Activated Stimulatory G Protein.” *Science* 364 (6438): 389–94.

Sadana, Rachna, and Carmen W. Dessauer. 2009. “Physiological Roles for G Protein-Regulated Adenylyl Cyclase Isoforms: Insights from Knockout and Overexpression Studies.” *Neuro-Signals* 17 (1): 5–22.

Shenoy, Sudha K., and Robert J. Lefkowitz. 2011. “β-Arrestin-Mediated Receptor Trafficking and Signal Transduction.” *Trends in Pharmacological Sciences* 32 (9): 521–33.

Sibley, D. R., R. H. Strasser, M. G. Caron, and R. J. Lefkowitz. 1985. “Homologous Desensitization of Adenylate Cyclase Is Associated with Phosphorylation of the Beta-Adrenergic Receptor.” *The Journal of Biological Chemistry* 260 (7): 3883–86.

Small, Kersten M., Kari M. Brown, Cheryl T. Theiss, Carrie A. Seman, Scott T. Weiss, and Stephen B. Liggett. 2003. “An Ile to Met Polymorphism in the Catalytic Domain of Adenylyl Cyclase Type 9 Confers Reduced beta2-Adrenergic Receptor Stimulation.” *Pharmacogenetics* 13 (9): 535–41.

Smith, F. Donelson, F. Donelson Smith, Lorene K. Langeberg, and John D. Scott. 2006. “The Where’s and When's of Kinase Anchoring.” *Trends in Biochemical Sciences*. https://doi.org/10.1016/j.tibs.2006.04.009.

Staehelin, M., and P. Simons. 1982. “Rapid and Reversible Disappearance of Beta-Adrenergic Cell Surface Receptors.” *The EMBO Journal* 1 (2): 187–90.

Steinberg, Florian, Matthew Gallon, Mark Winfield, Elaine C. Thomas, Amanda J. Bell, Kate J. Heesom, Jeremy M. Tavaré, and Peter J. Cullen. 2013. “A Global Analysis of SNX27-Retromer Assembly and Cargo Specificity Reveals a Function in Glucose and Metal Ion Transport.” *Nature Cell Biology* 15 (5): 461–71.

Stoeber, Miriam, Damien Jullié, Braden T. Lobingier, Toon Laeremans, Jan Steyaert, Peter W. Schiller, Aashish Manglik, and Mark von Zastrow. 2018. “A Genetically Encoded Biosensor Reveals Location Bias of Opioid Drug Action.” *Neuron* 98 (5): 963–76.e5.

Sunahara, R. K., C. W. Dessauer, and A. G. Gilman. 1996. “Complexity and Diversity of Mammalian Adenylyl Cyclases.” *Annual Review of Pharmacology and Toxicology* 36: 461–80.

Su, Y. F., T. K. Harden, and J. P. Perkins. 1979. “Isoproterenol-Induced Desensitization of Adenylate Cyclase in Human Astrocytoma Cells. Relation of Loss of Hormonal

Responsiveness and Decrement in Beta-Adrenergic Receptors." *The Journal of Biological Chemistry* 254 (1): 38–41.

Tang, W. J., and A. G. Gilman. 1991. "Type-Specific Regulation of Adenylyl Cyclase by G Protein Beta Gamma Subunits." *Science* 254 (5037): 1500–1503.

Taussig, R., J. Iniguez-Lluhi, and A. Gilman. 1993. "Inhibition of Adenylyl Cyclase by Gi Alpha." *Science*. https://doi.org/10.1126/science.8327893.

Temkin, Paul, Ben Lauffer, Stefanie Jäger, Peter Cimermancic, Nevan J. Krogan, and Mark von Zastrow. 2011. "SNX27 Mediates Retromer Tubule Entry and Endosome-to-Plasma Membrane Trafficking of Signalling Receptors." *Nature Cell Biology* 13 (6): 715–21.

Temkin, Paul, Wade Morishita, Debanjan Goswami, Kristin Arendt, Lu Chen, and Robert Malenka. 2017. "The Retromer Supports AMPA Receptor Trafficking During LTP." *Neuron*. https://doi.org/10.1016/j.neuron.2017.03.020.

Thomsen, Alex R. B., Bianca Plouffe, Thomas J. Cahill 3rd, Arun K. Shukla, Jeffrey T. Tarrasch, Annie M. Dosey, Alem W. Kahsai, et al. 2016. "GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling." *Cell* 166 (4): 907–19.

Tsvetanova, Nikoleta G., and Mark von Zastrow. 2014. "Spatial Encoding of Cyclic AMP Signaling Specificity by GPCR Endocytosis." *Nature Chemical Biology* 10 (12): 1061–65.

Tzavara, E. T., Y. Pouille, N. Defer, and J. Hanoune. 1996. "Diurnal Variation of the Adenylyl Cyclase Type 1 in the Rat Pineal Gland." *Proceedings of the National*

Academy of Sciences of the United States of America 93 (20): 11208–12.

Varandas, Katherine C., Roshanak Irannejad, and Mark von Zastrow. 2016. “Retromer Endosome Exit Domains Serve Multiple Trafficking Destinations and Regulate Local G Protein Activation by GPCRs.” *Current Biology: CB* 26 (23): 3129–42.

Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. 1983. “Characterization of an Altered Membrane Form of the Beta-Adrenergic Receptor Produced during Agonist-Induced Desensitization.” *The Journal of Biological Chemistry* 258 (22): 13900–908.

Wang, Hongbing, Victor V. Pineda, Guy C. K. Chan, Scott T. Wong, Louis J. Muglia, and Daniel R. Storm. 2003. “Type 8 Adenylyl Cyclase Is Targeted to Excitatory Synapses and Required for Mossy Fiber Long-Term Potentiation.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 23 (30): 9710–18.

Wang, Zhenshan, Carlos Balet Sindreu, Vicky Li, Aaron Nudelman, Guy C-K Chan, and Daniel R. Storm. 2006. “Pheromone Detection in Male Mice Depends on Signaling through the Type 3 Adenylyl Cyclase in the Main Olfactory Epithelium.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 26 (28): 7375–79.

Weis, William I., and Brian K. Kobilka. 2018. “The Molecular Basis of G Protein–Coupled Receptor Activation.” *Annual Review of Biochemistry*. https://doi.org/10.1146/annurev-biochem-060614-033910.

Wu, Z. L., S. A. Thomas, E. C. Villacres, Z. Xia, M. L. Simmons, C. Chavkin, R. D.

Palmiter, and D. R. Storm. 1995. “Altered Behavior and Long-Term Potentiation in Type I Adenylyl Cyclase Mutant Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (1): 220–24.

Xia, Zhengui, Eui-Ju Choi, Fan Wang, Christine Blazynski, and Daniel R. Storm. 1993. “Type I Calmodulin-Sensitive Adenylyl Cyclase Is Neural Specific.” *Journal of Neurochemistry* 60 (1): 305–11.

Zastrow, M. von, and B. K. Kobilka. 1992. “Ligand-Regulated Internalization and Recycling of Human Beta 2-Adrenergic Receptors between the Plasma Membrane and Endosomes Containing Transferrin Receptors.” *The Journal of Biological Chemistry* 267 (5): 3530–38.

Zou, Dong-Jing, Alexander T. Chesler, Claire E. Le Pichon, Andriy Kuznetsov, Xin Pei, Eugene L. Hwang, and Stuart Firestein. 2007. “Absence of Adenylyl Cyclase 3 Perturbs Peripheral Olfactory Projections in Mice.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 27 (25): 6675–83.

Chapter 2:

Differentially regulated endocytosis of Adenylyl Cyclase type 9 contributes to the B2AR-mediated signaling from endosomes

André M. Lazar conceived the project and performed all of the experiments and data analysis. André M. Lazar and Mark von Zastrow wrote the manuscript.

2.1 Abstract

G protein-coupled receptors (GPCRs), the largest family of signal-transducing receptors, are increasingly recognized to activate cognate G proteins after ligand-induced endocytosis. A critical knowledge gap is whether relevant G protein-linked effectors exist in the endosome membrane. We show that adenylyl cyclase type 9 (AC9) is such an effector, and undergoes rapid redistribution from the plasma membrane to endosomes in response to activation of the Gs-coupled β2-adrenoceptor (β2AR). AC9 trafficking is isoform-specific because adenylyl cyclase type 1 (AC1) remains in the plasma membrane under similar conditions, and it is induced by activation of Gs- but not Gi-coupled GPCRs. AC9 can localize to endosomes independently from GPCRs and its trafficking requires Gs rather than beta-arrestin. Moreover, AC9 contributes to the β2AR-elicited cAMP response from endosomes. Taken together, these findings reveal the dynamic coordination by membrane trafficking of receptor-G protein activation with a functionally relevant G-protein linked effector within the endocytic network.

2.2 Introduction

G-Protein Coupled Receptors (GPCRs) comprise the largest family of signaling receptors and an important class of therapeutic drug targets (Lefkowitz 2007). GPCRs are so-named because a major mechanism by which they mediate transmembrane signaling is through ligand-dependent activation of heterotrimeric G proteins that act as transducers of the intracellular signal (Spiegel 1987; Gilman 1987; Sunahara 1996). Canonical GPCR signaling cascades invariably require one additional component, an effector protein that is regulated by the G protein to convey the signal downstream (Dessauer, Posner, and Gilman 1996; Gilman 1987; Rosenbaum, Rasmussen, and Kobilka 2009). Such GPCR - G protein - effector cascades were thought for many years to operate exclusively in the plasma membrane, with the endocytic network considered only in the context of signal termination and proteolytic down-regulation of receptors (von Zastrow and Kobilka 1992; Sibley et al. 1986).

The mechanism of receptor regulation by membrane trafficking has been well described for the beta-2 adrenoceptor (β2AR) (Sibley et al. 1986; Waldo et al. 1983; von Zastrow and Kobilka 1992), a member of the largest class (family A) of mammalian GPCRs that activates downstream signaling by coupling to the G stimulatory protein (Gs) (Lefkowitz 2007). Phosphorylation of the receptor is required for arrestin binding (Lefkowitz 2007), which is critical for recruitment to clathrin-coated pits and dynamin-dependent endocytosis to the early endosome (Goodman et al. 1996). Arrestins have also been described to initiate G protein-independent downstream signaling cascades, including

from the endosome membrane, providing an early clue to the signaling potential of endosomes (Shukla, Xiao, and Lefkowitz 2011). There is an accumulating body of evidence for G protein-dependent signal initiation from endosomes (Irannejad et al. 2013; Lyga et al. 2016; Kotowski et al. 2011; Ferrandon et al. 2009; Calebiro et al. 2009; Vilardaga, Jean-Alphonse, and Gardella 2014; Lohse and Calebiro 2013; Irannejad et al. 2015; Stoeber et al. 2018; Thomsen et al. 2016; Slessareva et al. 2006). In mammalian cells, much of this evidence regards signaling via Gs, with ligand-dependent activation of both the β2AR and Gs explicitly demonstrated in endosomes (Irannejad et al. 2013).

A major class of Gs-regulated effectors are the adenylyl cyclases (ACs) (Dessauer, Posner, and Gilman 1996; Sadana, Rachna, and Dessauer 2009). Gs-linked activation of adenylyl cyclase produces cAMP, a second messenger molecule which has several downstream targets including Protein Kinase A (Taylor et al. 2013). Mammals express nine AC isoforms, all of which are sensitive to Gs but differ both in their expression across cell types and sensitivity to regulation by other pathways (Sunahara 1996). While the regulation of adenylyl cyclase catalytic activity has been well described, relatively little is known about the subcellular distribution of AC isoforms or consequences thereof.

G protein regulation of cAMP signaling requires GPCR, G protein and AC to be embedded in the same membrane bilayer (Gilman 1989). GPCRs and Gs are well known to undergo dynamic redistribution in the endocytic network (Irannejad et al. 2015;

Marrari et al. 2007) but much less is known about endocytic trafficking of ACs. Indeed, it has been generally assumed that GPCR-elicited cAMP production is restricted to the plasma membrane and relies on diffusion of cAMP for control of downstream targets. Nevertheless, adenylyl cyclase activity has long been recognized at internal membrane locations (Cheng and Farquhar 1976) and there is functional evidence that several AC isoforms contribute to endomembrane signaling (Ferrandon et al. 2009; Vilardaga, Jean-Alphonse, and Gardella 2014; Caldieri and Sigismund 2016; Cancino et al. 2014). However, to our knowledge, a specific AC isoform present in the endosome limiting membrane has not been identified, and dynamic regulation of ACs by membrane trafficking has remained largely undescribed. Here we demonstrate that β2AR-Gs activation triggers isoform-specific trafficking of adenylyl cyclase type 9 (AC9) and that AC9 mediates cAMP generation from endosomes.

2.3 Results

β2AR activation promotes accumulation of AC9 but not AC1 in endosomes

We chose an N-terminal Flag tagging strategy because AC1 function was previously shown to tolerate such a tag (Chen et al. 1997). In HEK293 cells, Flag-AC1 primarily localized at the plasma membrane (**Figure 2.1 (A)**), a distribution similar to that of an HA-tagged β2AR construct (HA-β2AR) in the absence of agonist (**Figure 2.1 (A)**). Application of the adrenergic agonist isoproterenol caused HA-β2AR redistribution to cytoplasmic punctae (**Figure 2.1 (A)**) that have been previously described as early endosomes (Irannejad et al. 2013; Temkin et al. 2011). In contrast, Flag-AC1 remained localized at the plasma membrane and did not visibly accumulate in these endosomes (**Figure 2.1 (A)**). We confirmed this observation across hundreds of cells captured by wide-field epifluorescence microscopy (**Figure 2.1 (M)**) and quantified the results in two ways. First, we determined the number of internal punctae per cell which were positive for HA-β2AR or Flag-AC1 (**Figure 2.1 (C)**). Second, we determined the fraction of cells visualized in each microscopic field that contained at least 10 such punctae (**Figure 2.1 (D)**).

We applied a similar tagging strategy to other AC isoforms and chose to focus on Flag-AC9 because it consistently expressed and localized to the PM similarly to Flag-AC1. We verified that Flag tagging also does not disrupt AC9 function (**Figure 2.1 (K, L)**). In the absence of agonist, Flag-AC9 localized at the plasma membrane along with HA-β2AR (**Figure 2.1 (B)**). Within minutes after application of isoproterenol,

Flag-AC9 redistributed to internal punctae that colocalized with HA-β2AR (**Figure 2.1 (B)**). Isoproterenol-induced accumulation of Flag-AC9 could be consistently observed in confocal optical sections (**Figure 2.1 (B)**) as well as by standard epifluorescence microscopy (sample data in **Figure 2.1 (N)** and quantified in **(C, D)**). Live-cell confocal microscopy of HEK293 cells expressing AC9-EGFP confirmed these observations.

Isoproterenol produced isoform-specific redistribution of AC9 in cells not expressing a recombinant β2AR construct (**Figure 2.1 (E, F, J)**) that was blocked by the beta-adrenergic antagonist alprenolol (**Figure 2.1 (G, H, J)**). This suggests that β2ARs expressed endogenously in HEK293 cells (Violin et al. 2008) are sufficient to drive AC trafficking and that this trafficking was not an off-target effect of isoproterenol. AC9 is endogenously expressed in HEK293 cells but at relatively low levels (Soto-Velasquez et al. 2018; Baldwin et al. 2019), and we were unable to detect endogenous AC9 in HEK293 cells using available antibodies. However, we did observe endogenous AC9 immunoreactivity in primary human airway smooth muscle cells that express AC9 in relatively high abundance (Billington et al. 1999). Endogenous AC9 immunoreactivity was localized to the plasma membrane under basal conditions in these cells, and redistributed to internal punctae after application of isoproterenol (**Figure 2.1 (I)**).

<u>AC9 is internalized from the plasma membrane and accumulates in early endosomes</u>

Early Endosome Antigen 1 (EEA1) is a marker of early endosomes where ligand-dependent activation of both β2AR and Gs has been demonstrated (Irannejad et

al. 2013). Treatment with isoproterenol resulted in a colocalization of Flag-AC9 with EEA1 (**Figure 2.2 (A)**), while AC1 remained at the plasma membrane under similar conditions (**Figure 2.2 (B)**). We verified this isoform-specific accumulation in early endosomes using a vesicle immunoisolation procedure based on anti-EEA1 pulldown (Hammond et al. 2010; Temkin et al. 2011; Cottrell et al. 2009). Both HA-β2AR and Flag-AC9 accumulated in the endosome fraction as a result of isoproterenol treatment, as detected by immunoblot analysis (**Figure 2.2 (C, D)**). By contrast, isoproterenol produced endosomal accumulation of HA-β2AR, but not Flag-AC1, with both isolations carried out in parallel (**Figure 2.2 (C, D)**). Documenting the efficiency of separation and fraction purity, the immunoisolated fraction recovered ~34% of total cellular EEA1 but ≤5% of Golgi, endoplasmic reticulum, or plasma membrane markers (**Figure 2.2 (G)**).

As an additional biochemical verification, we used surface biotinylation to assess protein depletion from the plasma membrane (Flesch et al. 1995; Whistler and von Zastrow 1998). Isoproterenol caused a pronounced depletion of Flag-AC9 but not Flag-AC1 from the surface-biotinylated fraction (**Figure 2.2 (E, F)**). Isoproterenol-induced depletion of HA-β2AR from the surface biotinylated fraction was observed in the same samples irrespective of the AC isoform coexpressed (**Figure 2.2 (E, F)**), with comparable loading confirmed in all experiments by immunoblot of lysate controls (**Figure 2.2 (E)**). These biochemical observations are consistent with the idea that AC9 undergoes depletion from the plasma membrane and accumulates in the early endosome fraction as a result of agonist stimulation of the β2AR.

AC9 internalization is promoted by Gs- but not Gi -coupled GPCRs

We next asked whether AC9 trafficking can be elicited by another Gs-coupled GPCR, focusing on the vasopressin-2 receptor (V2R) (Birnbaumer 2000). Unlike the β2AR, the V2R is not endogenously expressed in HEK293 cells. In cells expressing only Flag-AC9 but not HA-V2R, the V2R agonist arginine-vasopressin (AVP) had no effect and Flag-AC9 remained in the plasma membrane (**Figure 2.3 (F)**). In contrast, in cells coexpressing HA-V2R, AVP caused Flag-AC9 redistributed from the plasma membrane to internal punctae (**Figure 2.3 (A, D, E, J)**). Surface biotinylation experiments confirmed that AVP produced a dramatic depletion of both HA-V2R and Flag-AC9 from the plasma membrane (**Figure 2.3 (H, I)**).

We then investigated if AC9 trafficking to endosomes can be elicited by a Gi-coupled GPCR. To do so we focused on the μ-opioid receptor (MOR), a well characterized example (Kieffer and Evans 2009) not endogenously expressed in HEK293 cells. Agonist induced internalization of the MOR shares a similar mechanism with the β2AR, and MOR is delivered to β2AR-containing endosomes (Lauffer et al. 2010). Application of the μ-opioid agonist [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) resulted in robust accumulation of HA-MOR but not Flag-AC9 in endosomes (**Figure 2.3 (B, D, E, K)**), and we verified lack of AC9 internalization biochemically by surface biotinylation (**Figure 2.3 (H, I)**).

To begin to examine whether AC9 and GPCR trafficking are regulated by similar mechanisms, we focused on a previously described V2R mutant. This tail-truncation mutant retains its ability to activate Gs but lacks critical Ser/Thr residues in its cytoplasmic tail that are phosphorylated in response to AVP and promote V2R accumulation in early endosomes (Innamorati et al. 1997, 1998; Oakley et al. 1999). We confirmed that internalization of the truncated mutant receptor (HA-V2R-T) was detectably reduced relative to its wild type HA-V2R counterpart in the same cells (**Figure 2.3 (C, D, E, L)**). Despite this, AVP-induced activation of this truncated mutant receptor resulted in robust internalization of Flag-AC9 to internal punctae, as visualized by both confocal (**Figure 2.3 (C)**) and epifluorescence microscopy (**Figure 2.3 (D, E, L)**). Surface biotinylation experiments verified that HA-V2R-T can mediate AVP-induced internalization of Flag-AC9 to a similar degree as the wild-type receptor (**Figure (H, I)**). Taken together, these results provide the first indication that AC9 trafficking correlates with receptor coupling to Gs, rather than with receptor tail phosphorylation and arrestin-dependent endocytosis.

AC9 trafficking is not dependent on cytoplasmic cAMP or adenylyl cyclase activity

We first considered the possibility that AC9 trafficking is a downstream consequence of elevated cytoplasmic cAMP concentration produced by Gs-coupled GPCR activation. To test this, we applied the diterpene drug forskolin (FSK) to directly activate adenylyl cyclases and thereby increase cytoplasmic cAMP concentration independently from receptor or Gs activation. Whereas AC9 is relatively insensitive to activation by FSK,

other AC isoforms that are major contributors to the cAMP production in HEK293 cells (particularly AC3 and AC6) are, making FSK an effective stimulus of overall cAMP elevation (Soto-Velasquez et al. 2018; Baldwin et al. 2019). FSK did not cause detectable internalization of HA-β2AR, and Flag-AC9 remained in the plasma membrane of FSK-treated cells (**Figure 2.4 (A, C, D, I)**), with FSK also failing to stimulate detectable internalization of either HA-β2AR or Flag-AC9 measured using surface biotinylation (**Figure 2.4 (E, F)**). Fully consistent with its lack of trafficking in response to receptor or Gs activation, Flag-AC1 remained in the plasma membrane (**Figure 2.4 (B, C, D, J)**) and did not detectably internalize in the presence of FSK (**Figure (E, F)**). Adding 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor that further enhances the FSK-induced cytoplasmic cAMP elevation, also did not appreciably change the localization of HA-β2AR and Flag-AC9 (**Figure 2.4 (H)**).

To determine whether enzymatic activity of AC9 is required for its internalization, we mutated a conserved aspartic acid residue in the active site, which coordinates a catalytic magnesium ion and was shown previously to be necessary for cAMP production by AC6 (Gao et al. 2011; Tesmer et al. 1997). Verifying that this mutation (AC9-D442A) also inactivates AC9, Flag-AC9-D442A overexpression did not detectably increase the cAMP response measured in HEK293 cells measured by whole cell cAMP assay, but wild type Flag-AC9 did (**Figure 2.1 (L)**). Despite significant loss of activity, Flag-AC9-D442A robustly internalized and colocalized in endosomes with HA-β2AR in response to isoproterenol-induced activation (**Figure 2.4 (G)**), indicating that the

adenylyl cyclase activity of AC9 is not necessary for regulated internalization of this AC isoform.

Gs activation is sufficient to promote AC9 trafficking

Since AC9 internalization can be promoted by Gs-coupled GPCRs but is insensitive to overall cytoplasmic cAMP elevation, we next determined whether activation of Gs itself is sufficient to promote AC9 internalization. To test this we used a point mutant of Gs (HA-Gs-Q227L) which has been previously described as constitutively active (Masters et al. 1989). Coexpressing Flag-AC9 with wild type HA-Gs resulted in localization of both proteins to the plasma membrane (**Figure 2.5 (A, C, D, K)**). In contrast, coexpression of Flag-AC9 with HA-Gs-Q227L resulted in the localization of both proteins to internal punctae (**Figure 2.5 (A, C, D, K)**). This effect was specific to AC9 because AC1 remained localized to the plasma membrane when coexpressed with either HA-Gs or HA-Gs-Q227L (**Figure 2.5 (B, C, D, L)**). We verified by immunoisolation that both HA-Gs-Q227L and Flag-AC9 (**Figure 2.5 (G, H)**), but not Flag-AC1 (**Figure 2.5 (I, J)**), constitutively accumulate in EEA1-positive endosomes. Consistent with this finding, activation of endogenous Gs by ADP-ribosylation using cholera toxin (CTX) also produced receptor-independent internalization of Flag-AC9, while HA-β2AR and Flag-AC1 remained in the plasma membrane (**Figure 2.5 (M, N)**).

We confirmed that isoproterenol treatment resulted in accumulation of HA-Gs and Flag-AC9 in the endosome fraction (**Figure 2.5 (E, F)**). However, in cells co-expressing

HA-Gs-Q227L, Flag-AC9 had already robustly accumulated in this fraction, and no additional effect was observed upon isoproterenol treatment (**Figure 2.5 (E, F)**). Taken together, the results suggest that Gs activation is sufficient to promote AC9 internalization, and further underscores the idea that GPCR and AC9 trafficking are regulated by separate mechanisms.

AC9 trafficking requires Gs but not β-arrestin

Since receptor-independent activation of Gs, by mutation or by cholera toxin, was sufficient to promote AC9 accumulation in endosomes, we next considered whether Gs activation is necessary to promote this regulated trafficking process. To test this we examined a previously described line of HEK293 cells lacking all Gs function due to CRISPR-mediated ablation of the Gs alpha subunit (Stallaert et al. 2017). In these Gs-knockout (GsKO) cells, Flag-β2AR and AC9-EGFP colocalized at the plasma membrane in the absence of isoproterenol. While treatment with isoproterenol dramatically increased the number of internal punctae that were positive for Flag-β2AR, this was not the case for AC9-EGFP in these GsKO cells (**Figure 2.6 (A, C, D, I)**). This isoproterenol-induced AC9-EGFP trafficking defect could be rescued by expression of recombinant wild-type HA-Gs (**Figure 2.6 (B, C, D, J)**). Together with the results presented above, these data indicate that activation of Gs is both necessary and sufficient to drive AC9 internalization but is not needed for β2AR trafficking.

