UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of protease-activated receptor-4 trafficking and heterodimerization in modulating receptor signaling

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Thomas Horace Smith

Committee in charge:

Professor JoAnn Trejo, Chair
Professor Joan Heller Brown
Professor Tracy Handel
Professor Paul Insel
Professor William Joiner
Professor Sanford Shattil

2016

The Dissertation of Thomas Horace Smith is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2016

DEDICATION

To my parents, Horace and Jennifer Smith.

This work would not have been possible without your

unconditional support and encouragement.

Thanks Mom and Dad!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	alanine
α_{2A}-AR	α_{2A}-adrenergic receptor
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-2	adaptor protein complex-2
β_2AR	β_2-adrenergic receptor
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
Ca^{2+}	calcium
CCP	clathrin-coated pit
cDNA	complementary DNA
CHC	clathrin heavy chain
CME	clathrin-mediated endocytosis
C-tail	cytoplasmic tail
C-terminal	carboxyl-terminal

DMEM	Dulbecco's Modified Eagle Medium
δOR	δ-opioid receptor
DTT	diothiothreiotol
EC_{50}	half maximal effective concentration
ECL	extracellular loop
EEA1	early endosomal antigen-1
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
ESCRT	endosomal sorting complexes required for transport
FLAG	DYKDDDDK epitope tag
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GPIIb	glycoprotein IIb
GPIIIa	glycoprotein IIIb
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate

HA	YPYDVPDYA epitope tag
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse radish peroxidase
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
ICL	intracellular loop
IP	inositol phosphate
IP3	inositol 1,4,5-triphosphate
K	lysine
kb	kilobase
kDa	kilodalton
L	leucine
LAMP1	lysosomal-associated membrane protein-1
MEF	mouse embryonic fibroblast
μM	micromolar
μOR	μ-opioid receptor
mRNA	messenger RNA
nM	nanomolar

NS	not statistically significant
ns	non-specific siRNA
OD	optical density
P	proline
PAR	protease-activated receptor
PEI	polyethylenimine
PI	phosphatidylinositol
PI3P	phosphatidylinositol 3-phosphate
PIP2	phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
PLC-β2	phospholipase C-β2
PVDF	polyvinylidene fluoride
R	arginine
Rluc	*Renilla* luciferase
RPMI	Roswell Park Memorial Institute medium
S	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA	small interfering RNA
SNP	single nucleotide polymorphism
T	threonine
TM	transmembrane
Vps4	vacuolar protein sorting-associated protein 4
WT	wild-type
Y	tyrosine
YFP	yellow fluorescent protein

LIST OF FIGURES & TABLES

ACKNOWLEDGEMENTS

I would like to acknowledge Professor JoAnn Trejo for her support as the chair of my committee. Dr. Trejo's guidance has proved invaluable throughout my graduate studies. I would also like to acknowledge the current and former members of the Trejo lab. I have been extremely lucky to be a member of such a supportive lab.

Some figures in Chapter 1 Section 1.4, in part, are published in a review article: Lin H, Liu AP, Smith TH, Trejo J. "Cofactoring and Dimerization of Proteinase-Activated Receptors." *Pharmacological Reviews*. **65**, 1198-1213 (2013). The dissertation author designed the figures used and is a co-author of the manuscript.

The majority of Chapter 2 is published as: Smith TH, Coronel LJ, Li JG, Dores MR, Nieman MT, Trejo J. "Protease-activated Receptor-4 Signaling and Trafficking Is Regulated by the Clathrin Adaptor Protein Complex-2 Independent of β-arrestins." *Journal of Biological Chemistry*. **291(35),** 18453-64 (2016). The dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

The majority of the data in Chapter 3 is being prepared for publication: Smith TH, Li JG, Dores MD, Trejo J. "Functional consequences of PAR4 heterodimerization with P2Y12 on endocytic sorting and β-arrestin recruitment." *Journal TBD.* The dissertation author is the primary author who directed experiments and analyzed the data.

VITA

EDUCATION

2016 Doctor of Philosophy, Biomedical Sciences

University of California, San Diego (La Jolla, CA)

2009 Bachelor of Arts, Biochemistry & Molecular Biology

Hendrix College (Conway, AR)

PUBLICATIONS

Lin H, Liu AP, **Smith TH**, and Trejo J. "Dimerization and cofactoring between proteinase-activated receptors: implications for ligand and signaling specificity." *Pharmacological Reviews*. **65**, 1198-1213 (2013). Cover Illustration.