Arrestins are known to be endocytic adaptor proteins essential for regulated internalization of the β2AR (Goodman et al. 1996). We next asked whether arrestins also regulate AC9 trafficking. To test this we used a previously described line of HEK293 cells lacking both beta-arrestin isoforms (arrestins 2 and 3, or beta-arrestin-1 and beta-arrestin-2)(O'Hayre et al. 2017). In these arrestin 2/3 double-knockout cells (Arr DKO), HA-β2AR and AC9-EGFP localized to the plasma membrane in the absence of isoproterenol, as in wild type cells. However, after application of isoproterenol, HA-β2AR remained restricted to the plasma membrane while AC9-EGFP still robustly localized to internal punctae (**Figure 2.6 (E, G, H, K)**). Addback of Arrestin 3 (beta-arrestin-2) rescued this isoproterenol-induced internalization of HA-β2AR and AC9-EGFP still internalized (**Figure 2.6 (E, G, H, L)**). These data indicate that beta-arrestin, while necessary for regulated internalization of β2AR, is not necessary to regulate trafficking of AC9. Accordingly, although β2AR and AC9 colocalize to the early endosome as a result of agonist stimulation, the mechanistic basis for their regulated trafficking differs fundamentally.

<u>AC9 is sufficient to promote a β2AR-mediated signaling cascade from endosomes</u>

Both AC1 and AC9 are well known physiological effectors of beta-adrenergic signaling (Small et al. 2003; Tantisira 2005; Sadana, Rachna, and Dessauer 2009) and both are endogenously expressed in HEK293 cells, but neither AC1 nor AC9 is the dominant source of cAMP in these cells . Nevertheless, cAMP accumulation analysis after siRNA knockdown (**Figure 2.7 (C)**) indicated that both AC1 and AC9 make a statistically

significant contribution (**Figure 2.7 (D)**) to the overall cAMP response elicited by isoproterenol. Consistent with previous reports (Baldwin et al. 2019), AC9 knockdown did not have a detectable effect on the cellular response to forskolin whereas AC1 knockdown produced a detectable reduction (**Figure 2.7 (D)**). Considering the differences in AC9 relative to AC1 trafficking, we asked if these isoforms are differentially activated depending on the location of receptor activation.

We used a pharmacological approach to differentiate between receptor activation in endosomes and the plasma membrane. CGP12177 (CGP) is a high affinity β2AR antagonist that is well known to be membrane-impermeant (Staehelin et al. 1983) and thus selectively accesses receptors at the plasma membrane but not at the endosome. Alprenolol is a similarly high affinity antagonist that is relatively membrane-permeant, and thus can access receptors at both locations. Isoproterenol has previously been shown to promote β2AR activation in endosomes as well as the plasma membrane as detected using Nb80-EGFP, a conformational biosensor of β2AR activation (Irannejad et al. 2013). Verifying this, exposure of cells to isoproterenol (100 nM) for 30 minutes produced a clear Nb80-EGFP recruitment to β2AR-containing endosomes (**Figure 2.7 (A)**). Subsequent addition of an excess concentration of alprenolol (10 μM) rapidly reversed Nb80-EGFP recruitment to endosomes, while β2AR was still detected in these membrane compartments. (**Figure 2.7 (A)**). In contrast, an excess concentration of CGP (10 μM) did not detectably reverse recruitment of Nb80-EGFP to β2AR-containing endosomes induced by the 30 minute pretreatment with isoproterenol (100 nM) (**Figure**

2.7 (B)). These results indicate that sequential treatment with isoproterenol and then CGP can selectively activate β2ARs at the endosome.

The cellular cAMP response was measured in HEK293 cells that had been pretreated with isoproterenol and then exposed to either alprenolol or CGP. While both antagonists reduced the overall cAMP response, the inhibitory effect of alprenolol was significantly larger than that of CGP (**Figure 2.7 (G**, **I)**). However, in cells depleted of AC9, this 'signal gap' between the effects of alprenolol and CGP was eliminated (**Figure 2.7 (H, I)**). The statistically significant difference between the effects of CGP and alprenolol in control HEK293 cells suggests that there is a cAMP response originating from an internal pool of receptors. Further, since AC9 knockdown eliminates this difference (**Figure 2.7(C)**), we conclude that AC9 is a significant contributor to this internally-generated response. Consistent with the trafficking data, knockdown of AC1 in these cells had no significant effect on the signal gap between alprenolol and CGP (**Figure 2.7 (E, F, J)**).

To further validate these findings, we over-expressed Flag-AC9 or Flag-AC1 in previously described AC3/6 double knockout (AC3/6 DKO) HEK293 cells (Soto-Velasquez et al. 2018). Because AC3 and AC6 generate the majority of cAMP in HEK293 cells, these cells provide a lower background on which to assess expressed isoforms. Overexpression of Flag-AC9 in AC3/6 DKO cells significantly increased the isoproterenol-induced cAMP response, consistent with previously reported results

(Soto-Velasquez et al. 2018). A portion of this increase was not inhibited by CGP, whereas the entire increase in cellular cAMP elevation due to AC9 expression was eliminated by alprenolol (**Figure 2.7 (Q)**). Overexpression of Flag-AC1 in AC3/6 DKO cells also increased the isoproterenol-induced cAMP response as expected, but this increase was eliminated in the presence of either CGP or alprenolol (**Figure 2.7 (R)**). These data suggest that AC1 activity is restricted to the plasma membrane while AC9 is a significant contributor to β2AR-mediated signaling from the endosome.

To determine whether the internally-generated cAMP response is endocytosis dependent, we examined the effect of DYNGO-4a, a chemical inhibitor of dynamin known to block endocytosis of β2AR (Irannejad et al. 2013). DYNGO-4a had little effect on the cellular cAMP response to sequential treatment with isoproterenol and then alprenolol, and this was the case both in control and AC9 siRNA knockdown conditions (**Figure 2.7 (K, L)**). In contrast, in cells subjected to sequential treatment with isoproterenol and then CGP, DYNGO-4a further enhanced the inhibitory effect of CGP on the cellular cAMP response. This was the case only in control conditions and the additive effect was lost after AC9 knockdown (**Figure 2.7 (M, N)**).

This difference in sensitivity to endocytic blockade was additionally verified in cells overexpressing AC9 on the AC3/6 KO background. AC9 overexpression increased the overall isoproterenol-induced cAMP response, and it increased the CGP-resistant component without producing a detectable alp-resistant component. In the presence of

endocytic blockade by DYNGO-4a, the CGP-resistant component of the response was lost whereas inhibition by alprenolol was unchanged (**Figure 2.7 (S)**). In contrast, while AC1 overexpression also increased the isoproterenol-induced cAMP response in AC3/6 DKO cells, there was no detectable CGP-resistant component added or additional effect of DYNGO-4a (**Figure 2.7 (T)**). These data provide functional evidence indicating that AC9 is a major contributor to the CGP-insensitive fraction of the β2AR-mediated cAMP response. Furthermore, this response is endocytosis-dependent, suggesting that the CGP-insensitive response represents an endosome-initiated signal cascade. AC1, while it contributes a similar (or larger) amount to the overall cellular cAMP response, does not contribute detectably to the endosome-initiated component.

2. 4 Discussion

Membrane trafficking is a dynamically regulated system that is critical for homeostatic integrity of the cell. Endocytosis of signaling receptors has historically been viewed only as a mechanism to confer long-term homeostasis of signaling from the plasma membrane. This has been extensively described for the β2AR, with endocytosis controlling the surface availability of functional receptors and initiating receptor downregulation. According to this view, effectors function only at the plasma membrane and communicate to downstream targets solely by diffusion of second messengers. There is now compelling evidence that the endocytic network can function as a site of receptor-mediated signaling, and various GPCRs, including the β2AR, have been shown to activate Gs in endosomes (Irannejad et al. 2015; Vilardaga, Jean-Alphonse, and Gardella 2014; Lohse and Calebiro 2013). This raises the question of whether the endocytic network can differentially regulate G protein-linked effectors, enabling the spatial coordination of a signaling cascade with its downstream targets in the cell. We addressed this question by focusing on adenylyl cyclases as critical Gs-linked effectors, and demonstrate here the dynamic and regulated trafficking of AC9. AC9 is already known to be physiologically and clinically relevant to β2AR signaling (Sunahara 1996; Small et al. 2003; Tantisira 2005), and we show that AC9 is necessary and sufficient to promote the endosome-derived cAMP response in HEK293 cells.

Our results indicate that AC1 trafficking is not regulated in the same way as AC9, establishing that AC trafficking is isoform-selective. However, we do not presently know

if AC9 accumulation in endosomes is unique to this isoform or more widespread. We anticipate the latter because a distantly related AC isoform has been localized to endosomes in *D. discoideum (Kriebel et al. 2008)*, although whether or not these endosomes also contain the GPCR and cognate G protein has not been established. There is evidence that both AC2 and AC3 contribute functionally to the cAMP response elicited by GPCRs after endocytosis (Kriebel et al. 2008; Jean-Alphonse et al. 2017), but whether or not either isoform directly localizes to endosomes is presently unknown. Further, we note that soluble AC (sAC) also contributes to the endocytosis-dependent cAMP response (Inda et al. 2016). In this case the effect is almost certainly indirect because sAC is not known to be activated by Gs. To our knowledge, the present results provide the first direct demonstration of dynamic trafficking of an adenylyl cyclase to endosomes that also contain an upstream activating GPCR and cognate G protein transductor, but whether this behavior is unique remains to be determined.

One possible mechanism for adenylyl cyclase trafficking is that the receptor, G protein, and cyclase form both a signaling, and a trafficking unit. In this model, adenylyl cyclase internalization would be a direct physical consequence of arrestin-dependent recruitment of the receptor to clathrin coated pits. However, we demonstrate here that AC9 trafficking does not require beta-arrestins. Instead, we show that AC9 trafficking requires Gs and, further, that receptor independent activation of Gs is sufficient to induce AC9 internalization. We verify that the β2AR exhibits the opposite phenotype in that its trafficking requires beta-arrestin but not Gs. This indicates that the delivery of

AC9 and β2AR to endosomes, while both initiated by receptor activation, are regulated by distinct mechanisms. Future studies into the pathway of AC9 membrane trafficking and its coordination with, or divergence from, an activating receptor are required.

The endosome is increasingly recognized as a relevant site for GPCR signaling. This was initially thought to be restricted to G protein-independent signaling, particularly arrestin-mediated pathways (Shukla, Xiao, and Lefkowitz 2011), but endosomes are now understood to be sites of GPCR-mediated activation of heterotrimeric G proteins (Vilardaga et al. 2012; Thomsen et al. 2016). We identify a relevant adenylyl cyclase at endosomes and demonstrate that Gs can regulate both effector location in the cell and enzymatic activity. Spatiotemporal control of effector location by receptor activation has significant implications for therapeutic drug design. Given clear evidence that cAMP location in the cell is important for determination of downstream signaling consequences (Zaccolo and Pozzan 2002), it may be possible to achieve enhanced selectivity in drug action based on the subcellular location of second messenger production.

2.5 Acknowledgements

We thank M. Stoeber, B. Lobingier, and other members of the von Zastrow laboratory for useful advice and critical discussion. We thank J. Taunton, D. Mullins and R. Sunahara for valuable insight and suggestions. We thank Monica Soto-Velasquez and V. Watts for the generous gift of AC3/6 knockout cells. We thank the E. Roth group for their generous support and lab equipment, and Kyra Kurtz for assistance. This work was supported by grants from the US National Institutes of Health (DA010711 and DA012864 to M.v.Z.; GM60419 to C.W.D; HL124049 to A.B.S.). A.L. is an ARCS scholar.

2.6 Materials & Methods

Cell culture, expression constructs, and transfections

HEK 293 cells (ATCC) were cultured in complete growth Dulbecco's modified Eagle's medium (DMEM, Gibco) and supplemented with 10% fetal bovine serum (UCSF Cell Culture Facility). We note that AC9 most consistently internalized in cells that were not removed from the incubator for longer than 3 min during plating or transfection. HA-β2AR(Tang et al. 1999; von Zastrow and Kobilka 1992), HA-V2R (Rochdi et al. 2010), HA-MOR (Whistler and von Zastrow 1998), HA-V2R-T (Rochdi et al. 2010; Charest and Bouvier 2003), all described previously, were sub-cloned from Flag-tagged constructs. Nb80-EGFP was previously described (Irannejad et al. 2013). HA-G(alpha)s, G(beta-1), G(gamma-2) were gifts from Philip Wedegaertner. HA-G(alpha)s-Q227L, a previously described point mutant of Gs that is constitutively active (Masters et al. 1989), was made from the original construct using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) with the forward primer 5'-CGATGTGGGCGGCCTGCGCGATGAACGCCGC-3'. Flag-AC1, Flag-AC9 from the Dessauer Lab, were originally described by (Premont et al. 1996; Krupinski et al. 1989; Hacker et al. 1998; Paterson et al. 2000). Flag-AC9-D442A (Catalytic inactive mutant) was also made from the original construct using QuikChange Kit with the forward primer 5'-CCACTAGTCCAGTGTGGTGGAATTCGCCATGGACTACAAAGACGATGACGAC-3'. Transfections were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Cells were transfected 48 hours before experiments.

siRNA knockdown of AC1 and AC9 expression in HEK293 cells was carried out using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. Cells were transfected 72 hours before experiments. Knockdown of AC1 used the siRNA CCGGGCGGTTCAGACCTTCAA and AC9 knockdown used CTGGGCATGAGGAGGTTTAAA.

Primary cultures of human airway smooth muscle cells were established as described previously (Tsvetanova et al. 2016). Cells were passaged no more than 5 times using Trypsin-EDTA (Life Technologies) and maintained in 10% FBS in DMEM.

Gs knockout (Stallaert et al. 2017) and beta-arrestin-1/2 double knockout (O'Hayre et al. 2017) HEK293 cells were previously described . AC3 / AC6 double knockout HEK293 cells were also described previously (Soto-Velasquez et al. 2018) and were provided as a generous gift by Drs. Monica Soto-Valasquez and Val Watts (Purdue University). Cells were passaged using PBS-EDTA and maintained in 10% FBS in DMEM.

Cholera Toxin (Sigma) was administered to cells for 16 hours overnight treatment at 10 ng/ml concentration in 10% FBS in DMEM.

Antibodies

Antibodies used were rabbit anti-Flag (Sigma), mouse anti-Flag M1 (Sigma), mouse anti-Flag M2 (Sigma), mouse anti-HA 16B12 (Biolegend), rat anti-HA (Roche), goat anti-AC9 (Santa Cruz Biotech), mouse anti-Golgin-97 (Thermo), rabbit anti-calnexin (Cell Signaling), mouse anti-Sodium/Potassium ATPase (Fisher).

Fixed cell confocal imaging

Cells were transfected with the indicated construct(s) and then plated on glass coverslips coated with poly-L-lysine (0.0001%, Sigma) 24 hours later. For antibody feeding assays, cells were: (1) placed on ice and rinsed with ice-cold phosphate-buffered saline (PBS), (2) labeled by the addition of antibodies diluted 1:1000 in DMEM for 10 minutes, and (3) rinsed with room temperature PBS and allowed to traffic for 30 minutes by the addition of 37°C fresh media (DMEM + 10% fetal bovine serum) with or without a saturating concentration of β2AR agonist (10 μM isoproterenol, Sigma), V2R agonist (10 μM arginine-vasopressin, Sigma), MOR agonist (10 μM DAMGO, Sigma), or forskolin (10 μM, Sigma). For all assays, cells were rinsed with cold PBS and fixed by incubation in 3.7% formaldehyde (Fisher Scientific) diluted in modified BRB80 buffer (80mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 6.8) for 20 minutes at room temperature. Cells were then blocked in 2% Bovine Serum Albumin (Sigma) in PBS with permeabilization by 0.2% triton X-100 (Sigma). Primary antibody labeling was performed by the addition of antibodies diluted 1:1000 in blocking/ permeabilization buffer for one hour at room temperature. Secondary labeling was performed by addition

of the following antibodies diluted at 1:500 in blocking/ permeabilization buffer for 20 minutes at room temperature: Alexa Fluor 555 or 488 donkey anti-mouse (Invitrogen), Alexa Fluor 555 or 488 donkey anti-rabbit (Invitrogen), Alexa Fluor 488 or 555 goat anti-rat (Invitrogen), or Alexa Fluor 488 donkey anti-sheep (Life Technologies). Specimens were mounted using ProLong Gold antifade reagent (Life Technologies).

Fixed cells were imaged by spinning disc confocal microscope (Nikon TE-2000 with Yokogawa confocal scanner unit CSU22) using a 100X NA 1.45 objective. A 488 nm argon laser and a 568 nm argon/krypton laser (Melles Griot) were used as light sources.

Microscope image acquisition and image analysis

Spinning disc images were collected using an electron multiplying CCD camera (Andor iXon 897) operated in the linear range controlled by Micro-Manager software (https://www.micro-manager.org). Images were processed at full bit depth for all analysis and rendered for display by converting to RGB format using ImageJ software (http://imagej.nih.gov/ij) and linear look up table. The number of endosomes was quantified by thresholding images and the ParticleTracker ImageJ plugin.

Live-cell confocal imaging

Live cell imaging was carried out using Yokagawa CSU22 spinning disk confocal microscope with a ×100, 1.4 numerical aperture, oil objective and a CO2 and 37 °C

temperature-controlled incubator. A 488 nm argon laser and a 568 nm argon/krypton laser (Melles Griot) were used as light sources for imaging EGFP and Flag signals, respectively. Cells expressing both Flag-tagged receptor and the indicated nanobody–EGFP were plated onto glass coverslips. Receptors were surface labelled by addition of M1 anti-Flag antibody (1:1000, Sigma) conjugated to Alexa 555 (A10470, Invitrogen) to the media for 30 min, as described previously. Indicated agonist (isoprenaline, Sigma) or antagonist (CGP-12177, Tocris) (alprenolol, Sigma) were added and cells were imaged every 3 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4 (UCSF Cell Culture Facility). Time-lapse images were acquired with a Cascade II EM charge-coupled-device (CCD) camera (Photometrics) driven by Micro-Manager 1.4 (http://www.micro-manager.org).

Endosome Pulldown

Cells were transfected with the indicated construct(s) 48 hours before lysis and plated onto 60mm cell culture dishes 24 hours before lysis. Cells were allowed to traffic for 30 minutes by the addition of 37°C fresh media (DMEM + 10% fetal bovine serum) with or without a saturating concentration of the indicated agonist. Cells were then placed on ice, washed with ice-cold PBS, and scraped into an isotonic homogenization buffer (10mM HEPES, 100mM KCl, 25mM sucrose, Complete protease inhibitor (Roche), pH 7.2) and passaged 20 times through a 22 G BD PrecisionGlide Needle. Whole cell lysates were then spun down at 1000 G for 10 minutes at 4°C and the pellets discarded. The supernatant was then bound to Early Endosome Antigen 1 mouse antibody (1:250, Fisher Scientific) and anti-mouse IgG magnetic microbeads (Miltenyi Biotech) overnight.

Endosomes were then bound to magnetic columns which were blocked with 3% BSA and washed with PBS. Endosomes were then eluted with 0.1% Triton-X and characterized by western blot.

Surface Biotinylation

Cells were transfected with the indicated construct(s) 48 hours before lysis and plated onto 60mm cell culture dishes coated with poly-L-lysine (0.0001%, Sigma) 24 hours before lysis. Cells were allowed to traffic for 30 minutes by the addition of 37°C fresh media (DMEM + 10% fetal bovine serum) with or without a saturating concentration of the indicated agonist. Cells were then placed on ice, washed with ice-cold PBS, and then surface labeled with EZ-link Sulfo-NHS-biotin (Pierce) for 30 min, rocking at 4°C. Reaction was then quenched with tris buffered saline (TBS) twice for 10 min. Cells were then placed on ice, washed with ice-cold PBS, and scraped into an isotonic homogenization buffer (10mM HEPES, 100mM KCl, 25mM sucrose, Complete protease inhibitor (Roche), pH 7.2) and passaged 20 times through a 22 G BD PrecisionGlide Needle. Cell lysate was then bound to streptavidin agarose resin (Thermo) overnight. Resin was spun down and the supernatant discarded, resuspended and washed in ice-cold PBS, and characterized by western blot.

Luminescence-based rapid cAMP assay

HEK293 cells were transfected with a plasmid encoding a cyclic-permuted luciferase reporter construct, based on a mutated RIIβB cAMP-binding domain from PKA

(pGloSensor-20F, Promega), which produces rapid and reversible cAMP-dependent activation of luciferase activity in intact cells. Cells were plated in 24-well dishes containing approximately 200,000 cells per well in 500 µl DMEM without phenol red and no serum and equilibrated to 37 °C in a light-proof cabinet. An image of the plate was focused on a 512 × 512 pixel electron multiplying CCD sensor (Hamamatsu C9100-13), cells were equilibrated for 1 h in the presence of 250 µg ml^{-1} luciferin (Biogold), and sequential luminescence images were collected every 10 s to obtain basal luminescence values. The camera shutter was closed, the cabinet opened and the indicated concentration of isoprenaline was bath applied, with gentle manual rocking before replacing in the dark cabinet and resuming luminescence image acquisition. In endocytic manipulation experiments, cells were pre-incubated with 30 µM Dyngo-4a (abcam Biochemicals) for 15 min. Every 10 s, sequential images were acquired using Micro-Manager (http://www.micro-manager.org) and integrated luminescence intensity detected from each well was calculated after background subtraction and correction for vignetting using scripts written in MATLAB (MathWorks). In each multiwell plate, and for each experimental condition, a reference value of luminescence was measured in the presence of 5 µM forskolin, a manipulation that stimulates a moderate amount of receptor-independent activation of adenylyl cyclase. The average luminescence value—measured across duplicate wells—was normalized to the maximum luminescence value measured in the presence of 5 µM forskolin.

Statistical Analysis

Results are displayed as mean of results from each experiment or data set, as indicated in Figure legends. The statistical significance between conditions for experiments with two conditions was calculated using paired, two tailed t-tests. All statistical calculations were performed using Excel (Microsoft Office) or Prism (GraphPad). The threshold for significance was $p<0.05$ and the coding for significance is reported as follows: (n.s.) $p>0.05$, (*) $p\leq0.05$, (**) $p\leq0.01$.

2.7 References

Baldwin, Tanya A., Yong Li, Cameron S. Brand, Val J. Watts, and Carmen W. Dessauer. 2019. “Insights into the Regulatory Properties of Human Adenylyl Cyclase Type 9.” *Molecular Pharmacology* 95 (4): 349–60.

Billington, C. K., I. P. Hall, S. J. Mundell, J. L. Parent, R. A. Panettieri Jr, J. L. Benovic, and R. B. Penn. 1999. “Inflammatory and Contractile Agents Sensitize Specific Adenylyl Cyclase Isoforms in Human Airway Smooth Muscle.” *American Journal of Respiratory Cell and Molecular Biology* 21 (5): 597–606.

Birnbaumer, M. 2000. “Vasopressin Receptors.” *Trends in Endocrinology and Metabolism: TEM* 11 (10): 406–10.

Caldieri, Giusi, and Sara Sigismund. 2016. “Spatial Resolution of cAMP Signaling by Soluble Adenylyl Cyclase.” *The Journal of Cell Biology* 214 (2): 125–27.

Calebiro, Davide, Viacheslav O. Nikolaev, Maria Cristina Gagliani, Tiziana de Filippis, Christian Dees, Carlo Tacchetti, Luca Persani, and Martin J. Lohse. 2009. “Persistent cAMP-Signals Triggered by Internalized G-Protein–Coupled Receptors.” *PLoS Biology* 7 (8): e1000172.

Cancino, Jorge, Anita Capalbo, Antonella Di Campli, Monica Giannotta, Riccardo Rizzo, Juan E. Jung, Rosaria Di Martino, et al. 2014. “Control Systems of Membrane Transport at the Interface between the Endoplasmic Reticulum and the Golgi.” *Developmental Cell* 30 (3): 280–94.

Charest, Pascale G., and Michel Bouvier. 2003. “Palmitoylation of the V2 Vasopressin Receptor Carboxyl Tail Enhances Beta-Arrestin Recruitment Leading to Efficient

Receptor Endocytosis and ERK1/2 Activation." *The Journal of Biological Chemistry* 278 (42): 41541–51.

Cheng, H., and M. G. Farquhar. 1976. "Presence of Adenylate Cyclase Activity in Golgi and Other Fractions from Rat Liver. II. Cytochemical Localization within Golgi and ER Membranes." *The Journal of Cell Biology* 70 (3): 671–84.

Chen, Y., A. Harry, J. Li, M. J. Smit, X. Bai, R. Magnusson, J. P. Pieroni, G. Weng, and R. Iyengar. 1997. "Adenylyl Cyclase 6 Is Selectively Regulated by Protein Kinase A Phosphorylation in a Region Involved in Galphas Stimulation." *Proceedings of the National Academy of Sciences of the United States of America* 94 (25): 14100–104.

Cottrell, Graeme S., Benjamin E. Padilla, Silvia Amadesi, Daniel P. Poole, Jane E. Murphy, Markus Hardt, Dirk Roosterman, Martin Steinhoff, and Nigel W. Bunnett. 2009. "Endosomal Endothelin-Converting Enzyme-1: A Regulator of Beta-Arrestin-Dependent ERK Signaling." *The Journal of Biological Chemistry* 284 (33): 22411–25.

Dessauer, C. W., B. A. Posner, and A. G. Gilman. 1996. "Visualizing Signal Transduction: Receptors, G-Proteins, and Adenylate Cyclases." *Clinical Science* 91 (5): 527–37.

Ferrandon, Sébastien, Timothy N. Feinstein, Marian Castro, Bin Wang, Richard Bouley, John T. Potts, Thomas J. Gardella, and Jean-Pierre Vilardaga. 2009. "Sustained Cyclic AMP Production by Parathyroid Hormone Receptor Endocytosis." *Nature Chemical Biology* 5 (10): 734–42.

Flesch, Brigitte, Flesch Brigitte, Ntambi Elisabeth, and Neppert Jürgen. 1995.

"Biotinylation: A Nonradioactive Method for the Identification of Cell Surface Antigens in Immunoprecipitates." *Electrophoresis* 16 (1): 757–62.

Gao, Mei Hua, Tong Tang, Ngai Chin Lai, Atsushi Miyanohara, Tracy Guo, Rouying Tang, Amy L. Firth, Jason X. Yuan, and H. Kirk Hammond. 2011. "Beneficial Effects of Adenylyl Cyclase Type 6 (AC6) Expression Persist Using a Catalytically Inactive AC6 Mutant." *Molecular Pharmacology* 79 (3): 381–88.

Gilman, A. G. 1987. "G Proteins: Transducers of Receptor-Generated Signals." *Annual Review of Biochemistry* 56: 615–49.

———. 1989. "Transmembrane Signaling, G Proteins, and Adenylyl Cyclase." *Harvey Lectures* 85: 153–72.

Goodman, O. B., Jr, J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic. 1996. "Beta-Arrestin Acts as a Clathrin Adaptor in Endocytosis of the beta2-Adrenergic Receptor." *Nature* 383 (6599): 447–50.

Hacker, B. M., J. E. Tomlinson, G. A. Wayman, R. Sultana, G. Chan, E. Villacres, C. Disteche, and D. R. Storm. 1998. "Cloning, Chromosomal Mapping, and Regulatory Properties of the Human Type 9 Adenylyl Cyclase (ADCY9)." *Genomics* 50 (1): 97–104.

Hammond, John C., Robert E. McCullumsmith, Adam J. Funk, Vahram Haroutunian, and James H. Meador-Woodruff. 2010. "Evidence for Abnormal Forward Trafficking of AMPA Receptors in Frontal Cortex of Elderly Patients with Schizophrenia." *Neuropsychopharmacology: Official Publication of the American College of*

Neuropsychopharmacology 35 (10): 2110–19.

Inda, Carolina, Paula A. dos Santos Claro, Juan J. BonFigurelio, Sergio A. Senin, Giuseppina Maccarrone, Christoph W. Turck, and Susana Silberstein. 2016. "Different cAMP Sources Are Critically Involved in G Protein–coupled Receptor CRHR1 Signaling." *The Journal of Cell Biology* 214 (2): 181–95.

Innamorati, G., H. Sadeghi, A. N. Eberle, and M. Birnbaumer. 1997. "Phosphorylation of the V2 Vasopressin Receptor." *The Journal of Biological Chemistry* 272 (4): 2486–92.

Innamorati, G., H. M. Sadeghi, N. T. Tran, and M. Birnbaumer. 1998. "A Serine Cluster Prevents Recycling of the V2 Vasopressin Receptor." *Proceedings of the National Academy of Sciences of the United States of America* 95 (5): 2222–26.

Irannejad, Roshanak, Irannejad Roshanak, Nikoleta G. Tsvetanova, Braden T. Lobingier, and Mark von Zastrow. 2015. "Effects of Endocytosis on Receptor-Mediated Signaling." *Current Opinion in Cell Biology* 35: 137–43.

Irannejad, Roshanak, Jin C. Tomshine, Jon R. Tomshine, Michael Chevalier, Jacob P. Mahoney, Jan Steyaert, Søren G. F. Rasmussen, et al. 2013. "Conformational Biosensors Reveal GPCR Signalling from Endosomes." *Nature* 495 (7442): 534–38.

Jean-Alphonse, Frédéric G., Vanessa L. Wehbi, Jingming Chen, Masaki Noda, Juan M. Taboas, Kunhong Xiao, and Jean-Pierre Vilardaga. 2017. "β2-Adrenergic Receptor Control of Endosomal PTH Receptor Signaling via Gβγ." *Nature Chemical Biology* 13 (3): 259–61.

Kieffer, Brigitte L., and Christopher J. Evans. 2009. “Opioid Receptors: From Binding Sites to Visible Molecules in Vivo.” *Neuropharmacology* 56 Suppl 1: 205–12.

Kotowski, Sarah J., F. Woodward Hopf, Taban Seif, Antonello Bonci, and Mark von Zastrow. 2011. “Endocytosis Promotes Rapid Dopaminergic Signaling.” *Neuron* 71 (2): 278–90.

Kriebel, Paul W., Valarie A. Barr, Erin C. Rericha, Guofeng Zhang, and Carole A. Parent. 2008. “Collective Cell Migration Requires Vesicular Trafficking for Chemoattractant Delivery at the Trailing Edge.” *The Journal of Cell Biology* 183 (5): 949–61.

Krupinski, J., F. Coussen, H. A. Bakalyar, W. J. Tang, P. G. Feinstein, K. Orth, C. Slaughter, R. R. Reed, and A. G. Gilman. 1989. “Adenylyl Cyclase Amino Acid Sequence: Possible Channel- or Transporter-like Structure.” *Science* 244 (4912): 1558–64.

Lauffer, Benjamin E. L., Cristina Melero, Paul Temkin, Cai Lei, Wanjin Hong, Tanja Kortemme, and Mark von Zastrow. 2010. “SNX27 Mediates PDZ-Directed Sorting from Endosomes to the Plasma Membrane.” *The Journal of Cell Biology* 190 (4): 565–74.

Lefkowitz, R. J. 2007. “Seven Transmembrane Receptors: Something Old, Something New.” *Acta Physiologica* 190 (1): 9–19.

Lohse, Martin J., and Davide Calebiro. 2013. “Cell Biology: Receptor Signals Come in Waves.” *Nature* 495 (7442): 457–58.

Lyga, Sandra, Silvia Volpe, Ruth C. Werthmann, Konrad Götz, Titiwat Sungkaworn,

Martin J. Lohse, and Davide Calebiro. 2016. “Persistent cAMP Signaling by Internalized LH Receptors in Ovarian Follicles.” *Endocrinology*, February, en20151945.

Marrari, Yannick, Marykate Crouthamel, Roshanak Irannejad, and Philip B. Wedegaertner. 2007. “Assembly and Trafficking of Heterotrimeric G Proteins.” *Biochemistry* 46 (26): 7665–77.

Masters, S. B., R. T. Miller, M. H. Chi, F. H. Chang, B. Beiderman, N. G. Lopez, and H. R. Bourne. 1989. “Mutations in the GTP-Binding Site of GS Alpha Alter Stimulation of Adenylyl Cyclase.” *The Journal of Biological Chemistry* 264 (26): 15467–74.

Oakley, R. H., S. A. Laporte, J. A. Holt, L. S. Barak, and M. G. Caron. 1999. “Association of Beta-Arrestin with G Protein-Coupled Receptors during Clathrin-Mediated Endocytosis Dictates the Profile of Receptor Resensitization.” *The Journal of Biological Chemistry* 274 (45): 32248–57.

O’Hayre, Morgan, Kelsie Eichel, Silvia Avino, Xuefeng Zhao, Dana J. Steffen, Xiaodong Feng, Kouki Kawakami, et al. 2017. “Genetic Evidence That β-Arrestins Are Dispensable for the Initiation of β2-Adrenergic Receptor Signaling to ERK.” *Science Signaling* 10 (484). https://doi.org/10.1126/scisignal.aal3395.

Paterson, J. M., S. M. Smith, J. Simpson, O. C. Grace, A. A. Sosunov, J. E. Bell, and F. A. Antoni. 2000. “Characterisation of Human Adenylyl Cyclase IX Reveals Inhibition by Ca(2+)/Calcineurin and Differential mRNA Plyadenylation.” *Journal of Neurochemistry* 75 (4): 1358–67.

Premont, R. T., I. Matsuoka, M. G. Mattei, Y. Pouille, N. Defer, and J. Hanoune. 1996.

"Identification and Characterization of a Widely Expressed Form of Adenylyl Cyclase." *The Journal of Biological Chemistry* 271 (23): 13900–907.

Rochdi, Moulay D., Gabriel A. Vargas, Eric Carpentier, Geneviève Oligny-Longpré, Stanford Chen, Abraham Kovoor, Stephen E. Gitelman, Stephen M. Rosenthal, Mark von Zastrow, and Michel Bouvier. 2010. "Functional Characterization of Vasopressin Type 2 Receptor Substitutions (R137H/C/L) Leading to Nephrogenic Diabetes Insipidus and Nephrogenic Syndrome of Inappropriate Antidiuresis: Implications for Treatments." *Molecular Pharmacology* 77 (5): 836–45.

Rosenbaum, Daniel M., Søren G. F. Rasmussen, and Brian K. Kobilka. 2009. "The Structure and Function of G-Protein-Coupled Receptors." *Nature* 459 (7245): 356–63.

Sadana, Rachna, Sadana Rachna, and Carmen W. Dessauer. 2009. "Physiological Roles for G Protein-Regulated Adenylyl Cyclase Isoforms: Insights from Knockout and Overexpression Studies." *Neuro-Signals* 17 (1): 5–22.

Shukla, Arun K., Kunhong Xiao, and Robert J. Lefkowitz. 2011. "Emerging Paradigms of β-Arrestin-Dependent Seven Transmembrane Receptor Signaling." *Trends in Biochemical Sciences* 36 (9): 457–69.

Sibley, D. R., R. H. Strasser, J. L. Benovic, K. Daniel, and R. J. Lefkowitz. 1986. "Phosphorylation/dephosphorylation of the Beta-Adrenergic Receptor Regulates Its Functional Coupling to Adenylate Cyclase and Subcellular Distribution." *Proceedings of the National Academy of Sciences* 83 (24): 9408–12.

Slessareva, Janna E., Sheri M. Routt, Brenda Temple, Vytas A. Bankaitis, and Henrik

G. Dohlman. 2006. “Activation of the Phosphatidylinositol 3-Kinase Vps34 by a G Protein Alpha Subunit at the Endosome.” *Cell* 126 (1): 191–203.

Small, Kersten M., Kari M. Brown, Cheryl T. Theiss, Carrie A. Seman, Scott T. Weiss, and Stephen B. Liggett. 2003. “An Ile to Met Polymorphism in the Catalytic Domain of Adenylyl Cyclase Type 9 Confers Reduced ??2-Adrenergic Receptor Stimulation.” *Pharmacogenetics* 13 (9): 535–41.

Soto-Velasquez, Monica, Michael P. Hayes, Aktan Alpsoy, Emily C. Dykhuizen, and Val J. Watts. 2018. “A Novel CRISPR/Cas9-Based Cellular Model to Explore Adenylyl Cyclase and cAMP Signaling.” *Molecular Pharmacology* 94 (3): 963–72.

Spiegel, Allen M. 1987. “Signal Transduction by Guanine Nucleotide Binding Proteins.” *Molecular and Cellular Endocrinology* 49 (1): 1–16.

Staehelin, M., P. Simons, K. Jaeggi, and N. Wigger. 1983. “CGP-12177. A Hydrophilic Beta-Adrenergic Receptor Radioligand Reveals High Affinity Binding of Agonists to Intact Cells.” *The Journal of Biological Chemistry* 258 (6): 3496–3502.

Stallaert, Wayne, Emma T. van der Westhuizen, Anne-Marie Schönegge, Bianca Plouffe, Mireille Hogue, Viktoria Lukashova, Asuka Inoue, et al. 2017. “Purinergic Receptor Transactivation by the β2-Adrenergic Receptor Increases Intracellular Ca2+ in Nonexcitable Cells.” *Molecular Pharmacology* 91 (5): 533–44.

Stoeber, Miriam, Damien Jullié, Braden T. Lobingier, Toon Laeremans, Jan Steyaert, Peter W. Schiller, Aashish Manglik, and Mark von Zastrow. 2018. “A Genetically Encoded Biosensor Reveals Location Bias of Opioid Drug Action.” *Neuron* 98 (5): 963–76.e5.

Sunahara, R. K. 1996. “Complexity and Diversity of Mammalian Adenylyl Cyclases.” *Annual Review of Pharmacology and Toxicology* 36 (1): 461–80.

Tang, Yuting, Liaoyuan A. Hu, William E. Miller, Niels Ringstad, Randy A. Hall, Julie A. Pitcher, Pietro DeCamilli, and Robert J. Lefkowitz. 1999. “Identification of the Endophilins (SH3p4/p8/p13) as Novel Binding Partners for the β1-Adrenergic Receptor.” *Proceedings of the National Academy of Sciences* 96 (22): 12559–64.

Tantisira, K. G. 2005. “Molecular Properties and Pharmacogenetics of a Polymorphism of Adenylyl Cyclase Type 9 in Asthma: Interaction between -Agonist and Corticosteroid Pathways.” *Human Molecular Genetics* 14 (12): 1671–77.

Taylor, Susan S., Ping Zhang, Jon M. Steichen, Malik M. Keshwani, and Alexandr P. Kornev. 2013. “PKA: Lessons Learned after Twenty Years.” *Biochimica et Biophysica Acta* 1834 (7): 1271–78.

Temkin, Paul, Temkin Paul, Lauffer Ben, Jäger Stefanie, Cimermancic Peter, Nevan J. Krogan, and Mark von Zastrow. 2011. “SNX27 Mediates Retromer Tubule Entry and Endosome-to-Plasma Membrane Trafficking of Signalling Receptors.” *Nature Cell Biology* 13 (6): 717–23.

Tesmer, J. J., R. K. Sunahara, A. G. Gilman, and S. R. Sprang. 1997. “Crystal Structure of the Catalytic Domains of Adenylyl Cyclase in a Complex with Gsalpha.GTPgammaS.” *Science* 278 (5345): 1907–16.

Thomsen, Alex R. B., Bianca Plouffe, Thomas J. Cahill 3rd, Arun K. Shukla, Jeffrey T. Tarrasch, Annie M. Dosey, Alem W. Kahsai, et al. 2016. “GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling.” *Cell*

166 (4): 907–19.

Tsvetanova, Nikoleta G., Michelle Trester-Zedlitz, Billy W. Newton, Daniel P. Riordan, Aparna B. Sundaram, Jeffrey R. Johnson, Nevan J. Krogan, and Mark von Zastrow. 2016. “G Protein–Coupled Receptor Endocytosis Confers Uniformity in Responses to Chemically Distinct Ligands.” *Molecular Pharmacology* 91 (2): 145–56.

Vilardaga, Jean-Pierre, Thomas J. Gardella, Vanessa L. Wehbi, and Timothy N. Feinstein. 2012. “Non-Canonical Signaling of the PTH Receptor.” *Trends in Pharmacological Sciences* 33 (8): 423–31.

Vilardaga, Jean-Pierre, Frederic G. Jean-Alphonse, and Thomas J. Gardella. 2014. “Endosomal Generation of cAMP in GPCR Signaling.” *Nature Chemical Biology* 10 (9): 700–706.

Violin, Jonathan D., Lisa M. DiPilato, Necmettin Yildirim, Timothy C. Elston, Jin Zhang, and Robert J. Lefkowitz. 2008. “beta2-Adrenergic Receptor Signaling and Desensitization Elucidated by Quantitative Modeling of Real Time cAMP Dynamics.” *The Journal of Biological Chemistry* 283 (5): 2949–61.

Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. 1983. “Characterization of an Altered Membrane Form of the Beta-Adrenergic Receptor Produced during Agonist-Induced Desensitization.” *The Journal of Biological Chemistry* 258 (22): 13900–908.

Whistler, J. L., and M. von Zastrow. 1998. “Morphine-Activated Opioid Receptors Elude Desensitization by Beta-Arrestin.” *Proceedings of the National Academy of Sciences of the United States of America* 95 (17): 9914–19.

Zaccolo, Manuela, and Tullio Pozzan. 2002. “Discrete Microdomains with High Concentration of cAMP in Stimulated Rat Neonatal Cardiac Myocytes.” *Science* 295 (5560): 1711–15.

Zastrow, M. von, and B. K. Kobilka. 1992. “Ligand-Regulated Internalization and Recycling of Human Beta 2-Adrenergic Receptors between the Plasma Membrane and Endosomes Containing Transferrin Receptors.” *The Journal of Biological Chemistry* 267 (5): 3530–38.

2.8 Figures

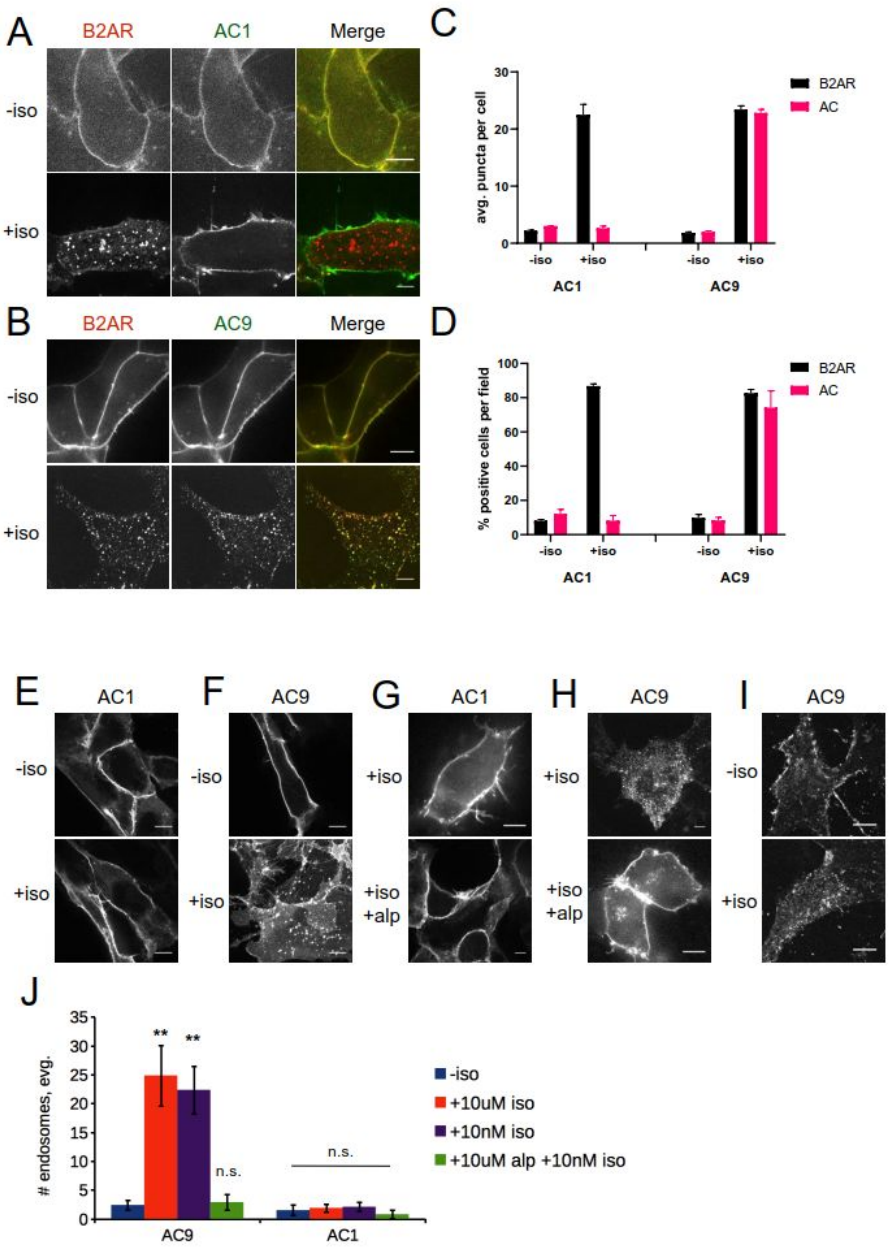

Figure 2.1: β2AR activation causes redistribution of AC9 but not AC1.
(A) Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC1 after treatment with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(B)** Representative confocal images of HEK293 cells coexpressing HA-β2AR and Flag-AC9 after treatment with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(C)** Quantification of internal puncta that are β2AR or AC1/9 positive, taken from wide field images (see: Figure 2.1M, 2.1N) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(D)** Quantification of cells with >10 internal puncta that are β2AR or AC1/9 positive, taken from wide field

images (see: Figure 2.1M, 2.1N) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(E-F)** Representative confocal imaging of HEK293 cells expressing Flag-AC1 **(E)** or Flag-AC9 **(F)** after treatment with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μM. **(G-H)** Representative confocal imaging of HEK293 cells expressing Flag-AC1 **(G)** or Flag-AC9 **(H)**. Cells were stimulated with 100 nM isoproterenol for 30 min with or without 15 min of pretreatment with 10 μM alprenolol. **(I)** Representative confocal images of primary culture human airway smooth muscle cells immunostained for endogenous AC9 after treatment with 10 μM isoproterenol or control for 30 min. Scale Bar is 16 μm. **(J)** Quantification of the number of endosomes in cells from **(Figure 2.1 E-H)** [mean±SEM; n=3 experiments, 25 cells per condition].

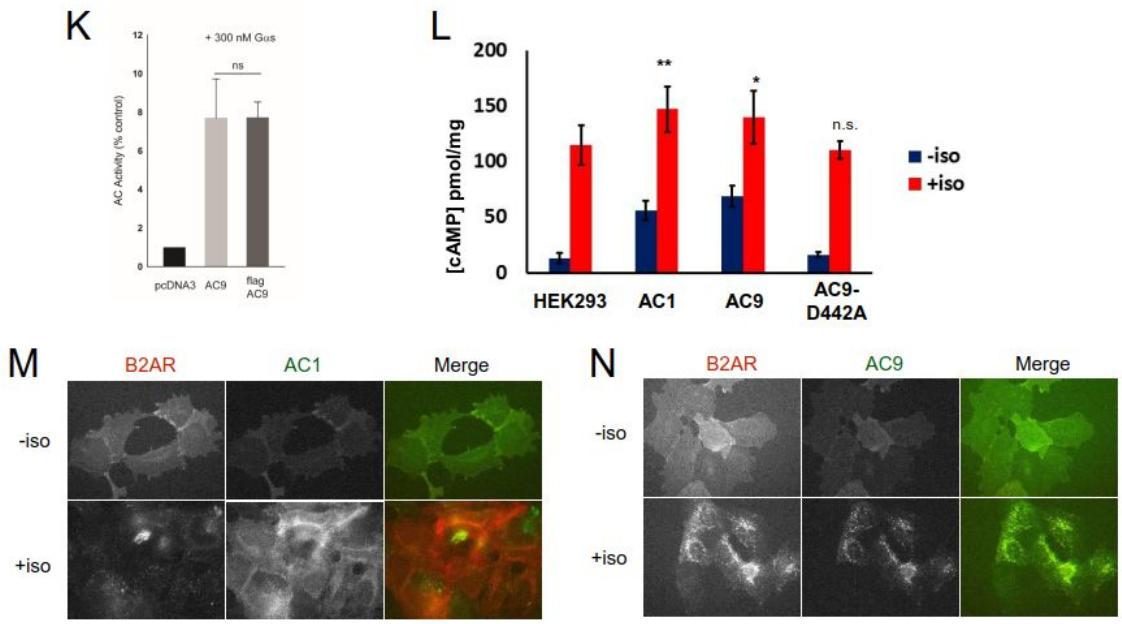

Figure 2.1: β2AR activation causes redistribution of AC9 but not AC1.
(K) Quantification of the in vitro cAMP response by pcDNA3 control, untagged AC9, and Flag-tagged AC9 to 300 nM Gs (alpha subunit). **(L)** Quantification of the cAMP response to 10μM isoproterenol in control HEK293 cells and cells overexpressing Flag-AC1, Flag-AC9, or Flag-AC9-D442A. [mean±SEM; n=3 experiments] **(M)** Wide field images of HEK293 cells coexpressing HA-β2AR and Flag-AC9, after treatment with 10 μm isoproterenol or control for 30 min. Scale bar is 16 μm. **(N)** Wide field images of HEK293 cells coexpressing HA-β2AR and Flag-AC1, after treatment with 10 μm isoproterenol or control for 30 min. Scale bar is 16 μm.