Kawamura T, Stephens, B, Qin L, Yin X, Dores MR, **Smith TH**, Grimsey N, Abagyan R, Trejo J, Kufareva I, Fuster MM, Salanga CL, and Handel TM. "A general method for site specific fluorescent labeling of recombinant chemokines." *PLOS One*. **9(1),** (2014).

Soto AG[*], **Smith TH**[*], Chen B[*], Bhattacharya S, Canto Cordova I, Kenakin T, Vaidehi N, and Trejo J. "N-linked glycosylation of protease-activated receptor-1 at extracellular loop 2 regulates G-protein signaling bias." *Proc Natl Acad Sci.* **112(27)**, E3600-8 (2015). [*]These authors contributed equally to this work.

Grimsey NJ, Aguilar B, **Smith TH**, Le P, SooHoo AL, Puthenveedu AM, Nizet V, and Trejo J. "Ubiquitin plays an atypical role in GPCR-induced p38 MAP kinase activation on endosomes." *Journal of Cell Biology*. **210(7)**, 1117-31 (2015).

Smith TH, Coronel LJ, Li JG, Dores MD, Nieman M, Trejo J. "Protease-activated Receptor-4 Signaling and Trafficking Is Regulated by the Clathrin Adaptor Protein Complex-2 Independent of β-arrestins." *Journal of Biological Chemistry*. **291(35),** 18453-64 (2016).

Abstracts

Smith TH and Trejo J. "Regulation of protease-activated receptor-4 signaling and trafficking." Presented in April 2012 at the FASEB Experimental Biology Meeting, San Diego, CA

Smith TH and Trejo J. "Functional consequences of PAR4 heterodimerization with P2Y12 on endocytic sorting and β-arrestin recruitment." Presented in December 2013 at the ASCB Meeting, New Orleans, LA

Soto AG, **Smith TH,** Chen B**,** and Trejo J. "Glycosylation of protease-activated receptor-1 regulates G12/13 versus Gq signal pathway bias." Presented in April 2014 at the FASEB Experimental Biology Meeting, San Diego, CA

Smith TH, Coronel LC, Li J, Dores MR, Nieman M, and Trejo J. "Signaling and intracellular trafficking of protease-activated receptor-4 is regulated by the clathrin adaptor protein complex-2 and a tyrosine-based sorting motif independent of β-arrestins." Presented in April 2016 at the FASEB Experimental Biology Meeting, San Diego, C

ABSTRACT OF THE DISSERTATION

Characterization of protease-activated receptor-4 trafficking and heterodimerization in modulating receptor signaling

by

Thomas Horace Smith

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor JoAnn Trejo, Chair

G protein-coupled receptors (GPCRs) are transmembrane proteins that allow cells to respond to extracellular stimuli. GPCR activation occurs when a ligand binds to the extracellular portion of the receptor. The ligand-bound receptor

undergoes a conformational change that allows the intracellular domain of the receptor to engage and activate signaling effectors. The protease-activated receptors (PARs) are a family of GPCRs that are activated by proteases such as thrombin. Unlike traditional GPCRs, which can return to their inactive state after signaling, PARs are irreversibly activated. Therefore, the eventual fate of an activated PAR is degradation. The internalization and endocytic sorting of PARs play key roles in their signal regulation.

Trafficking has been well-characterized for PAR1, the prototypical thrombin receptor. PAR4 was the last of the four thrombin receptor to be discovered, and has in large part been relatively under-studied. Recent studies have shown that PAR4 plays distinct roles that differ from those of PAR1, and as such may represent a potential drug target. Thus, understanding the mechanisms that regulate PAR4 signal regulation is important.

In this dissertation, I characterized the role that trafficking and heterodimerization play in regulating PAR4 signaling. I found that adaptor-protein complex-2 (AP-2) is a key mediator of PAR4 agonist-induced internalization, and that AP-2 binds to intracellular loop 3 (ICL3) of PAR4. Disruption of PAR4 internalization resulted in enhanced and prolonged ERK1/2 activation, suggesting that endocytosis may mediate PAR4 signal attenuation.