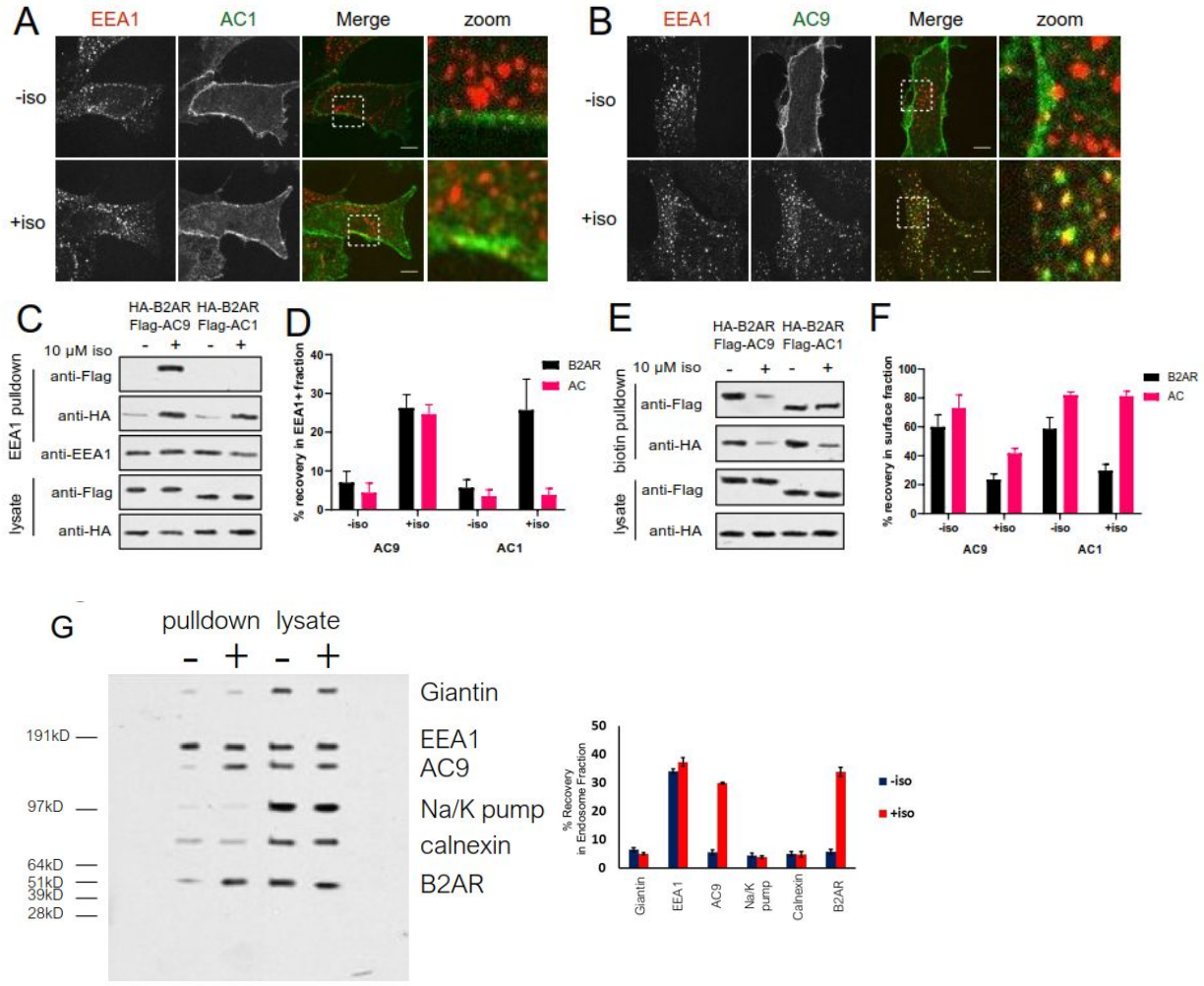

Figure 2.2: Surface AC9 is internalized to early endosomes upon adrenergic stimulation. (A-B) Representative confocal images of HEK293 cells expressing Flag-AC1 **(A)** or Flag-AC9 **(B)** after treatment with 10 μM isoproterenol or control for 30 min and stained for endogenous EEA1. Scale bar is 8 μm. **(C)** Representative western blot of a fraction isolated using antibodies to EEA1. Lanes 1-2 correspond to control HEK293 cells, lanes 3-4 to cells coexpressing Flag-AC9 and HA-β2AR, and lanes 5-6 to cells coexpressing Flag-AC1 and HA-β2AR. **(D)** Quantification of recovery of HA-β2AR, Flag-AC9 and Flag-AC1 in the endosome fraction relative to cell lysate. [mean±SEM; n=7 experiments]. ** P < 0.01 by two-tailed t-test. **(E)** Representative western blot of the surface exposed fraction isolated by surface labeling with Sulfo-NHS-biotin and purified with streptavidin. Lanes 1-2 correspond to cells coexpressing Flag-AC9 and HA-β2AR, and lanes 3-4 to cells coexpressing Flag-AC1 and HA-β2AR. **(F)** Quantification of recovery of HA-β2AR, Flag-AC9 and Flag-AC1 in the surface biotinylated fraction relative to total cell lysate. [mean±SEM; n=7 experiments]. ** P < 0.01 by two-tailed t-test. **(G)** Full western blot of the endosome fraction vs total cell lysate, probed for cellular markers Giantin (Golgi), Na/K pump (plasma membrane), calnexin (endoplasmic reticulum). Quantification of 3 such experiments.

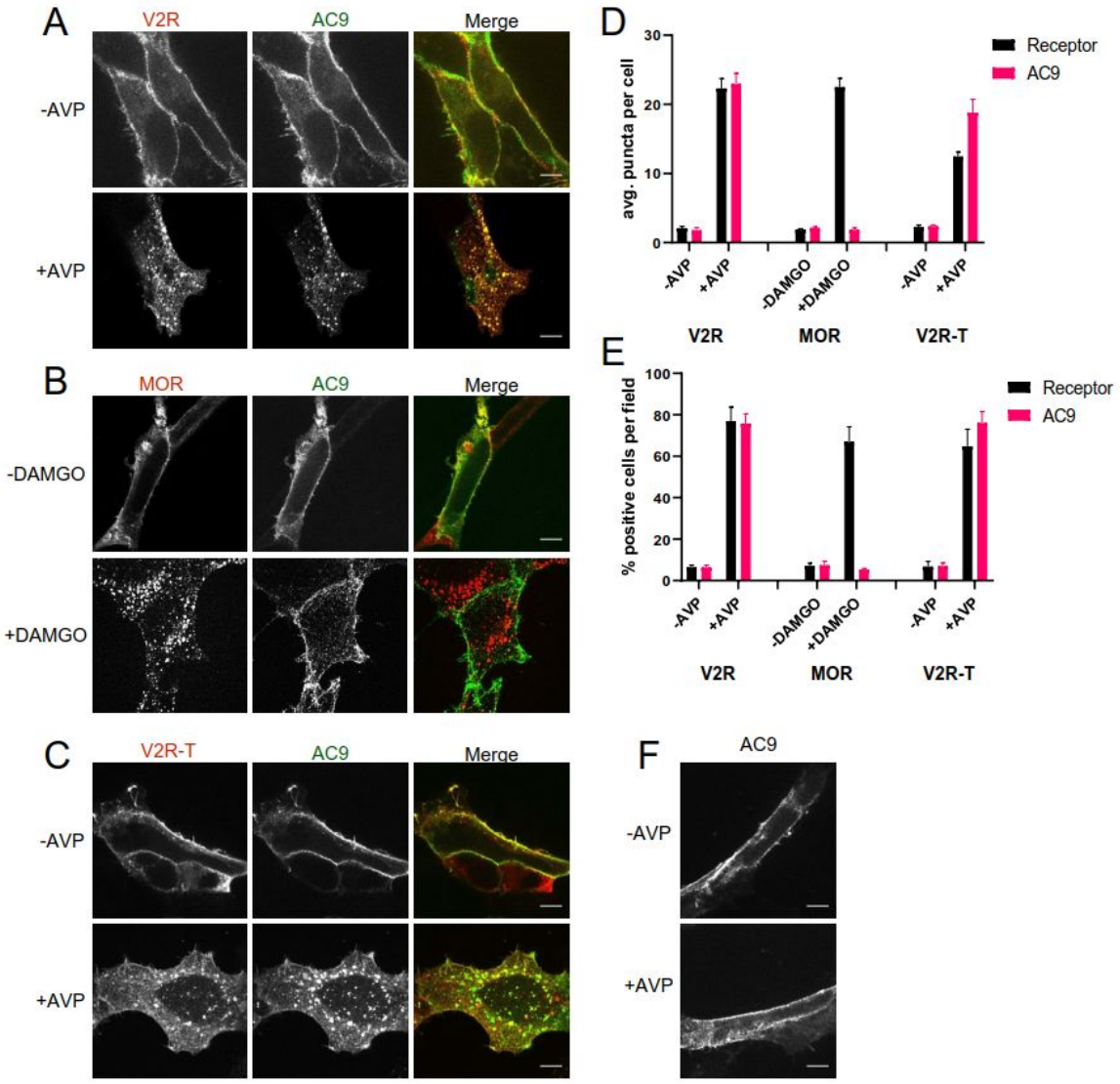

Figure 2.3: V2R, but not MOR, can cause AC9 to localize to early endosomes. **(A-C)** Representative confocal imaging of HEK293 cells coexpressing Flag-AC9 and HA-V2R **(A)**, HA-MOR **(B)**, or HA-V2R-trunc **(C)**, after treatment with 10 μM agonist (AVP or DAMGO) or control for 30 min. Scale Bar is 8 μm. **(D)** Quantification of internal puncta that are V2R, MOR, V2R-T, or AC1/9 positive, taken from wide field images (see: Figure 3J, 3K, 3L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(E)** Quantification of cells with >10 internal puncta that are V2R, MOR, V2R-T or AC1/9 positive, taken from wide field images (see: Figure 3J, 3K, 3L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(F)** Representative confocal imaging of HEK293 cells expressing Flag-AC9, after treatment with 10 μM AVP or control for 30 min. Scale Bar is 8 μm.

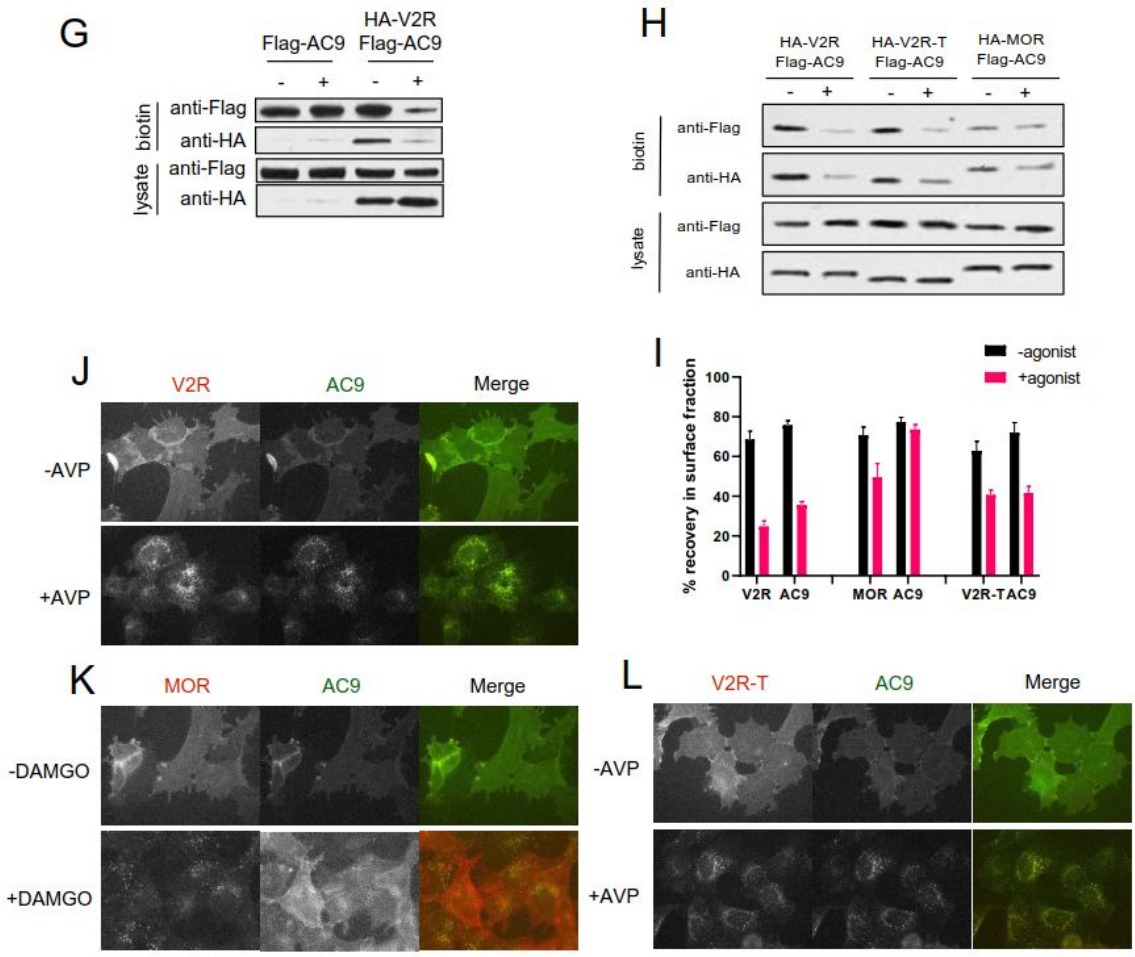

Figure 2.3: V2R, but not MOR, can cause AC9 to localize to early endosomes. **(G)** Representative western blot of the surface biotinylated fraction from HEK293 cells expressing Flag-AC9 (lanes 1-2) or coexpressing HA-V2R and Flag-AC9 (lanes 3-4) [mean±SEM; n=3 experiments]. **(H)** Representative western blot of the surface biotinylated fraction from HEK293 cells coexpressing HA-V2R and Flag-AC9 (lanes 1-2), HA-V2R-T and Flag-AC9 (lanes 3-4), or HA-MOR and Flag-AC9 (lanes 5-6). **(I)** Recovery of tagged protein in the surface biotinylated fraction relative to the total cell lysate as seen in **(H)** [mean±SEM; n=7 experiments]. ** $P < 0.01$ by two-tailed t-test. **(J)** Wide field images of HEK293 cells coexpressing HA-V2R and Flag-AC9, after treatment with 10 μm arginine vasopressin (AVP) or control for 30 min. Scale bar is 16 μm. **(K)** Wide field images of HEK293 cells coexpressing HA-MOR and Flag-AC9, after treatment with 10 μm [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) or control for 30 min. Scale bar is 16 μm. **(L)** Wide field images of HEK293 cells coexpressing HA-V2R-trunc and Flag-AC9, after treatment with 10 μm arginine vasopressin (AVP) or control for 30 min. Scale bar is 16 μm.

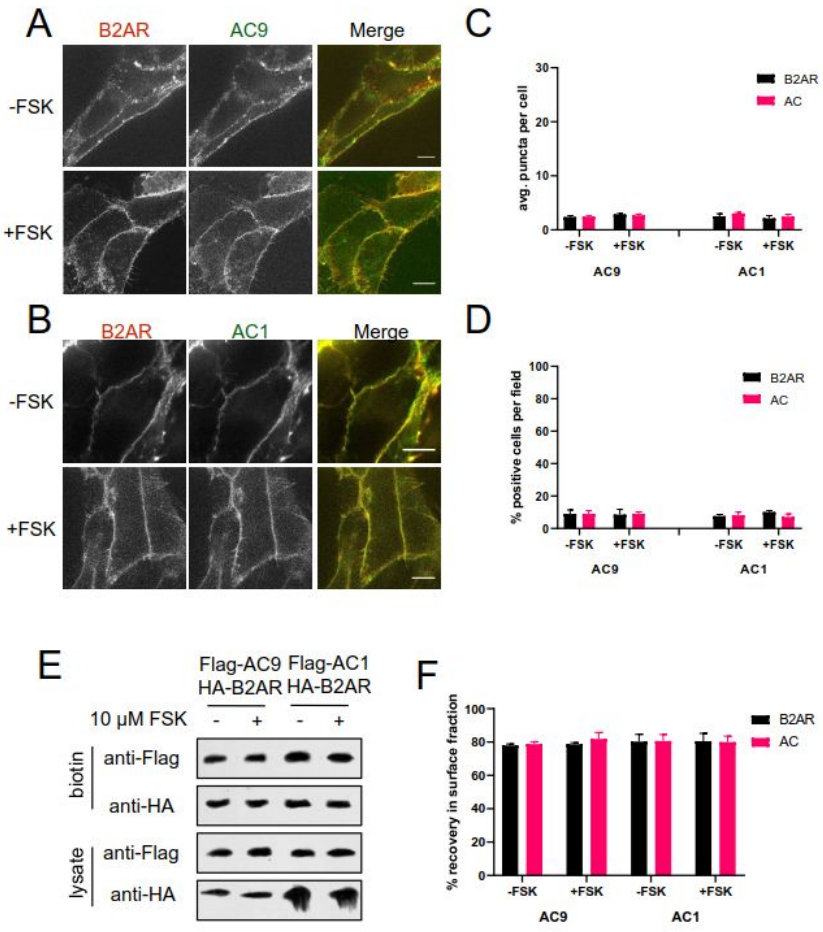

Figure 2.4: Forskolin-promoted cAMP accumulation is insufficient to drive AC9 internalization. (A) Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC9 were treated with 10 μM FSK or control for 30 min. Scale Bar is 8 μm. **(B)** Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC1 were treated with 10 μM FSK or control for 30 min. Scale Bar is 8 μm. **(C)** Quantification of internal puncta that are β2AR or AC1/9 positive, taken from wide field images (see: Figure 4I, 4J) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test. **(D)** Quantification of cells with >10 internal puncta that are β2AR or AC1/9 positive, taken from wide field images (see: Figure 4I, 4J) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test. **(E)** Representative western blot of the surface biotinylated fraction of cells from **(A,C)**. **(F)** Quantification of the percent loss from the surface biotinylated fraction relative to the total cell lysate as seen in **(E)** [mean±SEM; n=5 experiments].

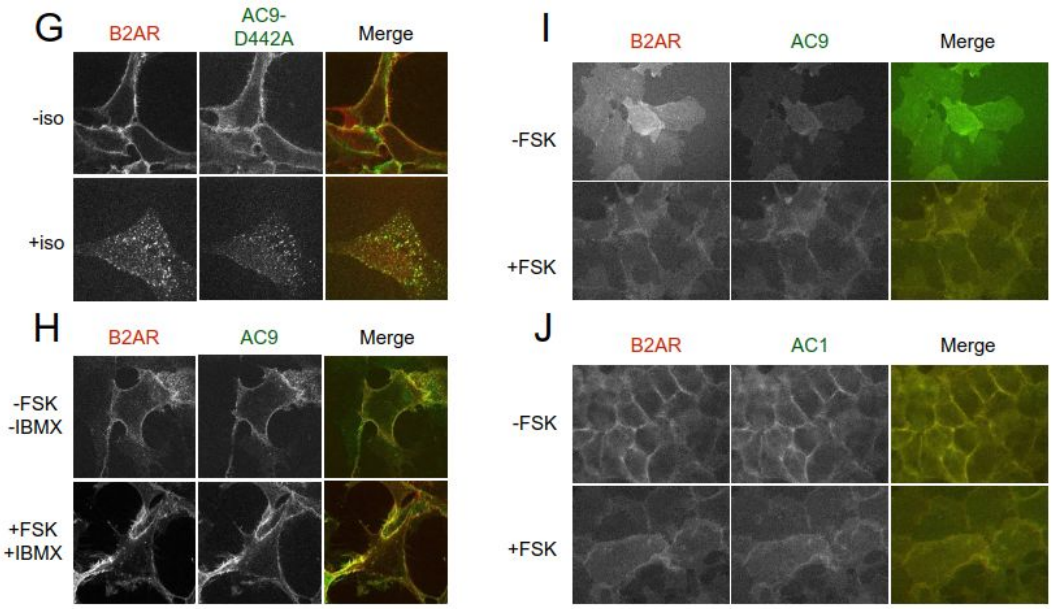

Figure 2.4: Forskolin-promoted cAMP accumulation is insufficient to drive AC9 internalization. (G) Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and the catalytic inactive mutant Flag-AC9-D442A were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(H)** Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC9 were treated with 10 μM FSK and 10 μM IBMX or control for 30 min. Scale Bar is 8 μm. **(I)** Representative wide-field imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC9 which were treated with 10 μM FSK or control for 30 min. **(J)** Representative wide-field imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC1 which were treated with 10 μM FSK or control for 30 min.

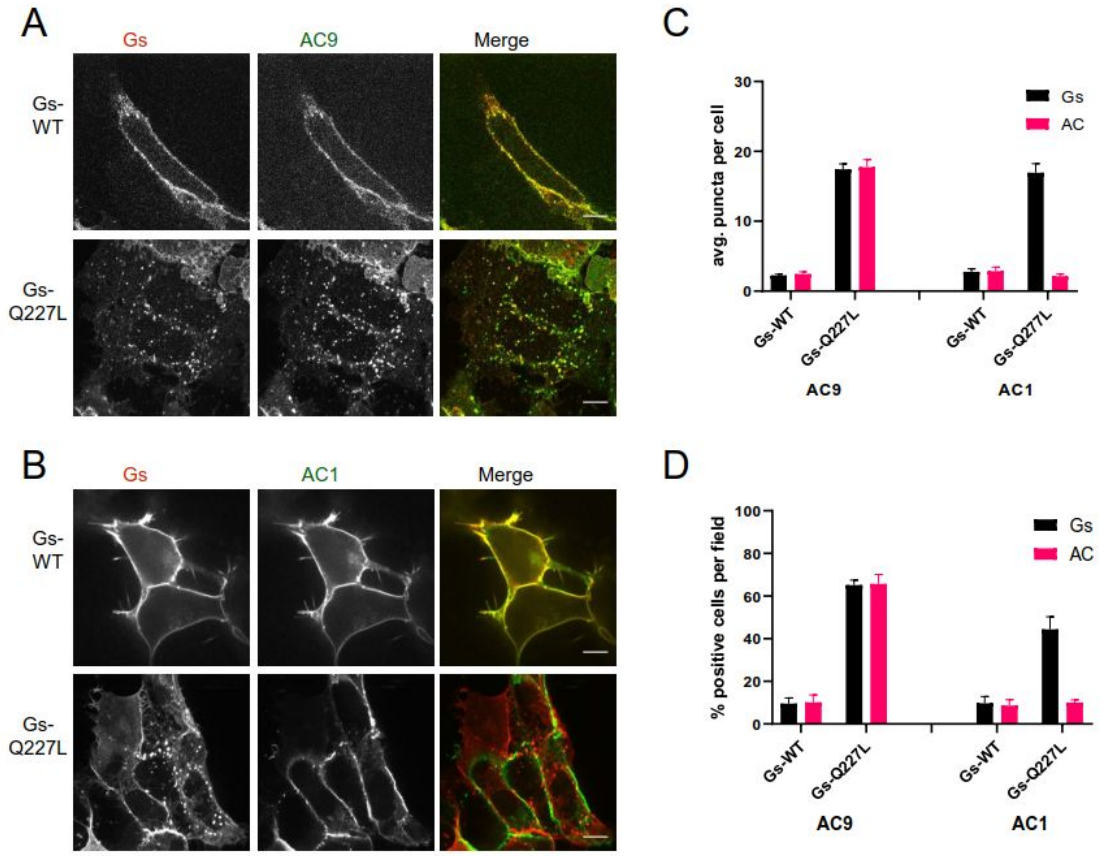

Figure 2.5: Gs activation is sufficient for AC9 to localize to early endosomes. **(A)** Representative confocal imaging of HEK293 cells cells coexpressing Flag-AC9, $G_s\beta$, G_s^{γ} and either HA-$G_s\alpha$ (HA-Gs) or HA-$G_s\alpha$-CA (HA-GsCA). **(B)** Representative confocal imaging of HEK293 cells coexpressing Flag-AC1, $G_s\beta$, G_s^{γ} and either HA-$G_s\alpha$ (HA-Gs) or HA-$G_s\alpha$-CA (HA-GsCA). **(C)** Quantification of internal puncta that are Gs or AC1/9 positive, taken from wide field images (see: Figure 5K, 5L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(D)** Quantification of cells with >10 internal puncta that are Gs or AC1/9 positive, taken from wide field images (see: Figure 5K, 5L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

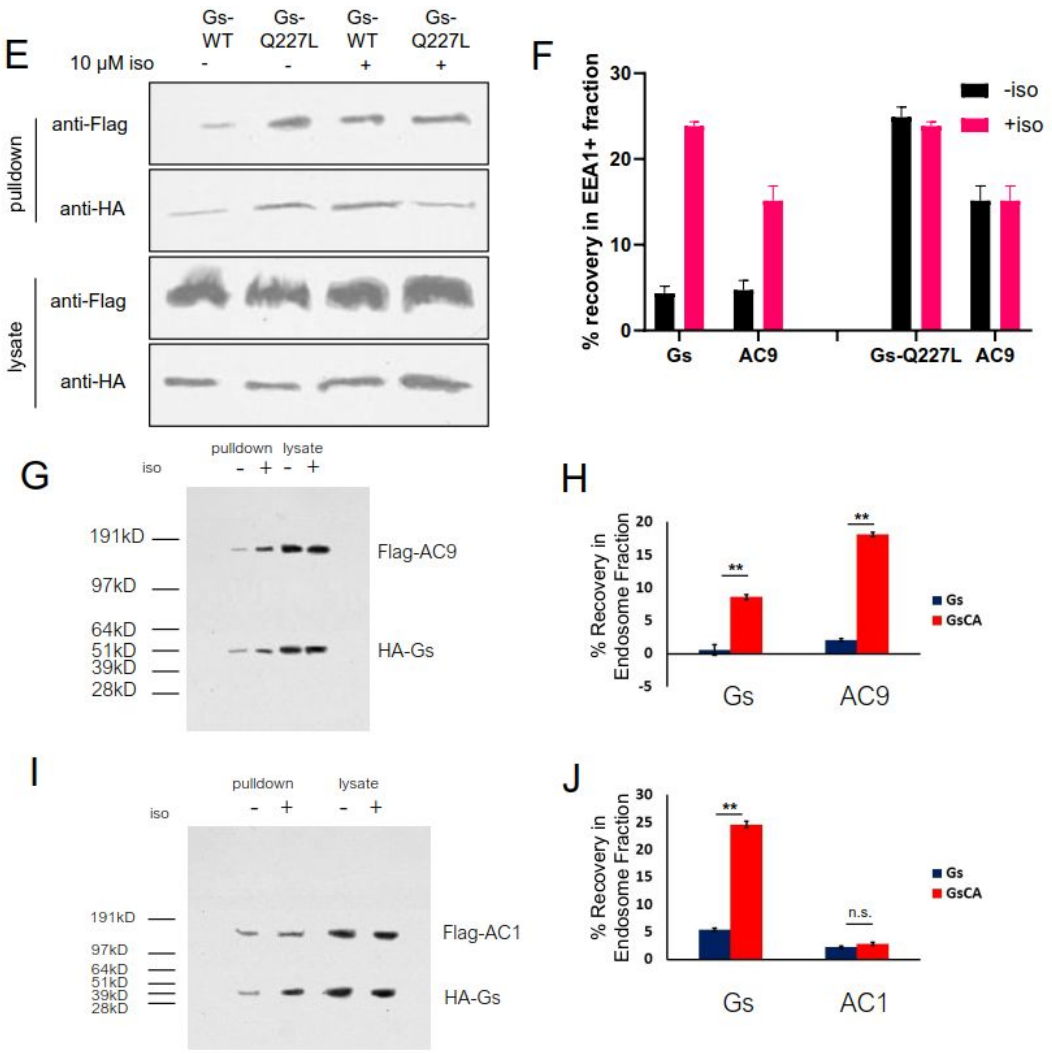

Figure 2.5: Gs activation is sufficient for AC9 to localize to early endosomes. **(E)** Representative western blot of an EEA1+ fraction from HEK293 cells coexpressing Flag-AC9 and HA-Gs (lanes 1 and 3) or Flag-AC9 and HA-GsCA (lanes 2 and 4) and after treatment with 10 μM isoproterenol (lanes 1-2) or control (lanes 3-4) for 30 min. **(F)** Quantification of the fraction of Flag-AC9 and HA-Gs/HA-GsCA recovered in the EEA1+ fraction **(E)** relative to total cell lysate. [mean±SEM; n=3 experiments] * $P < 0.05$ ** $P < 0.01$ by two-tailed t-test. **(G)** Representative western blot of an EEA1 positive endosome fraction from HEK293 cells coexpressing Flag-AC9, $G_s\beta$, G_s^{γ} and HA-Gs (lane 1) or HA-GsCA (lane 2). **(H)** Quantification of endosome enrichment of Flag-AC9 and HA-Gs or HA-GsCA as seen in **(G)** by normalizing to the EEA1 signal. [mean±SEM; n=5 experiments] ** $P < 0.01$ by two-tailed t-test. **(I)** Representative western blot of an EEA1 positive endosome fraction from HEK293 cells coexpressing Flag-AC1, $G_s\beta$, G_s^{γ} and HA-Gs (lane 1) or HA-GsCA (lane 2). **(J)** Quantification of endosome enrichment of Flag-AC1 and HA-Gs or HA-GsCA as seen in **(I)** by normalizing to the EEA1 signal. [mean±SEM; n=5 experiments] ** $P < 0.01$ by two-tailed t-test.

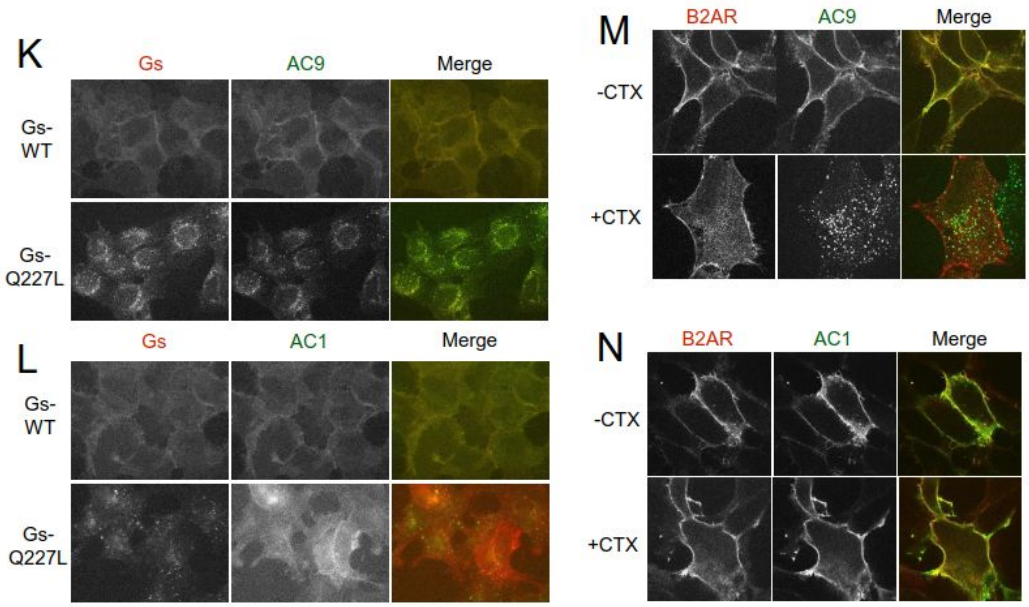

Figure 2.5: Gs activation is sufficient for AC9 to localize to early endosomes. **(K)** Representative wide-field imaging of HEK293 cells coexpressing HA-Gs (wild type) or HA-Gs-Q227L and Flag-AC9. **(L)** Representative wide-field imaging of HEK293 cells coexpressing HA-Gs (wild type) or HA-Gs-Q227L and Flag-AC1. **(M)** Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC9 were treated with 100 nM cholera toxin overnight. Scale Bar is 8 μm. **(N)** Representative confocal imaging of HEK293 cells coexpressing HA-β2AR and Flag-AC1 were treated with 100 nM cholera toxin overnight.

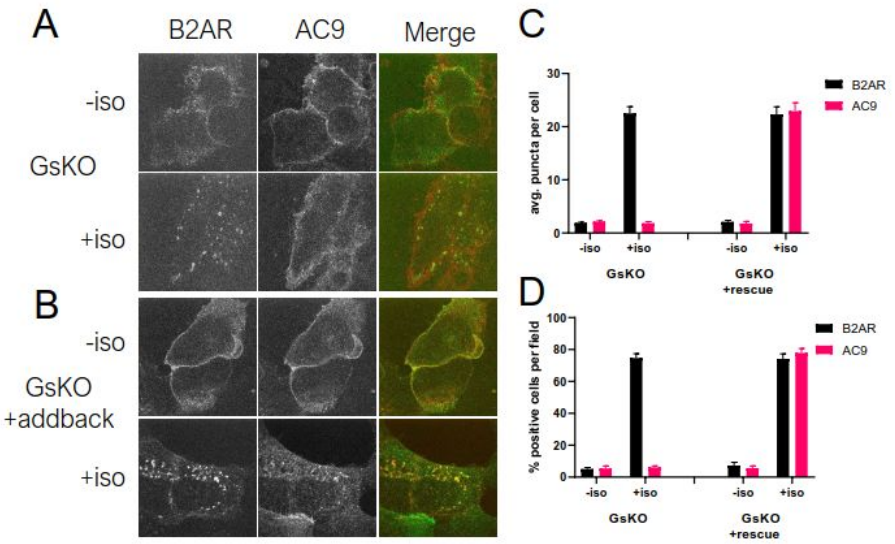

Figure 2.6: Gs activation is necessary for arrestin-independent endocytosis of AC9

(A-B) Representative confocal imaging of Gs knockout (GsKO) HEK293 cells coexpressing Flag-β2AR, AC9-EGFP, and either pcDNA3 **(A)** or wild-type HA-Gs rescue **(B)**. **(C)** Quantification of internal puncta that are β2AR or AC9 positive, taken from wide field images (see: Figure 6I, 6J) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(D)** Quantification of cells with >10 internal puncta that are β2AR or AC9 positive, taken from wide field images (see: Figure 6I, 6J) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

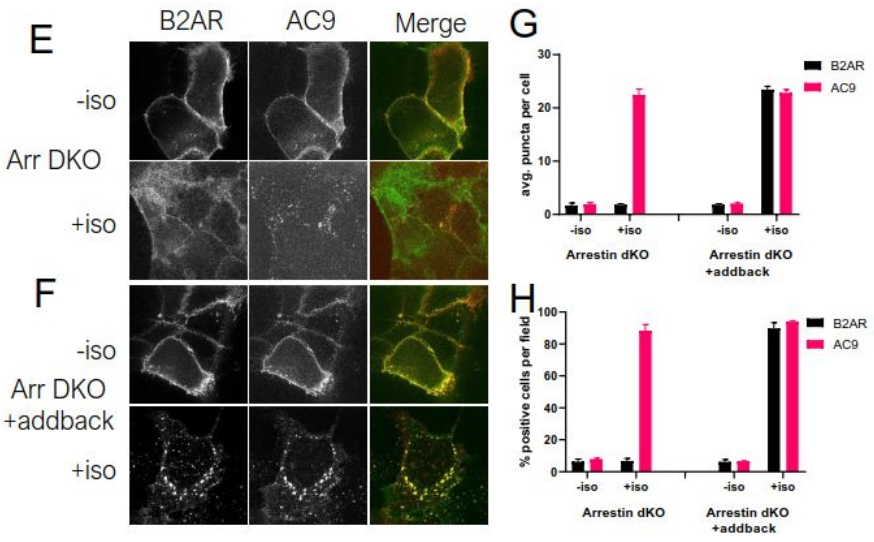

Figure 2.6: Gs activation is necessary for arrestin-independent endocytosis of AC9 (E-F) Representative confocal imaging of Arrestin2/3 double knockout (Arr dKO) cells coexpressing Flag-β2AR, AC9-EGFP, and either pcDNA3 **(D)** or HA-Arrestin 3 rescue **(F)**. **(G)** Quantification of internal puncta that are β2AR or AC9 positive, taken from wide field images (see: Figure 6K, 6L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test. **(H)** Quantification of cells with >10 internal puncta that are Gs or AC1/9 positive, taken from wide field images (see: Figure 6K, 6L) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test.

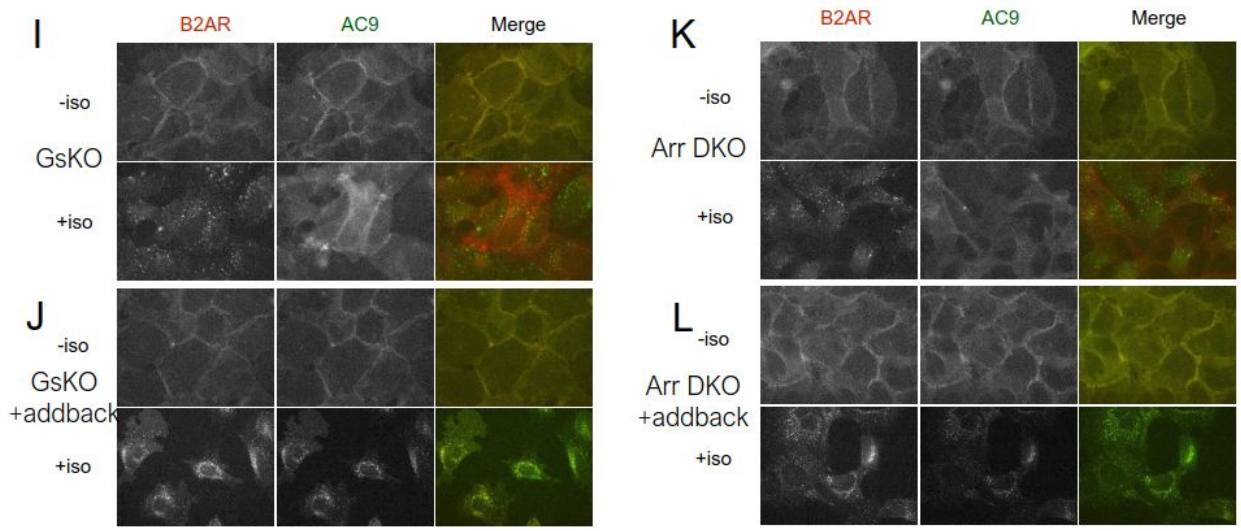

Figure 2.6: Gs activation is necessary for arrestin-independent endocytosis of AC9 (I) Representative wide-field imaging of Gs-knockout HEK293 cells coexpressing HA-β2AR and Flag-AC9 were treated with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(J)** Representative wide-field imaging of Gs-knockout HEK293 cells coexpressing Gs (wild type) HA-β2AR and Flag-AC9 were treated with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(K)** Representative wide-field imaging of Arrestin 2/3 double knockout HEK293 cells coexpressing HA-β2AR and Flag-AC9 were treated with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(L)** Representative wide-field imaging of Arrestin 2/3 double knockout HEK293 cells coexpressing Arrestin 2, HA-β2AR and Flag-AC9 were treated with 10 μM isoproterenol for 30 min.

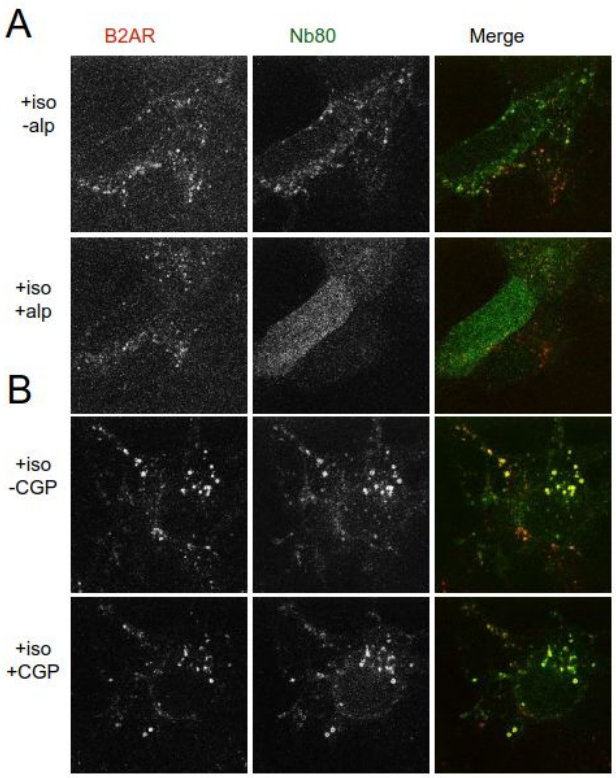

Figure 2.7: AC9 is a significant contributor to the β2AR-mediated cAMP response from endosomes. (A) Recruitment of conformational biosensors to β2AR-containing endosomes is reversed by application of the membrane permeable antagonist alprenolol for 20 min. Scale Bar is 8 μm. **(B)** Recruitment of conformational biosensors to β2AR-containing endosomes is unaffected by application of the membrane impermeable antagonist CGP12177 for 20 min. Scale Bar is 8 μm.

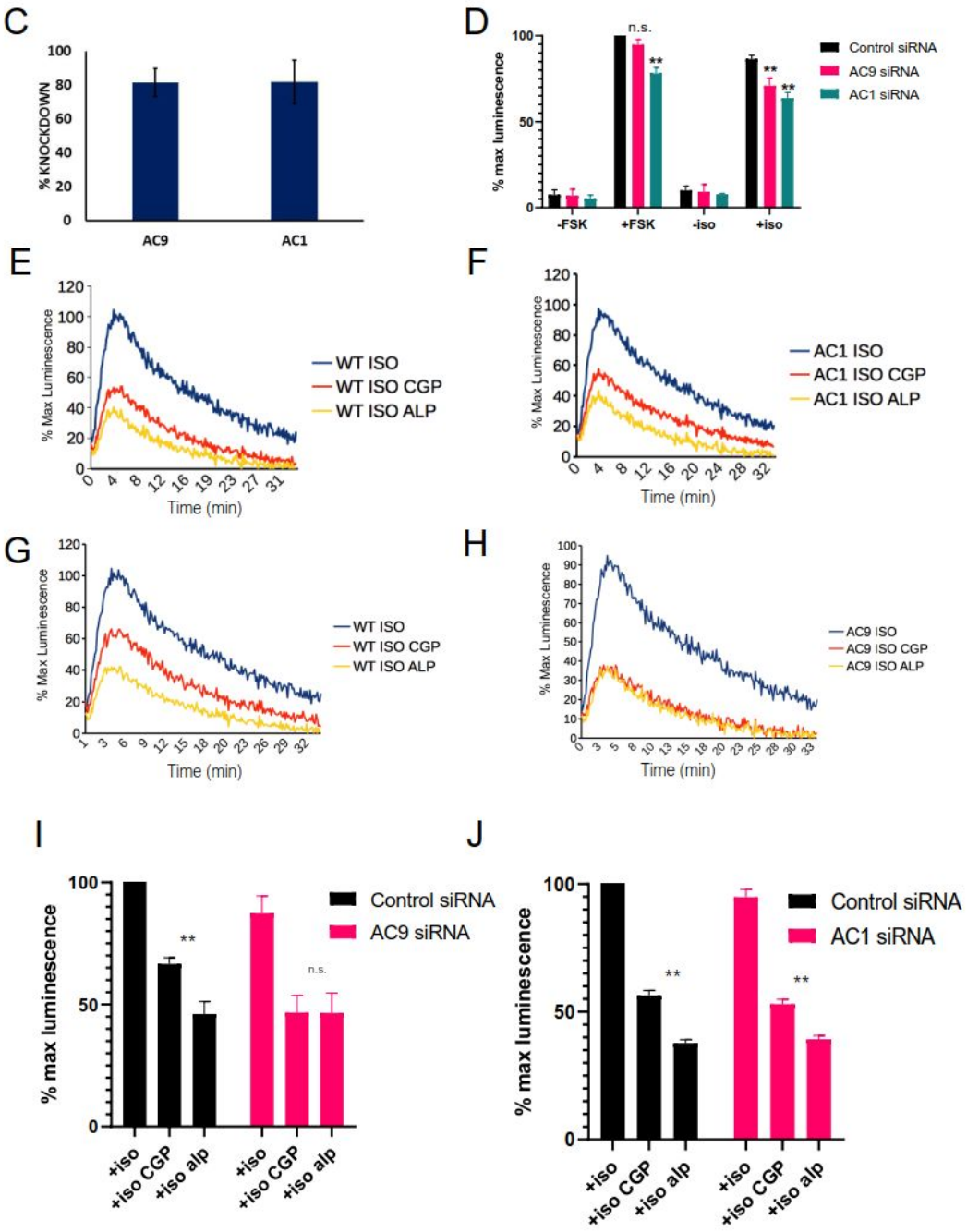

Figure 2.7: AC9 is a significant contributor to the β2AR-mediated cAMP response from endosomes. (C) Percent knockdown of AC9 and AC1 in HEK293 cells as determined by qPCR. [mean±SEM; n=3 experiments]. **(D)** Effect of siRNA knockdown of AC9 and AC1 expression in HEK293 cells on the cAMP response to 10 μM FSK and 10 μM isoproterenol stimulation [mean±SEM; n=4 experiments] * $P < 0.05$ by two-tailed t-test. **(E)** Representative normalized β2AR-mediated cAMP response in control HEK293 cells pretreated with 100nM isoproterenol and exposed to supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol). **(F)** Representative normalized β2AR-mediated cAMP response in AC1 knockdown HEK293 cells

pretreated with 100nM isoproterenol and exposed to supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol). **(G)** Representative normalized β2AR-mediated cAMP response in control HEK293 cells pretreated with 100nM isoproterenol and exposed to supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol). **(H)** Representative normalized β2AR-mediated cAMP response in AC9 knockdown HEK293 cells pretreated with 100nM isoproterenol and exposed to supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol). **(I)** Quantification of the maximum cAMP response in control and in AC9 siRNA knockdown HEK293 cells pretreated with 100nM isoproterenol and exposed to supersaturating conditions of membrane permeable antagonist (10μM alprenolol) or membrane impermeable antagonist (10μM CGP12177). [mean±SEM; n=4 experiments] **(J)** Quantification of the maximum cAMP response in control in AC1 siRNA knockdown HEK293 cells pretreated with 100nM isoproterenol and exposed to supersaturating conditions of membrane permeable antagonist (10μM alprenolol) or membrane impermeable antagonist (10μM CGP12177). [mean±SEM; n=4 experiments].

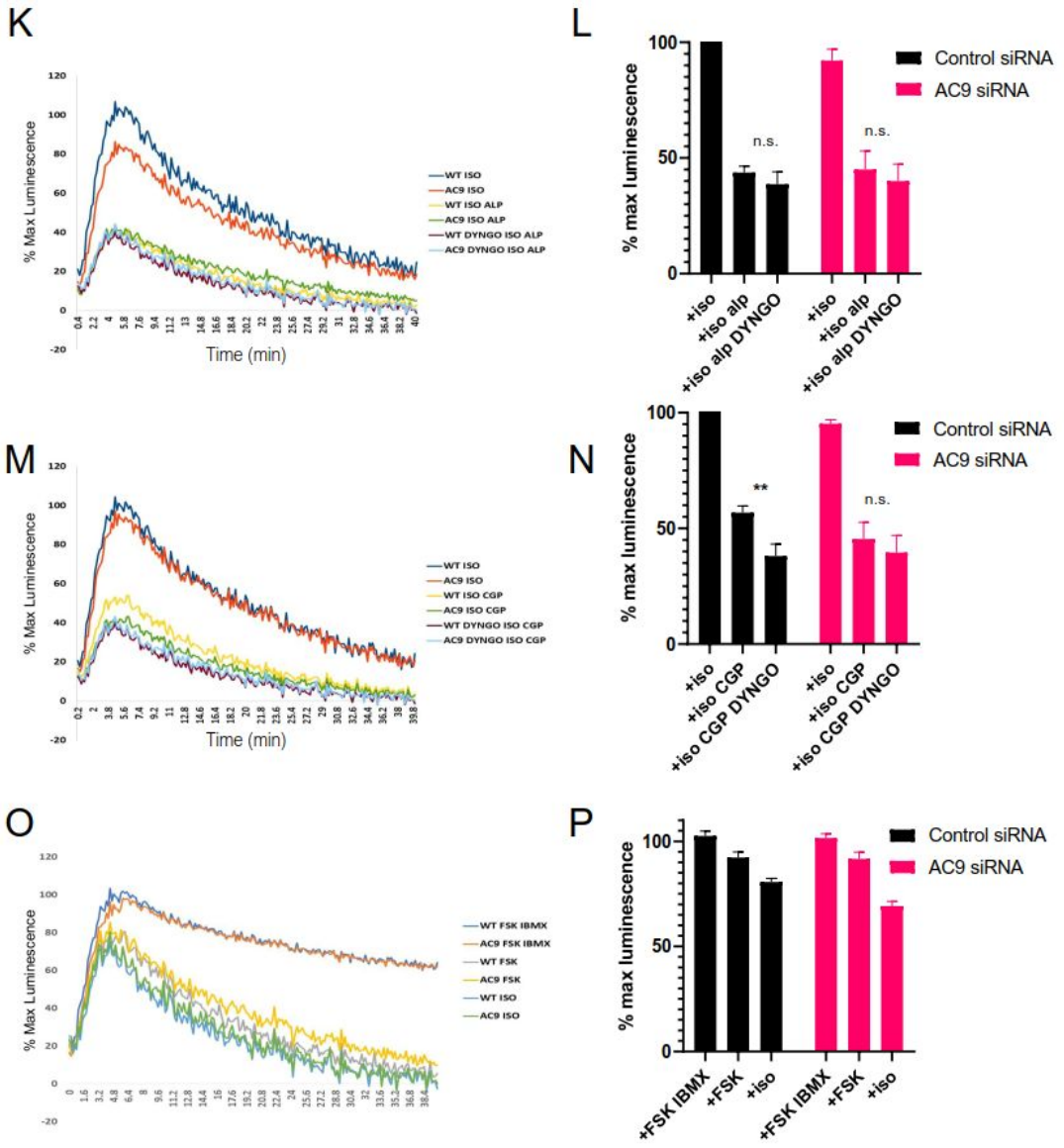

Figure 2.7: AC9 is a significant contributor to the β2AR-mediated cAMP response from endosomes. (K) Representative cAMP response to pretreatment with DYNGO-4a and 100nM isoproterenol, and exposure to 10 μM alprenolol in AC9 knockdown or control HEK293 cells. **(L)** Quantification of the maximum cAMP response as seen in **(A)** [mean±SEM; n=3 experiments]. **(M)** Representative cAMP response to pretreatment with DYNGO-4a and 100nM isoproterenol, and exposure to 10 μM CGP12177 in AC9 knockdown or control HEK293 cells. **(N)** Quantification of the maximum cAMP response as seen in **(C)** [mean±SEM; n=3 experiments]. **(O)** Representative cAMP response to stimulation with 10 μM FSK and 10 μM IBMX or control under conditions of siRNA knockdown of AC9 expression or control (ASD). **(P)** Quantification of the maximum cAMP response as seen in **(E)** [mean±SEM; n=3 experiments].

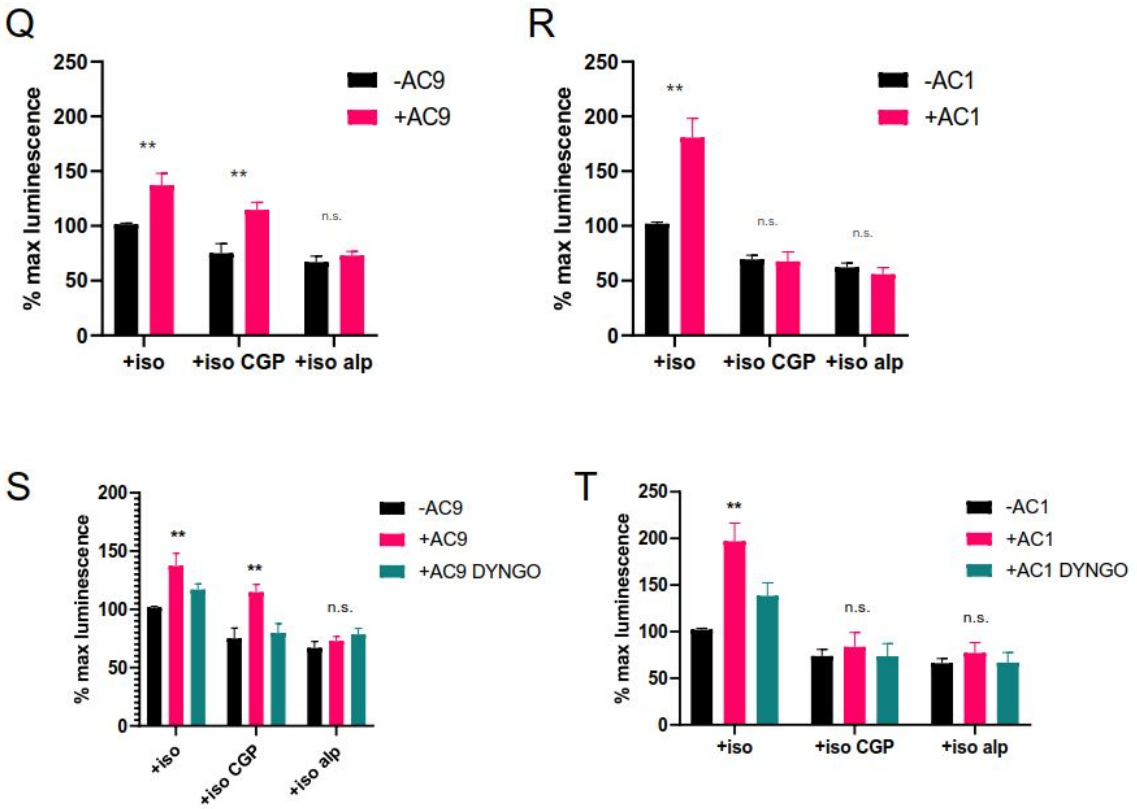

Figure 2.7: AC9 is a significant contributor to the β2AR-mediated cAMP response from endosomes. (Q) Quantification of the maximum cAMP response in AC3/6KO HEK293 cells overexpressing control or AC9. Cells were pretreated with 100nM isoproterenol and exposed to supersaturating conditions of membrane permeable antagonist (10μM alprenolol) or membrane impermeable antagonist (10μM CGP12177). [mean±SEM; n=4 experiments]. **(R)** Quantification of the maximum cAMP response in AC3/6KO HEK293 cells overexpressing control or AC1. Cells were pretreated with 100nM isoproterenol and exposed to supersaturating conditions of membrane permeable antagonist (10μM alprenolol) or membrane impermeable antagonist (10μM CGP12177). [mean±SEM; n=4 experiments]. **(S)** Quantification of the maximum cAMP response in AC3/6KO HEK293 cells overexpressing AC9. Cells were pretreated with DYNGO-4a and then 100nM isoproterenol, and supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol). **(T)** Quantification of the maximum cAMP response in AC3/6KO HEK293 cells overexpressing AC1. Cells were pretreated with DYNGO-4a and then 100nM isoproterenol, and supersaturating antagonist conditions (10μM CGP12177, 10μM alprenolol).