I also characterized the role of heterodimerization with the puringeric receptor P2Y12 in modulating PAR4 signaling. Previous studies had demonstrated that PAR4 and P2Y12 coordinate β-arrestin-dependent Akt

activation. My work strongly suggests that PAR4 and P2Y12 physically associate basally and co-internalize in response to activation of PAR4. I also demonstrate that β-arrestin is recruited to the co-internalized PAR4-P2Y12 complex. In contrast to the effect on ERK1/2 activation, disruption of PAR4 internalization diminished Akt activation. Taken together, the studies summarized in this dissertation highlight the importance of PAR4 internalization in modulating activity of functionally and spatially distinct signaling pathways.

CHAPTER 1: INTRODUCTION

1.1 Introduction

G protein-coupled receptors (GPCRs) are seven transmembrane spanning cell surface receptors that play essential roles in many physiological processes. GPCRs are also implicated in many disease states, making them attractive drug targets (1,2). A thorough understanding of the molecular mechanisms that dictate GPCR function will help to guide the design of future therapeutics. Signal regulation, which has been shown to be intimately linked with receptor trafficking, is an important determinant of the function of many GPCRs and their associated cellular responses (3). The focus of this dissertation research was to investigate the interplay between trafficking, dimerization, and signal regulation of protease-activated receptor-4 (PAR4), a poorly characterized GPCR for the pro-coagulant protease thrombin.

1.2 Protease-activated receptors

1.2.1 PARs and thrombin signaling

Thrombin is serine protease that is essential for mediating the vascular response to tissue injury (4). Under basal conditions, thrombin is present in the bloodstream in its inactive zymogen form, pro-thrombin. When vascular tissue injury occurs, the integral membrane protein tissue factor is exposed to the vascular lumen, where it initiates a cascade of proteolytic cleavage events that ultimately lead to cleavage of pro-thrombin and generation of active thrombin.

One important target of thrombin is fibrinogen, which is cleaved to yield fibrin (5). Fibrin molecules polymerize and are stabilized in a complex that is incorporated at sites of vascular injury, where it serves as a major structural component for platelet recruitment to the developing blood clot (5).

Thrombin is able to elicit cellular responses in a variety of different cell types (6). One of the more well-studied functions of thrombin is its ability to activate platelets. Platelets are anucleate cells that are produced in the bone marrow by progenitor cells called megakaryocytes (7). Although platelets lack nuclei, they do contain megakaryocyte-derived messenger RNA (mRNA) and behave like typical cells in many respects. Platelets respond to vascular tissue injury by acting as the key initiators of blood clots at sites of vascular injury (8). Defects in platelet abundance or function are associated with bleeding disorders. Thrombin has long been known to be a key mediator of platelet activation during vascular injury, however, for many years the mechanism by which a protease was able to elicit cellular responses was unknown. This mystery was solved by the discovery of the protease-activated receptor (PAR) family of G protein-coupled receptors (GPCRs).

1.2.2 PAR characterization and tissue distribution

The PARs are a unique family of GPCRs that are able to signal in response to cleavage by proteases. PAR1 was the first member of the family to be cloned and characterized. PAR1 was discovered in 1991 through a *Xenopus laevis* oocyte expression screen (9). This involved microinjecting Xenopus

oocytes with megakaryocyte-derived mRNA and assessing responsiveness to thrombin as measured by $^{45}Ca^{2+}$ release. mRNA from oocytes that responded to thrombin treatment was isolated, fractioned by size, and assayed for thrombin responsiveness in the oocyte expression system. A cDNA library was generated from the 4 kb fraction, as it was the most responsive to thrombin treatment. A single 3.4 kb cDNA clone was identified by screening pools from the cDNA library. This clone was sequenced and designated as the thrombin receptor PAR1. PAR1's thrombin cleavage site was mapped to Arg-41/Ser-42 in the extracellular N-terminal domain (9).