Chapter 3:

An acute CO2 gradient is sufficient to inhibit isoproterenol-induced AC9 internalization in HEK293 cells

André M. Lazar conceived the project and performed all of the experiments and data analysis. Mark von Zastrow performed experiments. André M. Lazar and Mark von Zastrow wrote the manuscript.

3.1 Abstract

G Protein Coupled Receptors (GPCR) are the largest group of signal-transducing receptors in mammals and are involved in a vast array of cellular responses. The endocytic network has long been recognized to regulate GPCR signaling, either by arrest or by delivery of signaling cascades to effector locations in the cell. However, membrane trafficking of adenylyl cyclase isoforms and the regulatory mechanisms involved is still a nascent field. Here we described the first observation that an acute negative CO2 gradient can inhibit AC9 endocytosis. We further show that this inhibition only recovers after 5 days, indicating a change in genetic expression. This unexpected cellular response to removal from the incubator for 3 minutes may have great implications on GPCR signaling studies and cell culture techniques more generally.

3.2 Introduction

This project has been unusual in many ways, and that is reflected in this chapter. No line of scientific investigation is ever truly finished, but in this case, this project is very much ongoing and there are several important questions still left. It would not be overstating it to say that this project has really only reached its starting point, which makes writing a thesis chapter about this topic so strange. However, no review of my work as a graduate student in the von Zastrow lab would be complete without a summary of the progress made here. This chapter is therefore written as more of a report of our progress rather than a conclusive document, and follows a fairly personal chronological account of experimentation.

Early progress in the data presented in Chapter 2 was rapid. Once a basic protocol was established for plating, transfecting, fixing and staining HEK293 cells with AC9, we were able to test many conditions and greatly expand our knowledge on the subject. Within two years of the project starting, we had put together enough data to try submitting an early manuscript. This project was moving so quickly and positive results were so forthcoming that others in the lab, including classmate Grace Peng, were attempting to direct their efforts towards exploring the burgeoning field of adenylyl cyclase trafficking.

By the time we had received reviewer comments from our initial manuscript submission, Grace had found that she had difficulty reproducing the basic experiment at the core of the paper. She was unable to consistently fix and stain Flag-AC9 in endosomes after

adrenergic stimulation, although she did consistently fix and stain HA-B2AR there under the same conditions. Grace and I began troubleshooting the protocol together for a few months with little insights or success, and she eventually moved to other projects. Having discontinued responding to reviewer comments at this point, I began to troubleshoot the protocol in earnest.

It was difficult to move forward on troubleshooting the protocol by myself, because the AC9 trafficking continued to consistently present in my hands. While we understand now that timing during plating and transfection is important, in the moment (late 2016 and early 2017) it seemed impossible that the same protocol, done side by side and with the same reagents, could produce different effects. Our inability to achieve consistent results in what was believed to be a well controlled experiment was extremely puzzling. Some in the lab cast a doubtful eye on the validity of my data, and the strain on my mental and physical health proved a significant roadblock.

In early 2017 I began working more closely with Mark, who like Grace had difficulty showing consistent AC9 internalization. Important to note is that it was inconsistent, not nonexistent. Experiments had internal consistency in that if we observed AC9 internalization or not, it was typically in all cells. We came to term this as ‘positive’ or ‘negative’ results. The classification of an experiment being ‘positive’ or ‘negative’ began with Grace, but was fully explored when working with Mark. We rigorously tested every reagent and step of the protocol, reducing or eliminating variables along the way. It

ultimately took us two more years (summer 2019) to reach a point at which we had consistent results in both our hands and gotten a postdoc in the lab, Braden Lobingier, to recapitulate them independently.

If the definition of insanity is repeating something and expecting a different result, the work that Mark and I did on this project may qualify. Seemingly identical experiments were performed over and over again with different results, and every time we thought we'd found a clue, success eluded us. We speculated jokingly about magic, or juju, or cooties, largely because Mark often produced positive results under my watchful eye, but negative ones when I wasn't observing him. We now know this was because his timing was crisper under observation, not magic. This has been the most confusing experience in my, and many others, scientific career, but the implications of our findings on GPCR signaling and cell culture techniques may be quite large. Our discovery of a sensitivity to acute exposure to a negative CO2 gradient is a novel phenomenon that will be a fascinating field to explore.

3.3 Results

Initial protocol

Day 0: Prepare slides: flame each slide and place in 12-well plate. Coat with 1:1000 Poly-L Lysine (in water) solution for 15 min. Aspirate and dry for 15 min at least. Wash 3X with water and dry 15 min at least. Plate HEK293 cells on slides at 40-50% confluence.

Day 1: Transfect cells (per well):

-300ng AC9 + 300ng B2AR + 100uL optimem

-1uL lipofectamine 2000 + 100uL optimem

Day 2: Exchange media

Day 3: Fix and Stain.

1. *Surface label receptor with HA antibody for 10 minutes.*
2. *Stimulate for 30 min with 10uM isoproterenol.*
3. *Wash 1x cold PBS.*
4. *Fix cells with 4% formaldehyde in modified BRB80 buffer (80mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 6.8), 20 min.*
5. *Wash 1x cold PBS.*
6. *Block non-specific labeling in 2% BSA (in Triton-X 1:1000 in TBS), 10 min.*
7. *Incubate in primary antibody solution, 60 min.*
    a. *Rabbit anti-Flag for Flag-AC9*
    b. *Mouse anti-HA for HA-B2AR*
8. *Wash 1x cold PBS.*

9. *Incubate in secondary antibody solution, 20 min*
    a. *Donkey anti rabbit 488 for Flag AC9*
    b. *Donkey anti-mouse 647 for HA G(alpha)s*
10. *Wash 1x cold PBS.*
11. *Dip in water and aspirate, dry lightly on kimwipe (cell side up) and then fix onto slide with proLong gold anti fade mounting media (cell side down).*

Troubleshooting the transfection and fixation steps of the protocol

We began by troubleshooting the protocol step by step. Our initial hypothesis was a technical issue with antibody staining. This is not uncommon in fixation experiments, especially when dealing with relatively small membrane structures like endosomes. However, our controls for antibody staining showed no improvement in the ability of others to reproduce the AC9 trafficking results, nor diminish my ability to consistently demonstrate isoproterenol-induced AC9 internalization. These early experiments were when we initially characterized as experiments being 'negative' or 'positive' for isoproterenol-induced AC9 trafficking (**Figure 3.1**).

The next most likely factor was then the fixation step. It was typical for those of us who were focused on membrane trafficking at the endosome to fix cells in formaldehyde diluted in BRB80. This is an established buffer that has been described as promoting microtubule integrity, which can be useful for maintaining structure of the endosome membranes, particularly those with retromer (recycling) tubules. However, initial

experiments by others (Grace Peng, Mark von Zastrow) used formaldehyde diluted in PBS, which is sufficient for receptor fixation at the plasma membrane. We found that using 4% formaldehyde in BRB80 during the fixation step rather than in PBS greatly improved yield of AC9-positive endosomes (**Figure 3.2**).

Although we established that the use of BRB80 over PBS was necessary to improve the consistency of 'positive' experiments, both in my hands and others, it did not eliminate the issues we had with consistency. Another area we were able to optimize in the fixation protocol was at the permeabilization step. Permeabilizing a cell membrane with detergent (0.1% Triton-X) in milk was the established protocol in our lab. We found that increased concentration of detergent helped with consistency, and we optimized the protocol with 0.2% Triton-X diluted in 2% Bovine Serum Albumin (BSA) in Tris-Buffered Saline (TBS) (**Figure 3.3**). While we saw incremental improvements in the quality of the images that could be captured with this optimized protocol, the consistency issue persisted.

<u>Timing of Transfection consistently affected AC9 internalization</u>

Having optimized the protocol post-fixation, we next asked whether there was a relevant factor while the cells were still live. Working backwards, we focused first on the transfection step. While transfection protocols are established by the manufacturer of transfection reagents, there is some inherent variation between subjects. We extensively troubleshot each step in the transfection protocols, to the point where two

subjects would do the same transfection side by side and would switch who did one step to isolate a particular step that might be the deciding factor.

It was during this troubleshooting that we discovered the first consistent way to achieve consistent AC9 trafficking in someone else's hands and block it in my hands. This experiment involved a side by side transfection in which subject A and subject B prepare transfection reagents for their own use and the other subject. Subject A would apply the transfection mix prepared by A to cells plated into two separate wells in a 12-well plate (Plate 1). Subject A would then apply transfection mix prepared by B to two separate wells. Subject A would then place the Plate 1 in the incubator. Subject B would have done the same with transfection reagents prepared by B and A to a separate Plate 2. Once both plates were in the incubator, they were removed and A would take Plate 2, and B would take Plate 1. A would then use the reagents prepared by B and apply them to the two wells in the 12-well plate originally used by B (See Table 1 for reference).

What we found was that for the first time, 'positive' results for isoproterenol-induced AC9 trafficking correlated not with which subject performed the experiment, but surprisingly, who performed the transfection first on each plate (**Figure 3.4**). In the experiment described above, on both Plate 1 and 2, the person preparing the transfection reagent had no effect (**Figure 3.4**). All wells transfected by Subject A on Plate 1 were 'positive', while wells transfected by Subject B on Plate 1 were 'negative'

(**Figure 3.4**). Similarly, Subject B produced 'positive' results on Plate 2, and Subject A produced 'negative' results on Plate 2 (**Figure 3.4**). We concluded from this experiment, performed 5 separate times with consistent results, that the timing of the transfection was relevant. Although it wasn't clear at the time, this was our first clue that the transfection reagents helped buffer an event that is triggered by taking the cells out of the incubator into a cell culture hood.

Early experiments with AC9-EGFP stables

Throughout this process, we were constantly trying to reduce the number of variables in the protocol. We eventually cloned AC9 from a Flag-tag vector into an EGFP vector. That plasmid was then transfected into HEK293 parental cells and maintained under selection until it AC9-EGFP was stably expressed in these cells. The motivation for developing this cell line as a tool was to eliminate the time consuming and resource intensive steps of transfection, permeabilization and antibody staining. Cells could be plated, stimulated, and fixed at low cost (both monetary and time), accelerating a lengthy protocol.

Early validation of the stable AC9-EGFP cell line was initially confusing, though ultimately informative. In my hands, AC9 did not exhibit robust internalization with these cells at first (**Figure 3.5 (A)**). However, AC9 did internalize in these cells when the experiment was performed by another (Mark). This was a welcome but unexpected reversal of the typical dynamic. With the Subject A/B, Plate 1/2 experiment in mind, I

tested whether the transfection reagents were somehow able to buffer any negative effects of removing the cells from the incubator. I found that transfecting these stable cell lines with empty pcDNA3 vector seemed to improve things in my hands (**Figure 3.5 (B)**). I also transfected these stable cells with Flag-AC9 and observed colocalization of the Flag-tagged construct and the EGFP-tagged construct, although the stably expressed AC9-EGFP exhibited higher expression (**Figure 3.5 (C)**).

Even more unexpected, this changed back to the original dynamic after a few experiments, in which the stable cells were positive for AC9 internalization in my hands but not in Marks. However, we found that if I split my cells into two flasks and gave one to Mark, the first experiment he would perform with those would be positive. Subsequent experiments with Mark passaging the cells in between experiments were negative. This led to our hypothesis that it was the way that I passaged my cells that was maintaining the cells in a state that promoted trafficking. A third party (Kyra) made the outside observation that the timing of my cell passaging / plating of cells onto coverslips was much faster than others (Mark). Based on this, we asked whether this was a relevant factor to AC9 internalization.

<u>Updated protocol</u>

Day 0: Plate AC9-EGFP stable cell line (HEK293)

1. *Flame coverslips and place each one in a separate well.*
2. *Coat coverslips with 0.1% Poly-L Lysine in deionized water for 15 minutes.*

3. *Aspirate and let dry for 15 minutes.*
4. *Wash 3X with deionized water and let dry for 15 minutes.*
5. *Remove flask from incubator and aspirate.*
6. *Wash cells in flask with 3mL PBS-EDTA.*
7. *Apply 1mL PBS-EDTA to cells in flask and incubate 3 minutes.*
8. *Resuspend cells in 9mL DMEM (+10% FBS).*
9. *Plate cells onto coverslips.*

Day 2: Fix cells.

1. *Stimulate for 30 min with 10uM isoproterenol.*
2. *Wash 1x cold PBS.*
3. *Fix cells with 4% formaldehyde in modified BRB80 buffer (80mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 6.8), 20 min.*
4. *Wash 1x cold PBS.*
5. *Dip in water and aspirate, dry lightly on kimwipe (cell side up) and then fix onto slide with proLong gold anti fade mounting media (cell side down).*

<u>Stables that never leave the incubator</u>

Throughout the (now simplified) experiment, typically one would take the cells out of the incubator briefly to check confluency, exchange media to promote growth, and in general monitor the health of the cells through a light microscope. With the idea that taking cells out of the incubator could be a factor as well as the timing of execution of the protocol, we asked whether these normal observations could actually be a

Shroedinger's cat scenario in which observation of the cells was disrupting them. Consistent with this, we found that never removing the cells from the incubator seemed to improve things (**Figure 3.6 (A)**) both in my hands and others (Mark).

0, 1, 2, 3 minutes out of the incubator

Now armed with the more concrete hypothesis that removing cells from the incubator could be a relevant factor, we attempted to determine the parameters of the phenomenon. Cells were placed in separate 12-well plates and incubated for 24 hours. Each plate was then removed from the incubator and placed in a normal cell culture hood for 1, 2, or 3 min (an additional plate never left the incubator) before being returned to the same incubator. As seen in confocal optical sections images in (**Figure 3.6 (B)**), 3 minutes out of the incubator appeared to block isoproterenol-induced AC9 trafficking in these AC9-EGFP stable HEK293 cells.

We quantified this across 10 fields per condition, over 3 experiments. Each field had at least 20 cells. Two methods of quantification were used: counting the number of internal puncta per cell (**Figure 3.6 (C)**), and then the percent cells per field that were 'positive', that is, they had more than 10 endosomes (**Figure 3.6 (D)**). The former was a raw metric demonstrating AC9 internalization. The latter is a more qualitative metric that attempts to demonstrate that when an experiment is 'positive', almost all the cells present this phenotype; it's not a matter of picking and choosing desirable cells.

Notably, 2 minutes out of the incubator seemed to reduce AC9 internalization marginally in this cell line, but cells still averaged greater than 10 endosomes, qualifying them as 'positive' for AC9 trafficking. But 3 minutes out of the incubator dramatically reduced AC9 internalization (**Figure 3.6 (C)**). A window of 1 minute between the 2 and 3 minute time points was then established, perhaps unprecedented in biology.

CO2 incubator, Hepes, Optimem

We speculated several causes behind this novel phenomenon in which cells that were removed from the incubator and then replaced after 2 minutes, but not 3 minutes, presented an isoproterenol-induced AC9 trafficking phenotype. Chief amongst these were: 1. Temperature gradient, 2. pH gradient, and 3. CO2 gradient, since temperature, humidity and CO2 are controlled by the incubator, and pH gradients have been a noted source of cell distress before. Initial experiments with pH control by the addition of Hepes during the 3 min room air challenge showed little effect, but treatment with the transfection reagent Opti-MEM did (**Figure 3.7 (A)**).

However, CO2 gradients proved a fruitful direction. The gauge for the CO2 source to a separate incubator was locked so that the incubator was not able to supplement the levels of CO2 at room air. We referred to this as a CO2 negative (-CO2) environment, although of course it was at room levels. But this allowed a controlled system to compare incubation with elevated CO2 and without. Cells were either left in the normal incubator or removed and placed in the -CO2 incubator before being returned to the

normal incubator after 3 minutes. We found that this was sufficient to block isoproterenol-induced AC9 trafficking in wide-field epifluorescence images (**Figure 3.7 (D)**). Quantification of the endosomes per cell and positive cells per field supported these observations (**Figure 3.7 (E, F)**).

Application of the pH buffer Hepes or the transfection reagent Opti-MEM increased the number of endosomes per cell (**Figure 3.7 (D, E)**). Only Opti-MEM produced AC9 trafficking that qualified as 'positive' by our metric of more than 10 endosomes per cell on average (**Figure 3.7 (D, F)**). However, this was not a full recovery of the effect. This investigation is currently ongoing and we are (and will be) characterizing this further.

5 day cycle

One way we have characterized this phenomenon is recovery over long time periods. Cells were grown on coverslips in separate 12-well plates. These plates were removed for 3 minutes to room air (cell culture hood) before being returned to a normal incubator. Cells were then incubated for various lengths of time: 12, 24, 36, 48, 72, 96, and 120 hours. After this long term incubation, cells were stimulated with isoproterenol and then fixed as per the updated protocol. Cells were imaged by wide field epifluorescence microscopy and quantification of AC9 internalization was performed as described above. We found that AC9 trafficking exhibited a local minimum after 48 hours of incubation and slowly recovered after another 72 hours, for a total of 120 hours (**Figure 3.8**).

3.4 Discussion

Our data suggests that AC9 trafficking is controlled by a regulatory mechanism that is sensitive to a relatively acute event. Three minutes exposure to a negative CO2 gradient is sufficient to inhibit isoproterenol-induced AC9 internalization over a five day period. This is not true for the B2AR, and we have already described other fundamental differences in the regulatory mechanisms governing AC9 and B2AR trafficking from the plasma membrane to the endosome in Chapter 2. Here we begin to characterize this unprecedented sensitivity to removal from the incubator for a short period of time, which produces an effect that lasts for up to 120 hours.

This is a remarkable discovery in HEK293 cells, which rapidly grow and double every 12-24 hours on average. Inhibition of AC9 trafficking over several generations of cell division suggests genetic expression changes. We interpret this data as either the increase in expression of an inhibitory factor in response to the negative CO2 gradient presented by room air relative to the incubator, or the rapid degradation of a necessary component of the AC9 trafficking regulatory mechanism and slow recovery of expression over several days. RNAseq experiments are the obvious next step, with an option for mass spectrometry as well. AC9 expression appeared fairly stable across conditions and replicates in this experiment, but we did not control for proteolysis or continued expression of AC9.

This also provides us with the first consistent method to block AC9 endocytosis in conditions that B2AR endocytosis is unaffected. We have previously shown that AC9 can internalize in a receptor-independent manner due to Gs activation. This will allow us to test the cAMP response in cells that are under conditions which inhibit the trafficking of B2AR, AC9, or both. The unique properties of AC9 are of note: it's high basal activity, autoinhibitory domain, and requisite of high concentrations of Gs. AC9 might not require an upstream GPCR at the endosome to contribute to functionally relevant signaling. Further exploration into the trafficking determinants of AC9 and the signaling implications are currently active areas of investigation in the lab.

As of the writing of this thesis, we now have a better understanding of the situation. More specifically, we found that a negative CO2 gradient can inhibit AC9 trafficking within 3 minutes. This is less of an explanation and more of a first clue. Standard protocol for plating cells involves counting the cells so that they can be plated at a certain concentration ('confluence') for consistent results. Our results indicate that this can block AC9 trafficking if it takes longer than 3 minutes, which it usually does. This could potentially mean that a lot of experiments that involve cell culture might not be properly controlled. An investigation of exactly what causes this and the implications on cell biology has begun and will continue after my time in the lab. It is possible that some or all of this story will be written up in a paper or submitted in some form to bioRxiv, but at the very least it will be recorded here. The clue that we have now is exciting in its absolutely unprecedented nature and its wide implications on cell culture.

As far as we can tell through extensive discussion with others in the field (and across fields in UCSF generally), this is a completely novel phenomenon. While fascinating, it is still poorly characterized. More work is necessary to better understand this sensitivity to CO2 gradient. Other cellular responses to stress such as temperature (Underhill and Smales 2007), ionic distress, and even oxygen (Ast and Mootha 2019) have been noted with varying levels of understanding. However, while control of CO2 concentration in incubators is known to be necessary for homeostasis, the acute nature of the phenomenon described here and its long term effects are unprecedented.

This has been a baffling project thus far and it took years of painstaking troubleshooting to get to even this first clue. Despite the difficulty in even defining the parameters involved, we are motivated to continue investigating this unknown system. AC9 is known to be a B2AR effector in human airway smooth muscle tissue including the throat and lungs. We speculate that AC9 trafficking's CO2 sensitivity has biological and clinical relevance in this context, but as of now the phenomenon is still poorly defined.

3.5 Acknowledgements

We thank Grace Peng, Miriam Stoeber, Braden Lobingier, Roshanak Irannejad, Erica Sanchez, and Lea Ripoll for their extensive time and effort in this project. They shared invaluable insights and performed experiments without which this project would not have progressed. We thank J. Taunton, D. Mullins and R. Sunahara for valuable insight and suggestions.We thank Kyra Kurtz for her participation in experimentation as well as making the first observation of the difference in timing between subjects. We thank Tanya Baldwin and Carmen Dessauer for their perspectives and independent testing of these results. This work was supported by grants from the US National Institutes of Health (DA010711 and DA012864 to M.v.Z.; GM60419 to C.W.D). André Lazar is an ARCS scholar.

3.6 Materials & Methods

Cell Culture + Constructs

HEK 293 cells (ATCC) were cultured in complete growth Dulbecco's modified Eagle's medium (DMEM, Gibco) and supplemented with 10% fetal bovine serum (UCSF Cell Culture Facility). We note that AC9 most consistently internalized in cells that were not removed from the incubator for longer than 3 min during plating or transfection. HA-β2AR (Tang et al. 1999; von Zastrow and Kobilka 1992) described previously, was sub-cloned from Flag-tagged constructs. Transfections were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Cells were transfected 48 hours before experiments.

Antibodies

Primary antibodies used were rabbit anti-Flag (Sigma), mouse anti-Flag M1 (Sigma), mouse anti-Flag M2 (Sigma), mouse anti-HA 16B12 (Biolegend), rat anti-HA (Roche).

Fixed cell confocal imaging

Cells were transfected with the indicated construct(s) and then plated on glass coverslips coated with poly-L-lysine (0.0001%, Sigma) 24 hours later. For antibody feeding assays, cells were: (1) placed on ice and rinsed with ice-cold phosphate-buffered saline (PBS), (2) labeled by the addition of antibodies diluted 1:1000 in DMEM for 10 minutes, and (3) rinsed with room temperature PBS and allowed to traffic for 30 minutes by the addition of 37°C fresh media (DMEM + 10% fetal bovine

serum) with or without a saturating concentration of β2AR agonist (10 μM isoproterenol, Sigma), V2R agonist (10 μM arginine-vasopressin, Sigma), MOR agonist (10 μM DAMGO, Sigma), or forskolin (10 μM, Sigma). For all assays, cells were rinsed with cold PBS and fixed by incubation in 3.7% formaldehyde (Fisher Scientific) diluted in modified BRB80 buffer (80mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 6.8) for 20 minutes at room temperature. Cells were then blocked in 2% Bovine Serum Albumin (Sigma) in PBS with permeabilization by 0.2% triton X-100 (Sigma). Primary antibody labeling was performed by the addition of antibodies diluted 1:1000 in blocking/ permeabilization buffer for one hour at room temperature. Secondary labeling was performed by the addition of the following antibodies diluted at 1:500 in blocking/ permeabilization buffer for 20 minutes at room temperature: Alexa Fluor 555 or 488 donkey anti-mouse (Invitrogen), Alexa Fluor 555 or 488 donkey anti-rabbit (Invitrogen), Alexa Fluor 488 or 555 goat anti-rat (Invitrogen), or Alexa Fluor 488 donkey anti-sheep (Life Technologies). Specimens were mounted using ProLong Gold antifade reagent (Life Technologies).

Fixed cells were imaged by spinning disc confocal microscope (Nikon TE-2000 with Yokogawa confocal scanner unit CSU22) using a 100X NA 1.45 objective. A 488 nm argon laser and a 568 nm argon/krypton laser (Melles Griot) were used as light sources.