In the years following the discovery of PAR1, two other members of the PAR family were characterized – PAR2, which signals in response to cleavage by trypsin, and PAR3, which is cleaved by thrombin but does not signal (10,11). However, knockout mice lacking the PAR1 gene exhibited no bleeding defects and platelets collected from these animals were still responsive to thrombin, suggesting the existence of an additional thrombin receptor (12). The identification of PAR4 as the last member of the PAR family was initiated by screening expressed sequence tag (EST) databases for sequences with homology to the known sequences of PAR1-3 (13). One EST was identified that shared homology with transmembrane region 4 (TM4) of the three existing PARs. A DNA probe was generated from the EST tag and used to screen a cDNA library derived from the Daudi, a lymphoma cell line. A 4.9 kb transcript was identified from this screen and designated as PAR4. Hydropathy analysis

confirmed that PAR4 contained the seven transmembrane domains that are hallmarks of GPCRs. Overall, PAR4 shares approximately 33% sequence homology with PAR1-3, and as expected, it contains a protease cleavage site – Arg-47/Gly-48 – at its N-terminus. Responsiveness to thrombin was assessed by measuring phosphatidylinositol 4,5-bisphosphate (PIP_2 or simply PI in this context) hydrolysis and inositol phosphate (IP) accumulation, which confirmed that thrombin was able to activate PAR4, although with a much higher EC_{50} than PAR1. The location of the thrombin cleavage site was confirmed by mutagenesis – a mutant human receptor, designated as PAR4 R47A, in which the critical arginine (R), Arg-47, was mutated to alanine (A), failed to signal in response to thrombin. A hexapeptide corresponding to the newly exposed amino terminus was able to activate wild-type PAR4 as well as the R47A mutant. This finding was consistent with previous studies on PAR1 and PAR2 that demonstrated peptides corresponding to their unmasked N-termini were also able to induce signaling independent of receptor cleavage (13).

PARs are expressed in a variety of tissue and cell types. The PAR4 gene has been detected by Northern blot analysis in the lung, pancreas, thyroid, testis, placenta, skeletal muscle, prostate, colon, and lymph nodes (13,14). The role, if any, of PAR4 in many of these tissues has yet to be determined, with much of the research focused on PAR4's role in the cardiovascular system. In addition to platelets, PAR4 is also expressed in human leukocytes, endothelial cells, and smooth muscle cells (15-18).

Studies in rats have demonstrated that PAR4 is linked to inflammation. Treatment of rat mesenteric venules with thrombin or the PAR4-specific activating peptide caused an increase in leukocyte recruitment to the peritoneal cavity. This finding implicates PAR4 as a key mediator of thrombin-induced effects on leukocytes associated with inflammation (15). PAR4 activation was also found to induce edema formation in rat paws (19). PAR4 is expressed in dorsal root ganglion neurons of rats, and has been shown to play a possible role in nociception. PAR4-specific agonist peptide treatment elicited analgesia against mechanical and thermal pain stimuli in rats (20). However, animal studies of PAR4 function are not necessarily translatable to human. The distribution of PARs in many cell types varies between species. For example, PAR4 activation mediates responses in mouse endothelium and rat aorta, but human preparations of these tissues were not responsive to PAR4 stimulation alone (21).

The most well-characterized function for PAR4 in human physiology is its critical role in platelet activation. Human platelets express PAR1 and PAR4, and PAR4's decreased affinity for thrombin relative to PAR1 initially led some to believe that PAR4 was simply a "back-up" thrombin receptor (14). However, further investigation revealed that PAR4 is responsible for a unique subset of events associated with platelet activation. Studies in human platelets demonstrated that PAR1 and PAR4 mediate two temporally distinct signaling responses associated with platelet activation (22). Platelets stimulated with the

PAR1-specific peptide agonist SFLLRN exhibited a rapid and transient signaling response, as measured by Ca^{2+} flux. Conversely, treatment with the PAR4-specific peptide agonist AYPGKF elicited a delayed, protracted signaling response (22). Thus, PAR1 is thought to be responsible for the early stages of platelet activation while PAR4 is hypothesized to mediate the later stages of activation which are important for formation of a stable blood clot.

1.2.3 PAR mechanism of activation

The majority of GPCRs are activated by binding of a soluble ligand to the receptor's extracellular domain or transmembrane regions. PARs utilize a unique mechanism of activation that is initiated by proteolytic cleavage of the receptor's N-terminus at a specific site. Following cleavage, the newly exposed N-terminus acts as a "tethered ligand" and binds intramolecularly to extracellular loop 2 (ECL2) of its cognate receptor. This binding causes a conformational change in the receptor that facilitates G protein coupling. Unlike other GPCRs, which can dissociate from their ligand after activation, PARs are irreversibly activated (Fig 1.1).