Statistical Analysis

Results are displayed as mean of results from each experiment or data set, as indicated in Figureure legends. The statistical significance between conditions for experiments

with two conditions was calculated using paired, two tailed t-tests. All statistical calculations were performed using Excel (Microsoft Office) or Prism (GraphPad). The threshold for significance was $p<0.05$ and the coding for significance is reported as follows: (n.s.) $p>0.05$, (*) $p\leq0.05$, (**) $p\leq0.01$.

3.7 References

Ast, Tslil, and Vamsi K. Mootha. 2019. “Oxygen and Mammalian Cell Culture: Are We Repeating the Experiment of Dr. Ox?” *Nature Metabolism*, August. https://doi.org/10.1038/s42255-019-0105-0.

Tang, Yuting, Liaoyuan A. Hu, William E. Miller, Niels Ringstad, Randy A. Hall, Julie A. Pitcher, Pietro DeCamilli, and Robert J. Lefkowitz. 1999. “Identification of the Endophilins (SH3p4/p8/p13) as Novel Binding Partners for the β1-Adrenergic Receptor.” *Proceedings of the National Academy of Sciences* 96 (22): 12559–64.

Underhill, Michèle F., and C. Mark Smales. 2007. “The Cold-Shock Response in Mammalian Cells: Investigating the HeLa Cell Cold-Shock Proteome.” *Cytotechnology* 53 (1-3): 47–53.

Zastrow, M. von, and B. K. Kobilka. 1992. “Ligand-Regulated Internalization and Recycling of Human Beta 2-Adrenergic Receptors between the Plasma Membrane and Endosomes Containing Transferrin Receptors.” *The Journal of Biological Chemistry* 267 (5): 3530–38.

3.8 Figures

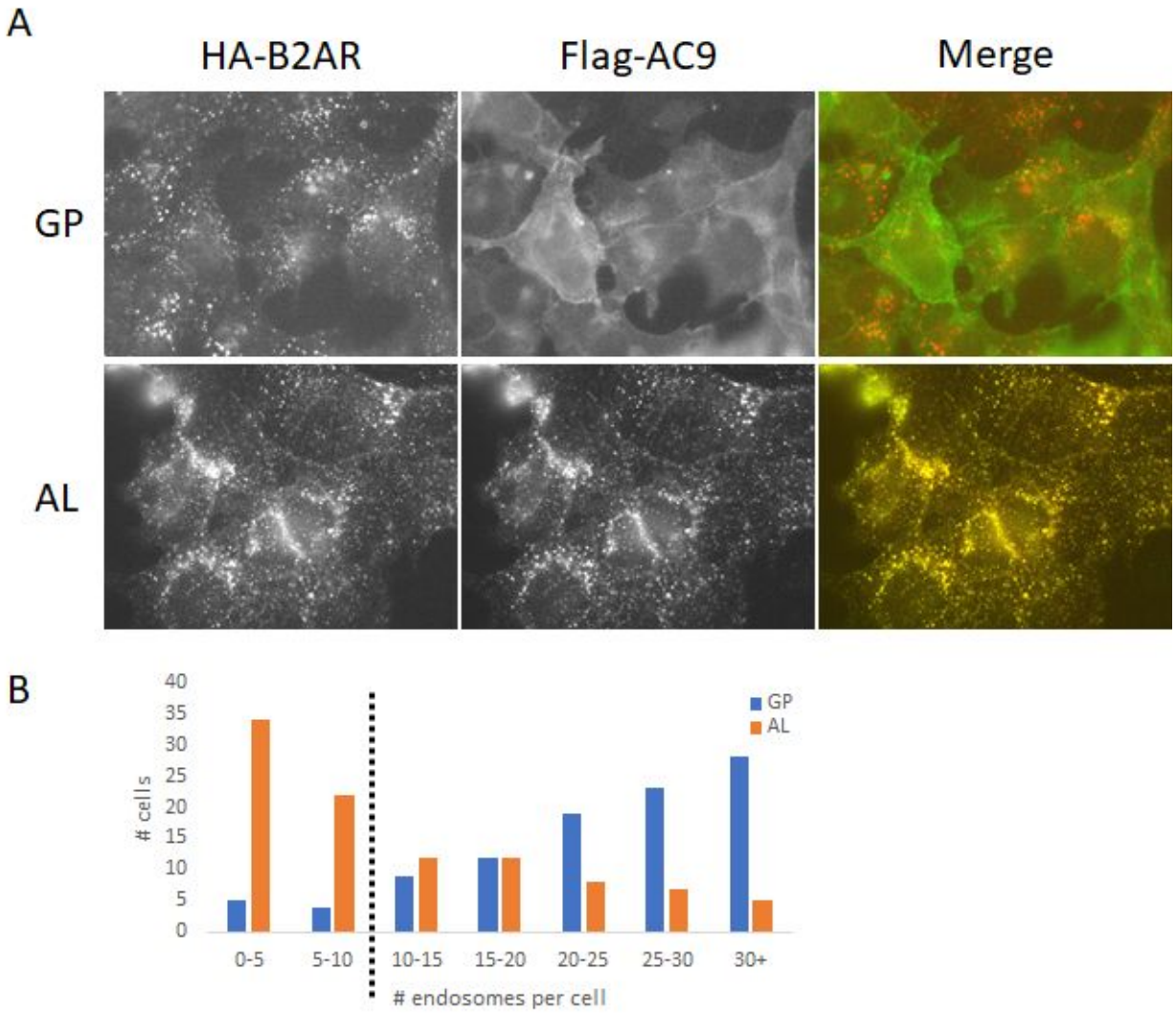

Figure 3.1: AC9 trafficking is consistent within an experiment but not across subjects.
(A) Representative epifluorescence imaging of HEK293 cells coexpressing HA-B2AR and Flag-AC9 that were treated with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(B)** Quantification of internal puncta that are β2AR or AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

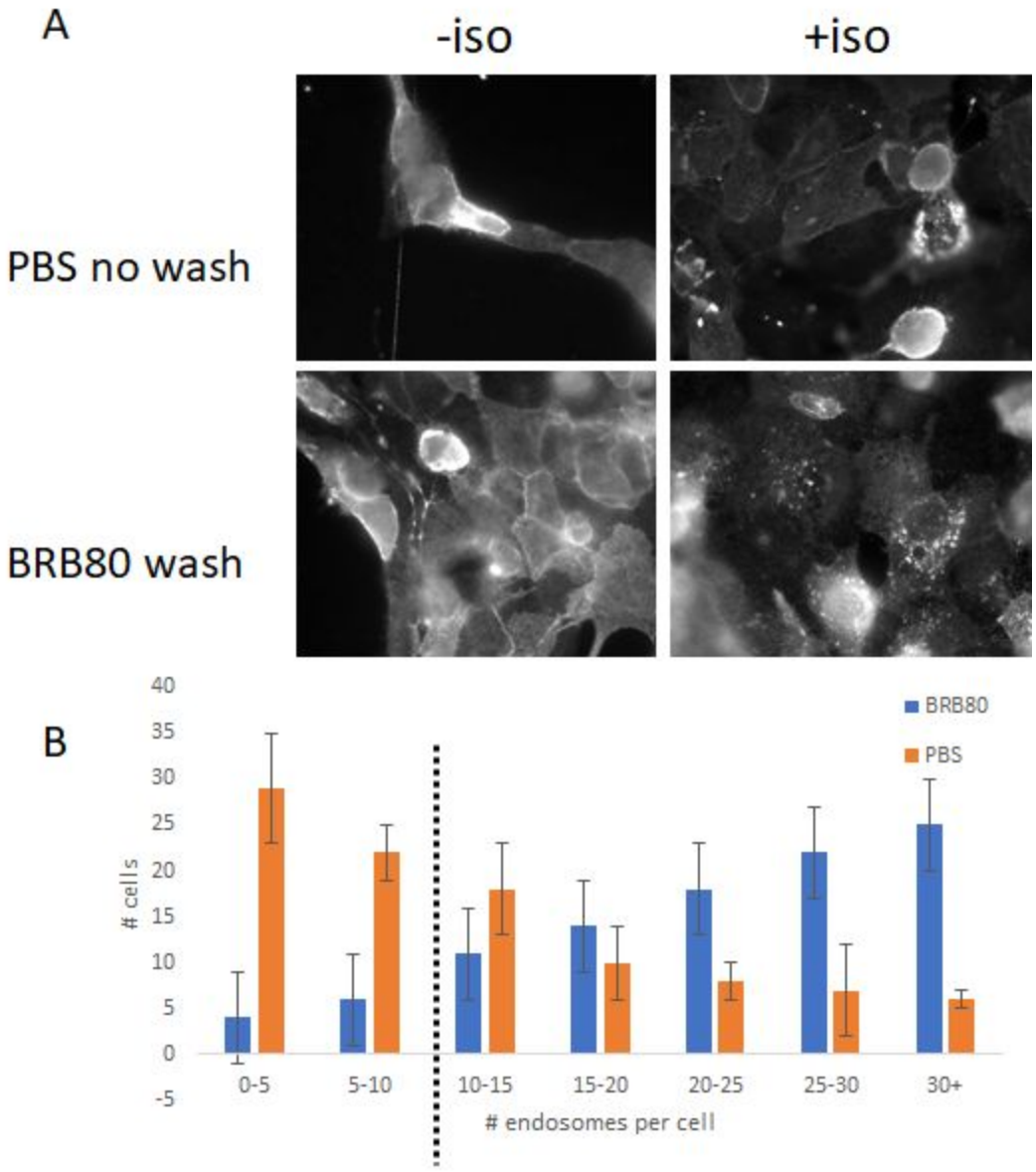

Figure 3.2: BRB80 improves fixation of Flag-AC9 in HEK293 cells.
(A) Representative epifluorescence imaging of HEK293 cells expressing Flag-AC9 that were treated with 10 μM isoproterenol or control for 30 min and fixed with formaldehyde in BRB80 or PBS. Scale Bar is 8 μm. **(B)** Quantification of internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

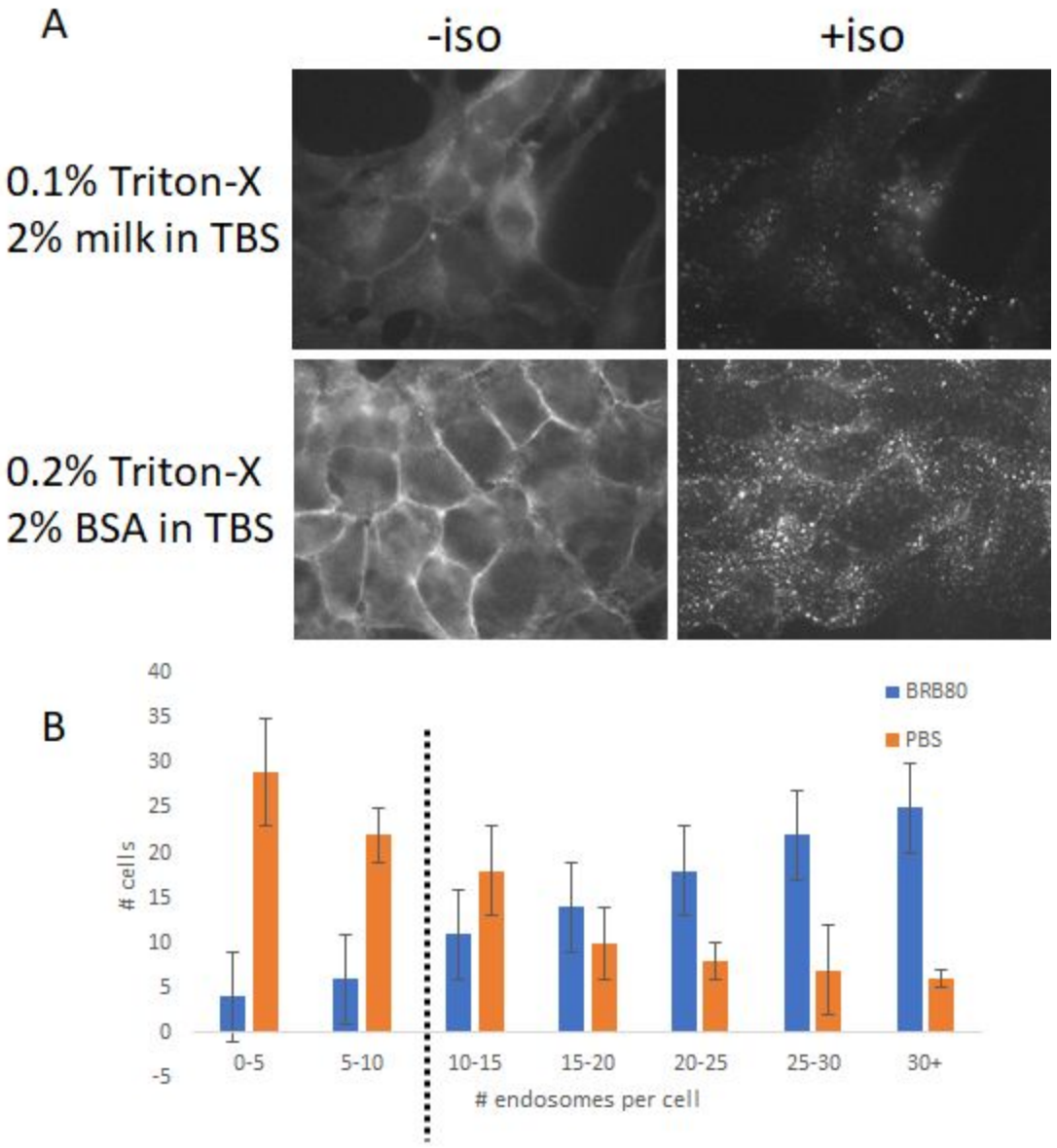

Figure 3.3: 0.2% Triton-X in 2% BSA-TBS improves fixation of Flag-AC9 in HEK293 cells.

(A) Representative epifluorescence imaging of HEK293 cells expressing Flag-AC9 that were treated with 10 μM isoproterenol or control for 30 min and permeabilized with 0.1% Triton-X in 2% milk (TBS) or 0.2% Triton-X in 2% BSA (TBS). Scale Bar is 8 μm. **(B)** Quantification of internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

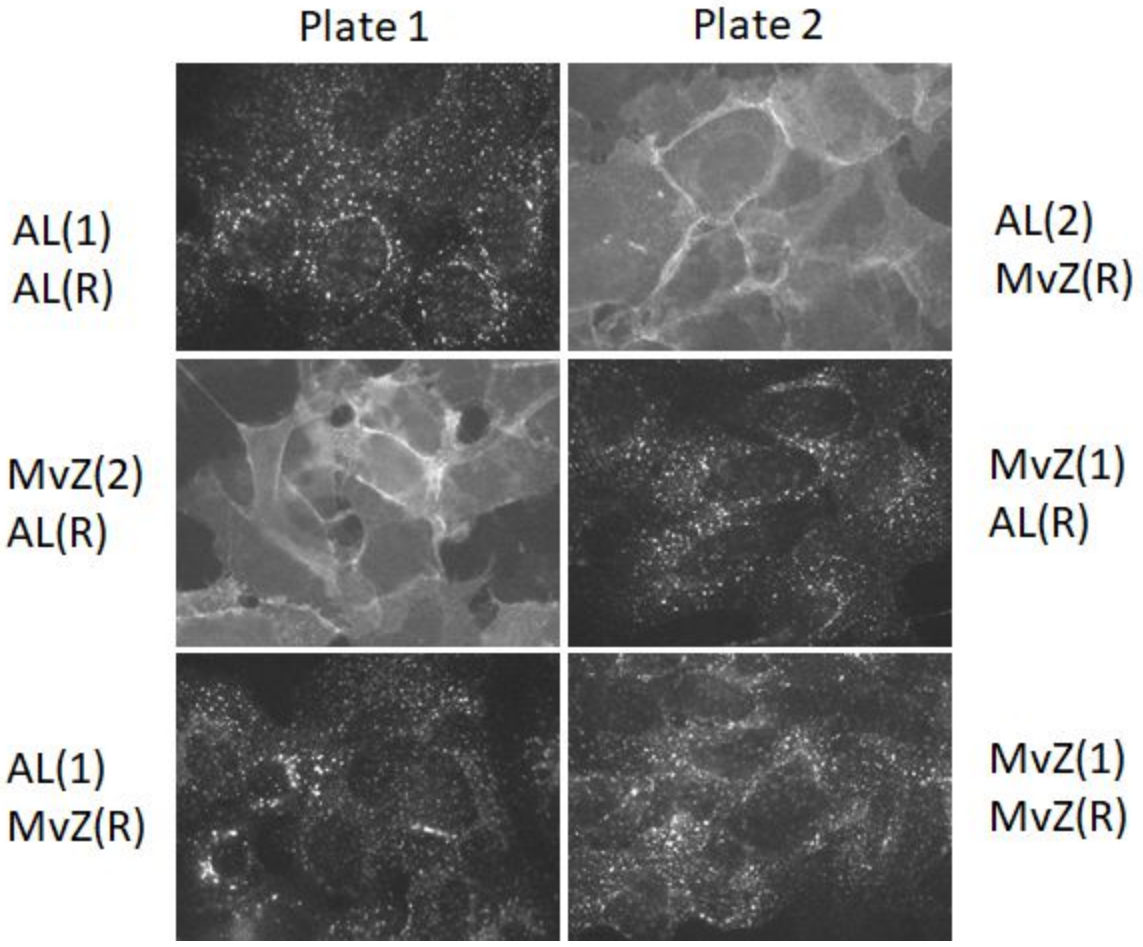

Figure 3.4: Timing of transfection is relevant to AC9 trafficking.
Representative epifluorescence imaging of HEK293 cells expressing Flag-AC9 that were treated with 10 μM isoproterenol for 30 min. Cells in Plate 1 were transfected by AL first and MvZ second, with reagents prepared by AL or MvZ as indicated. Plate 2 was transfected by MvZ first and AL second. Scale Bar is 8 μm.

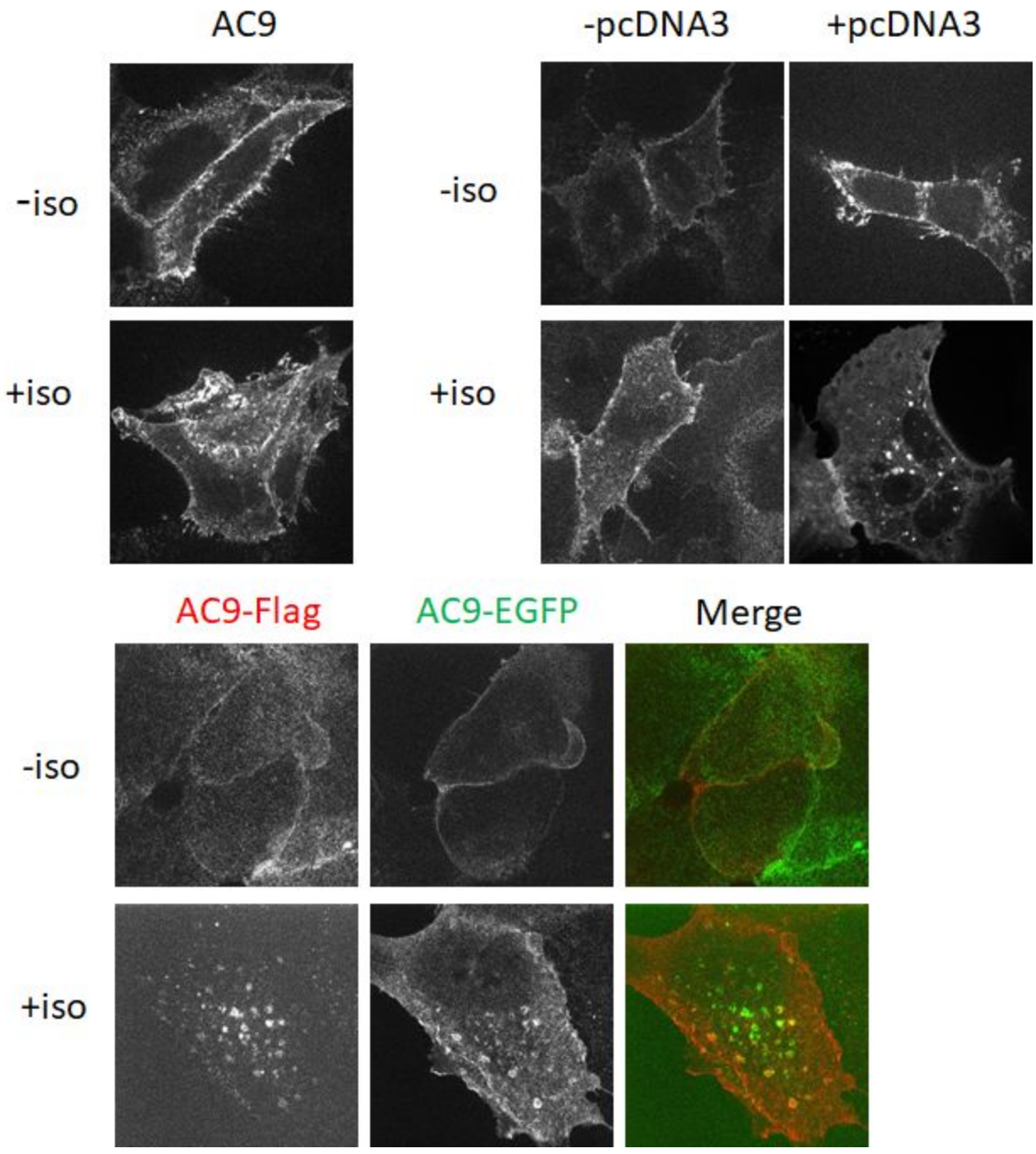

Figure 3.5: Initial testing of the AC9-EGFP stable cell line.
(A) Representative confocal imaging of AC9-EGFP stable HEK293 cells that were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(B)** Representative confocal imaging of AC9-EGFP stable HEK293 cells transfected with vehicle or pcDNA3 vector were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(C)** Representative confocal imaging of AC9-EGFP stable HEK293 cells transfected with Flag-AC9 were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm.

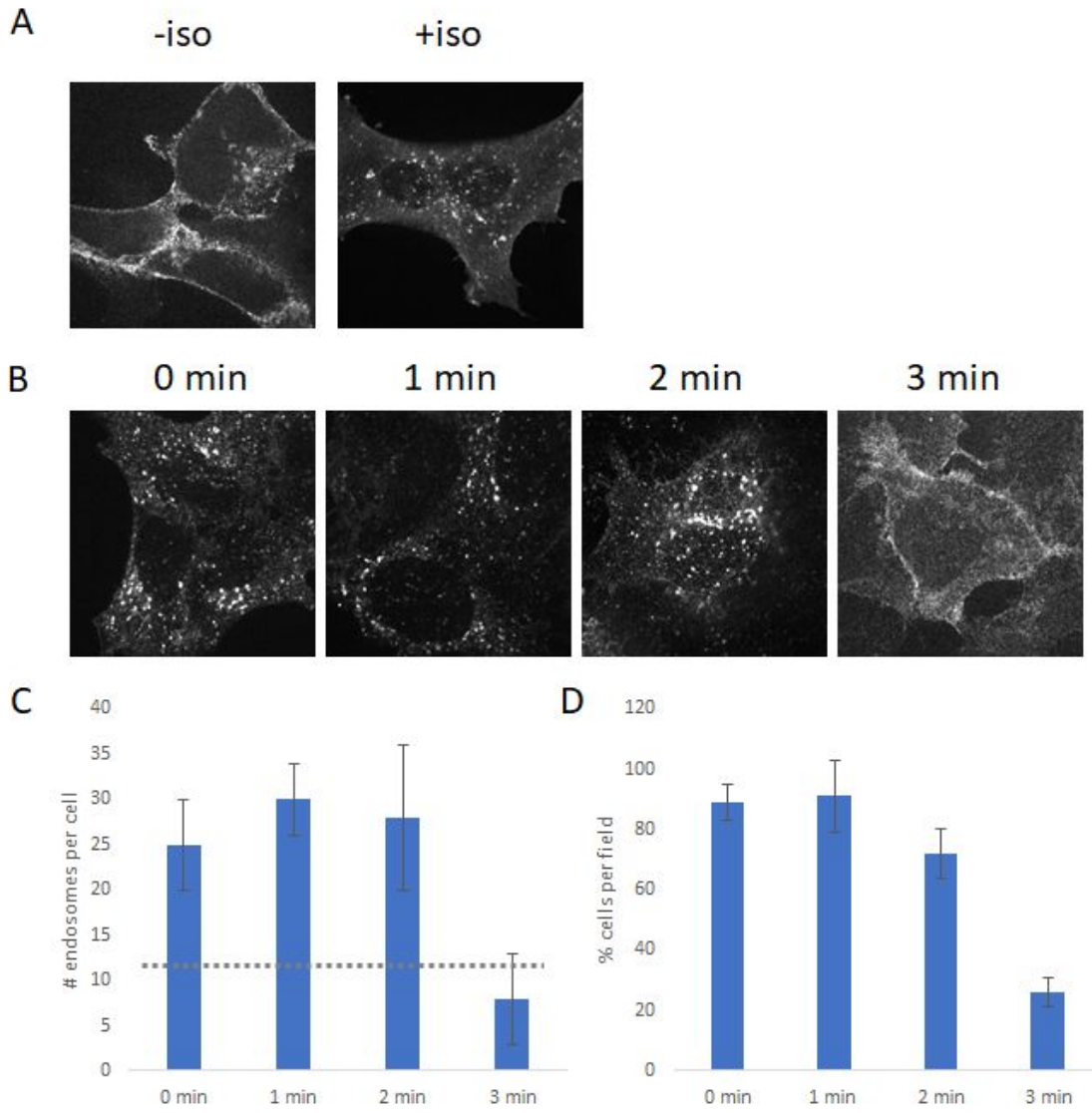

Figure 3.6: Removal from incubator for 3 minutes inhibits AC9 trafficking in AC9-EGFP stable cells.

(A) Representative confocal imaging of AC9-EGFP stable HEK293 cells which never left the incubator were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(B)** Representative confocal imaging of AC9-EGFP stable HEK293 cells were treated with 10 μM isoproterenol for 30 min. Cells were removed from the incubator to a cell culture hood for 0, 1, 2, or 3 minutes and then returned to the incubator 48 hours before stimulation. Scale Bar is 8 μm. **(C)** Quantification of internal puncta that are AC9 positive, taken from wide field images (B) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(D)** Quantification of cells with >10 internal puncta that are AC9 positive, taken from wide field images (B) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

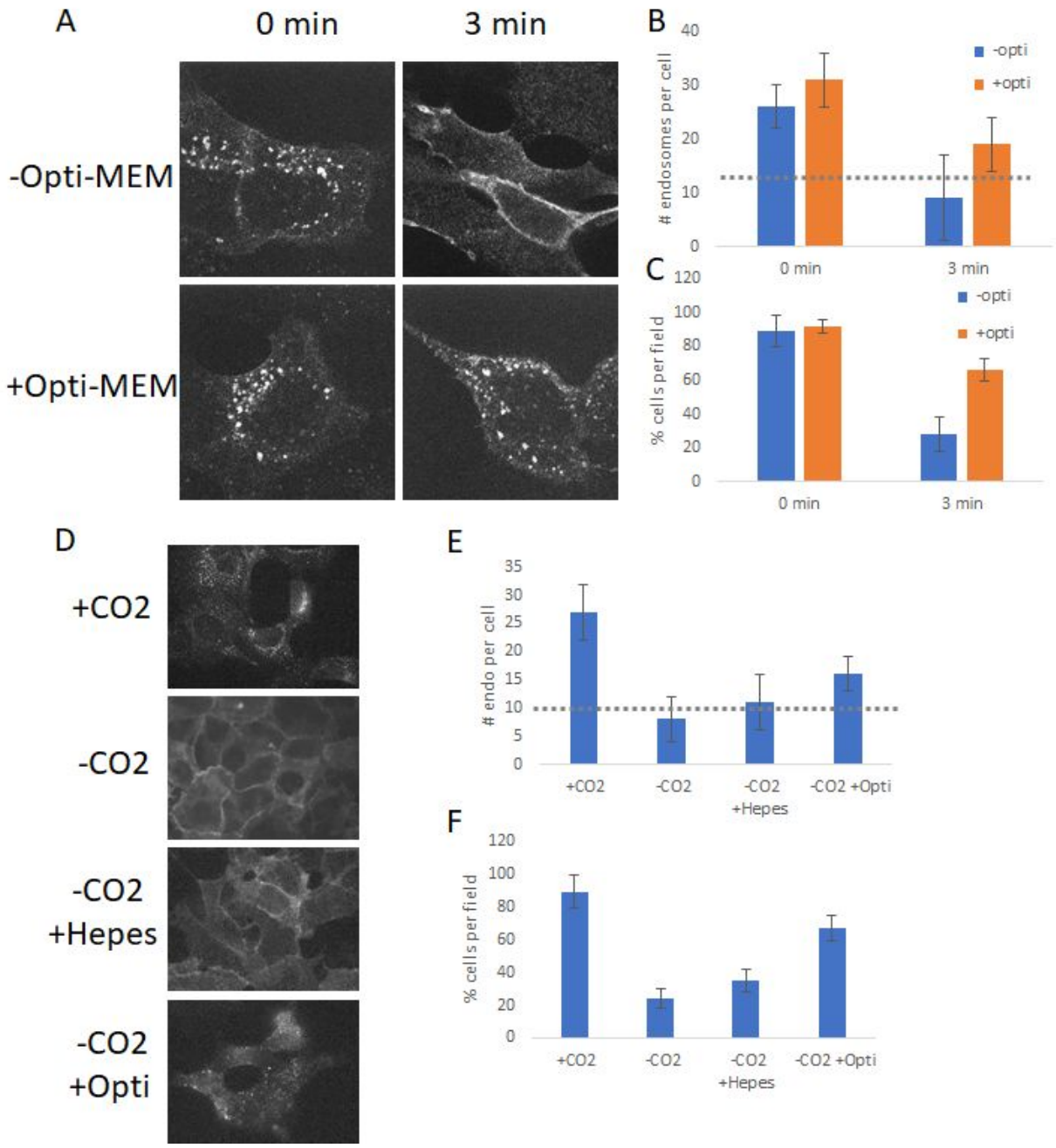

Figure 3.7: A negative CO2 gradient is sufficient to inhibit AC9 trafficking.
(A) Representative confocal imaging of AC9-EGFP stable HEK293 cells removed from the incubator for 0 or 3 minutes and Opti-MEM or control (48 hours prior) were treated with 10 μM isoproterenol or control for 30 min. Scale Bar is 8 μm. **(C)** Quantification of internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test. **(C)** Quantification of cells with >10 internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+

cells per condition]. ** P < 0.01 by two-tailed t-test. **(D)** Representative confocal imaging of AC9-EGFP stable HEK293 cells removed from a normal incubator to a CO2-incubator 3 minutes and treated with control, 10mM Hepes, or Opti-MEM (48 hours prior) were treated with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(E)** Quantification of internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test. **(F)** Quantification of cells with >10 internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** P < 0.01 by two-tailed t-test.

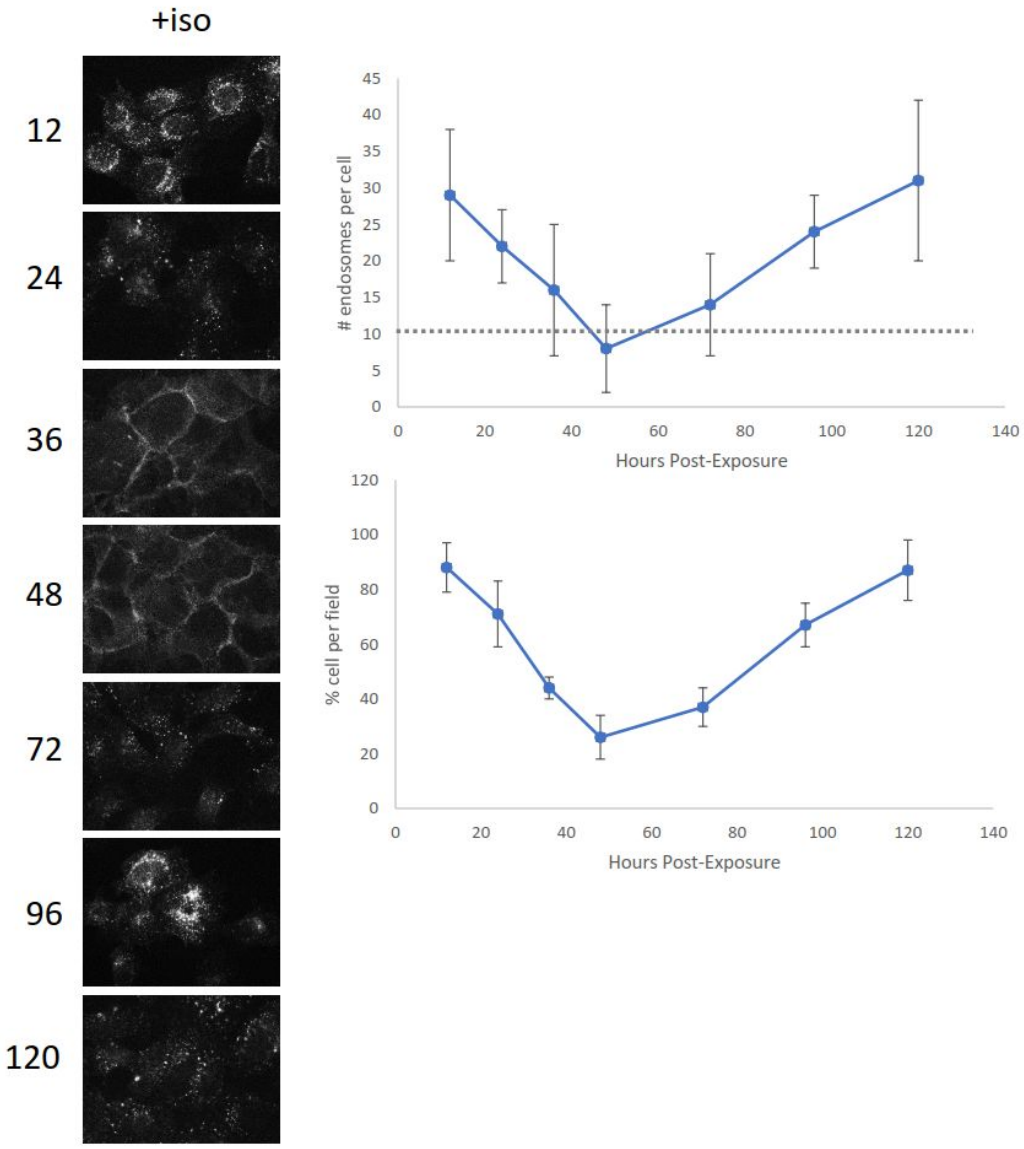

Figure 3.8: Acute exposure to room air inhibits AC9 for 5 days.
(A) Representative confocal imaging of AC9-EGFP stable HEK293 cells removed from the incubator for 3 minutes were incubated for 12, 24, 336, 48, 72, 96 or 120 hours before treatment with 10 μM isoproterenol for 30 min. Scale Bar is 8 μm. **(C)** Quantification of internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test. **(C)** Quantification of cells with >10 internal puncta that are AC9 positive, taken from wide field images (A) [mean±SEM; n=3 experiments, 10 visual fields and 200+ cells per condition]. ** $P < 0.01$ by two-tailed t-test.

Chapter 4:

Discussion

4.1 Dynamic trafficking of a Gs effector to the endosome

The early model of GPCR-mediated signaling and regulation by membrane trafficking emerged before a more complex understanding of the cell could provide necessary context. It was not unreasonable at the time to explain the observed cycle of desensitization and resensitization as membrane trafficking of the receptor from the plasma membrane to the endosome and back. Early biochemical testing indicated that receptor phosphorylation and internalization was coincident with an arrest of the initial acute cAMP response in the cell. Given the fairly small distances involved in the cell types used, it was reasonable that the system could rely on the fast diffusion of a large cAMP gradient from the plasma membrane to various effectors throughout the cell.

As an increasingly large number of GPCRs were catalogued, it became clear that cAMP alone is not enough for effector specificity. AKAPs scaffolding of AC isoforms and effectors allows a system that funnels several signal pathways through a single molecule to achieve input-output specificity. This growing understanding of local cAMP signaling as a proximally coordinated process is part of the logical foundation for endosome-based signaling. Studies of GPCR-mediated signaling in larger cell types such as neurons provided the first indication that delivery by the endocytic network may actually be required for the transmission of certain signals. Endosomal signaling is a necessity in cells in which it is energetically inefficient to rely on the diffusion of a small molecule gradient.

With several studies suggesting that the endosome is the site of GPCR and G protein activation, a critical question emerged: is there a Gs-regulated effector at the endosome? The data provided in this thesis demonstrate that AC9 is one such effector. We also show that this process is isoform specific, as AC1 remained restricted to the plasma membrane under all conditions tested in the same cells. However, we do not claim that AC9 is the only endosome-associated isoform. Given evidence suggesting AC2's involvement in PTHR signaling (Ferrandon et al. 2009) and AC5/6 association with AKAP79/150 (the only AKAP known thus far to enter endosomes) (Keith et al. 2012), we deem it likely that AC9 is not unique in this way. Rather, our observation of endosome-localized AC9 probably represents merely the first of many observations of differential AC localization. As AC isoform regulation includes cell type expression and co-expression with relevant AKAPs, a comprehensive model may take extensive exploration of relevant AC isoform localization in a variety of cell lines. However, we acknowledge that studies of AC localization have historically be hindered by a lack of good antibodies, low endogenous expression, and difficulty in cloning these very large proteins.

We also showed that not only is AC9 present at the endosome, but that it undergoes dynamically regulated endocytosis from the plasma membrane as a result of upstream Gs-coupled receptor activation. More specifically, activation of Gs and its engagement with AC9 is necessary and sufficient to drive AC9 internalization. This has several implications, the first being that adenylyl cyclases can exhibit isoform-specific trafficking.

This suggests that the endocytic network is not limited to just to deliver receptor and G protein to effectors around the cell (possibly coordinated by AKAPs). Rather, the endosome can constitute and deliver a complete GPCR-mediated signaling cascade with spatial and effector specificity.

The endosome is increasingly understood to be the site of GPCR-mediated signaling rather than the pool of inactive receptors. The existence of a Gs-regulated effector at the endosome is a significant step forward in the evolving model of the endosome. The revelation that the endocytic network differentially regulates AC isoforms and receptors and coordinates the necessary components for a cAMP response is massive. An overreliance on the assumption that cAMP diffusion is sufficient for robust Gs-regulated signal transduction must be challenged. Instead, a model in which the endocytic network and scaffolding proteins such as AKAPs are key spatial regulators of local cAMP signaling can be favored.

4.2 Distinct regulatory mechanism of AC9 trafficking

Data presented in this thesis strongly indicates that AC9 trafficking is regulated by a mechanism that is distinct from the B2AR. This was demonstrated in several ways in Chapter 2: genetic inhibition of receptor internalization (endocytic mutant V2R-T) did not affect AC9 trafficking, genetic and chemical activation of Gs was sufficient to drive AC9 internalization (but not B2AR), and arrestin knockout blocked receptor trafficking but had no effect on AC9. The acute negative CO2 gradient described in Chapter 3 was also shown to inhibit trafficking of AC9 but not B2AR. All of these data would be inconsistent with a model in which AC9 internalizes as a consequence of receptor endocytosis, either by tethering in a physical complex or by bulk membrane flow. Rather, they support a model in which AC9 localization is dynamically regulated, in which Gs plays a key role both in promoting catalytic activity and in accumulation in early endosomes.

While it is clear that B2AR and AC9 are regulated by distinct mechanisms, both proteins undergo regulated endocytosis from the plasma membrane to the endosome as a result of ligand binding of the receptor. Further studies into the relative kinetics of these processes are an immediate priority to the von Zastrow lab. Whether AC9 also enters clathrin coated pits and what, if any, adaptor proteins are involved in the accumulation of AC9 in B2AR-containing CCPs, is also of great interest. A comparison across GPCRs is also appropriate. Class B receptors such as the V2R and PTHR have been described in a ‘megaplex’ (Thomsen et al. 2016) with G protein and arrestin. The B2AR is a class

A GPCR, which does not associate in a stable complex with arrestin at the endosome. A comparison of long-term GPCR cargo sorting and AC isoform trafficking should prove informative. What role arrestin has in scaffolding or inhibiting GPCR-mediated cAMP response generated at the endosome will be an important question in the field.

One of the primary reasons we investigated AC9 membrane trafficking was that it was shown to be depleted from the plasma membrane due to retromer knockdown (Steinberg et al. 2013). We hypothesize that AC9 can undergo retromer-dependent recycling back to the plasma membrane, although this may be cell line specific. Although not formally presented in this thesis, we have observed AC9 and Gs in tubules extending from the endosome. Whether these tubules are retromer (recycling) tubules or not has not yet been rigorously investigated. Identification of a retromer-binding motif on AC9 or some binding partner, and observation of B2AR, Gs, and AC9 cargo sorting into retromer tubules, are active areas of interest in the von Zastrow lab.

The role of Gs in both promoting AC activity and in membrane trafficking of AC9 is a rather surprising one. We hypothesize that while Gs activation is both necessary and sufficient for AC9 trafficking, other binding partners such as AKAPs are more intimately involved in the membrane trafficking decisions. AC9 is most commonly associated with the plasma membrane localized AKAP, Yotiao. Yotiao is critical for coordination of adrenergic receptor signaling through AC9 with potassium channels in cardiac tissue (Li et al. 2012), but its role and localization patterns are not well described in other cell

lines. It is possible that Yotiao is involved in the accumulation of AC9 in endocytic pits, or that AC9 dynamically associates with another AKAP during this process. AC9 has not been previously associated with the only known AKAP to enter endosomes, AKAP79/150 (Keith et al. 2012). However, AC5 and AC6 have both been associated with AKAP79/150. It is possible that AC9 heterodimerization with AC5/6 (Baldwin et al. 2019) is required for AKAP79/150-dependent recruitment to the endosome. It is also possible that AC9 does not require engagement with an AKAP to enter endosomes. It has been proposed that GPCR endocytosis promotes a sustained cAMP response in part through separation from plasma membrane associated PDEs (Tsvetanova and von Zastrow 2014), and it is possible that AC9 has a similar relationship with Yotiao.

4.3 AC9 activity at the endosome

We also presented data in Chapter 2 that demonstrated that AC9 is a major contributor to the B2AR-mediated cAMP response from the endosome in HEK293 cells. Comparison of the antagonistic effects of the membrane permeant alprenolol and membrane impermeant CGP12177 enabled the isolation of a B2AR-mediated signal from an internal compartment. The CGP-insensitive 'signal gap' was shown to be eliminated by AC9 knockdown and by endocytic blockade with the dynamin inhibitor DYNGO-4a. Overexpression of AC9 (but not AC1) in AC3/6 double knockout cells was sufficient to produce a detectable CGP-insensitive cAMP response. These data were all consistent with the idea that B2AR mediates a cAMP response from the endosome, which is amplified in large part by AC9 in HEK293 cells.

AC9 activity has several unique characteristics relative to the other AC isoforms, including insensitivity to activation by forskolin, as well as insensitivity to direct inhibition by Protein Kinase A, Protein Kinase C, $G_{\beta\gamma}$, and Gi (Baldwin et al. 2019). AC9 has also been described to have a relatively high basal activity and requires higher concentration of Gs for maximal activity (Baldwin et al. 2019), but is unique amongst AC isoforms in that it has an autoinhibitory domain in its C terminus (Pálvölgyi et al. 2018). It is possible that once AC9 undergoes regulated endocytosis from the plasma membrane, it does not require receptor or even Gs engagement to continue propagating a cAMP response from the endosome.

Our data suggests that AC9 is the major contributor to the B2AR-mediated cAMP response from endosomes. However, this does not preclude other AC isoform involvement at the endosome. As mentioned previously, B2AR is a class A GPCR which is rapidly dephosphorylated at the endosome leading to arrestin dissociation. However, Class B receptors like the V2R continue to associate with arrestin and even form a supercomplex with Gs as well. It is probable that GPCR identity and its consequential binding partners (including AKAPs) may be a determinant for which AC isoform is active in a cell at any given time. Comparison of endosomal signaling across GPCRs and with disruption of arrestin binding is an active area of investigation in the von Zastrow lab.

A thorough investigation of how AC isoform localization affects the overall cellular response is necessary. It has been shown that the location of cAMP production affects responses in genetic expression (Tsvetanova and von Zastrow 2014). It has also been shown that AKAP localization and association with specific AC isoforms is important in several cell types (Keith et al. 2012; Esseltine and Scott 2013; Delint-Ramirez et al. 2015; Gros et al. 2006; Dessauer 2009). Isoform-specific membrane trafficking through the endocytic network is a significant puzzle piece in the growing understanding of spatial and effector specificity of cAMP signaling.

4.4 Inconsistency (a theme with AC9)

Cell signaling is notoriously difficult to study due to inconsistency and noise in data, and AC9 is a perfect example of this. Initial cloning of AC9 produced constructs of different length (Hacker et al. 1998; Iourgenko et al. 1997). Originally described as sensitive to inhibition by calmodulin and PKC but not $G_{\beta\gamma}$ or Gi (Dessauer et al. 2017), recent studies *in vitro* indicate that AC9 is not directly regulated by any of these mechanisms (Baldwin et al. 2019). However, heterodimerization with AC5/6 (Baldwin et al. 2019) may result in indirect regulation by some of these molecules and may explain the inconsistent reports of AC9 regulation.

Furthering this previously noted inconsistency in the literature is the subject of AC9 trafficking as discussed in Chapter 3. We devoted almost 3 years to troubleshooting the fixation protocol and ultimately discovered that AC9 trafficking is sensitive to inhibition by an acute exposure to a negative CO2 gradient. Other groups (including unpublished work) have noticed similar issues with standard cell culture techniques, describing sensitivity to temperature and oxygen gradient (Ast and Mootha 2019). It is not unreasonable that cells can quickly detect changes in their environment, but this appears to have been greatly underestimated by the cell biology field as a whole. The importance of cAMP signaling on physiological responses and drug development may force the scientific community to address these sensitivities. A wide array of cellular responses have been affected by any number of these phenomena unbeknownst to the researchers performing the assays.

4.5 References

Ast, Tslil, and Vamsi K. Mootha. 2019. “Oxygen and Mammalian Cell Culture: Are We Repeating the Experiment of Dr. Ox?” *Nature Metabolism*, August. https://doi.org/10.1038/s42255-019-0105-0.

Baldwin, Tanya A., Yong Li, Cameron S. Brand, Val J. Watts, and Carmen W. Dessauer. 2019. “Insights into the Regulatory Properties of Human Adenylyl Cyclase Type 9.” *Molecular Pharmacology*. https://doi.org/10.1124/mol.118.114595.

Delint-Ramirez, Ilse, Debbie Willoughby, Gerald R. V. Hammond, Laura J. Ayling, and Dermot M. F. Cooper. 2015. “Palmitoylation Targets AKAP79 Protein to Lipid Rafts and Promotes Its Regulation of Calcium-Sensitive Adenylyl Cyclase Type 8.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.a111.243899.

Dessauer, Carmen W. 2009. “Adenylyl Cyclase–A-Kinase Anchoring Protein Complexes: The Next Dimension in cAMP Signaling.” *Molecular Pharmacology* 76 (5): 935–41.

Dessauer, Carmen W., Val J. Watts, Rennolds S. Ostrom, Marco Conti, Stefan Dove, and Roland Seifert. 2017. “International Union of Basic and Clinical Pharmacology. CI. Structures and Small Molecule Modulators of Mammalian Adenylyl Cyclases.” *Pharmacological Reviews* 69 (2): 93–139.

Esseltine, Jessica L., and John D. Scott. 2013. “AKAP Signaling Complexes: Pointing towards the next Generation of Therapeutic Targets?” *Trends in Pharmacological*

Sciences 34 (12): 648–55.

Ferrandon, Sébastien, Timothy N. Feinstein, Marian Castro, Bin Wang, Richard Bouley, John T. Potts, Thomas J. Gardella, and Jean-Pierre Vilardaga. 2009. “Sustained Cyclic AMP Production by Parathyroid Hormone Receptor Endocytosis.” *Nature Chemical Biology* 5 (10): 734–42.

Gros, Robert, Qingming Ding, Jozef Chorazyczewski, J. Geoffrey Pickering, Lee E. Limbird, and Ross D. Feldman. 2006. “Adenylyl Cyclase Isoform–Selective Regulation of Vascular Smooth Muscle Proliferation and Cytoskeletal Reorganization.” *Circulation Research*, October. https://doi.org/10.1161/01.RES.0000245189.21703.c0.

Hacker, B. M., J. E. Tomlinson, G. A. Wayman, R. Sultana, G. Chan, E. Villacres, C. Disteche, and D. R. Storm. 1998. “Cloning, Chromosomal Mapping, and Regulatory Properties of the Human Type 9 Adenylyl Cyclase (ADCY9).” *Genomics* 50 (1): 97–104.

Iourgenko, V., B. Kliot, M. J. Cann, and L. R. Levin. 1997. “Cloning and Characterization of a Drosophila Adenylyl Cyclase Homologous to Mammalian Type IX.” *FEBS Letters* 413 (1): 104–8.

Keith, Dove J., Jennifer L. Sanderson, Emily S. Gibson, Kevin M. Woolfrey, Holly R. Robertson, Kyle Olszewski, Rujun Kang, Alaa El-Husseini, and Mark L. Dell’acqua. 2012. “Palmitoylation of A-Kinase Anchoring Protein 79/150 Regulates Dendritic Endosomal Targeting and Synaptic Plasticity Mechanisms.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 32 (21):

7119–36.

Li, Yong, Lei Chen, Robert S. Kass, and Carmen W. Dessauer. 2012. “The A-Kinase Anchoring Protein Yotiao Facilitates Complex Formation between Adenylyl Cyclase Type 9 and the IKs Potassium Channel in Heart.” *The Journal of Biological Chemistry* 287 (35): 29815–24.

Pálvölgyi, Adrienn, James Simpson, Ibolya Bodnár, Judit Bíró, Miklós Palkovits, Tamás Radovits, Paul Skehel, and Ferenc A. Antoni. 2018. “Auto-Inhibition of Adenylyl Cyclase 9 (AC9) by an Isoform-Specific Motif in the Carboxyl-Terminal Region.” *Cellular Signalling* 51 (November): 266–75.

Steinberg, Florian, Matthew Gallon, Mark Winfield, Elaine C. Thomas, Amanda J. Bell, Kate J. Heesom, Jeremy M. Tavaré, and Peter J. Cullen. 2013. “A Global Analysis of SNX27-Retromer Assembly and Cargo Specificity Reveals a Function in Glucose and Metal Ion Transport.” *Nature Cell Biology* 15 (5): 461–71.

Thomsen, Alex R. B., Bianca Plouffe, Thomas J. Cahill 3rd, Arun K. Shukla, Jeffrey T. Tarrasch, Annie M. Dosey, Alem W. Kahsai, et al. 2016. “GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling.” *Cell* 166 (4): 907–19.

Tsvetanova, Nikoleta G., and Mark von Zastrow. 2014. “Spatial Encoding of Cyclic AMP Signaling Specificity by GPCR Endocytosis.” *Nature Chemical Biology* 10 (12): 1061–65.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature

08/19/2019

Date