One important distinction between PAR4 and PAR1 is their different affinities for thrombin. With an EC_{50} of 0.2 nM, PAR1 is known as a high-affinity thrombin receptor, whereas PAR4 has a much lower affinity for thrombin, with an EC_{50} of 5 nM (13,23). PAR1's high affinity for thrombin has been attributed to the presence of a hirudin-like sequence in the receptor's N-terminus. This domain allows PAR1 to interact with thrombin's exosite I, which is a groove of positively

charged residues essential for strong interaction with several of the protease's substrates (24). PAR4 lacks a hirudin-like domain, and as a result is unable to form a strong association with thrombin via exosite I. Instead, PAR4 utilizes a cluster of anionic residues in its N-terminus to interact with cationic domains of thrombin at or near exosite I (25).

PARs have the capacity to couple to multiple different G protein subtypes, including $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ (26). PAR4 specifically couples to $G\alpha_{12/13}$ and $G\alpha_q$. PAR4-mediated $G\alpha_{12/13}$ signaling leads to activation of Rho, which causes Rho-kinase-dependent cytoskeletal changes. In platelets, $G\alpha_{12/13}$-induced Rho-kinase activation induces shape change, a critical event in platelet activation. In mouse endothelial cells, PAR4-induced Rho activation has been shown to induce vascular and colonic paracellular permeability (27). PAR4 coupling to $G\alpha_q$ activates phospholipase C-β2 (PLC-β2), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP_3). IP_3 induces intracellular Ca^{2+} mobilization, which leads to the activation of several downstream effectors such as protein kinase C (PKC) and extracellular signal-regulated kinase-1 and 2 (ERK1/2) (28). In platelets, $G\alpha_q$ signaling by PAR4 is responsible for integrin activation and the release of dense granules that contain various factors that are critical for sustained platelet activation and recruitment of additional platelets to the developing clot (4,29).

1.3 Regulation of PAR signaling

1.3.1 Desensitization

Cells exercise tight control over the magnitude and duration of an activated GPCR's signaling response. Maintaining proper signal fidelity is important for normal cell physiology, and aberrant signaling is associated with cardiovascular diseases and cancer (30). According to the canonical model, GPCR desensitization is initiated by phosphorylation of serines and threonines located primarily within the cytoplasmic tail (C-tail) domain of the receptor. Subsequent recruitment of β-arrestin causes uncoupling of the G proteins from the activated receptor, thereby attenuating signaling (Fig 1.2*B*) (31-33).

Several studies have investigated the desensitization mechanisms of PAR1 and PAR2. PAR1 undergoes robust phosphorylation within minutes of activation by thrombin (34,35). A PAR1 phospho-deficient mutant lacking all cytoplasmic serine and threonines did not undergo agonist-induced phosphorylation and failed to terminate $G\alpha_q$ signaling, as measured by PI hydrolysis (34). PAR1 phosphorylation appears to be mediated by G protein-coupled receptor kinases (GRKs) -3 and -5, as overexpressing either of these kinases resulted in enhanced PAR1 phosphorylation and suppressed signaling (36). β-arrestins have been shown to be essential for desensitization of PAR1 signaling (37). Intriguingly, β-arrestin-mediated desensitization of PAR1 was demonstrated to occur independent of receptor phosphorylation.

PAR2 also undergoes significant phosphorylation following agonist stimulation. The C-tail of PAR2 contains several serine and threonine residues, mutation of which results in a phospho-deficient PAR2 mutant that fails to undergo agonist-induced phosphorylation (38). Like PAR1, the PAR2 phospho-deficient mutant also exhibits defects in signal termination (38). PAR3 is cleaved by thrombin, but has not been shown to elicit autonomous signaling responses in most cell types, but rather appears to function as a co-factor for thrombin-activation of other receptors. Thus, the mechanisms responsible for PAR3 signal termination have not been determined. The initial studies to characterize PAR4 signal regulation focused on identifying the role of phosphorylation. Intriguingly, however, PAR4 does not appear to undergo agonist-induced phosphorylation, and a PAR4 mutant lacking all C-tail serines and threonines did not exhibit defects in signal termination (34). Beyond the studies of PAR4 phosphorylation, there has not been any further examination of the mechanism that regulates PAR4 signaling, which is one focus of this dissertation research.

1.3.2 Internalization

The majority of GPCRs undergo regulated endocytosis following their activation at the plasma membrane. GPCR internalization most often occurs by clathrin-mediated endocytosis (CME) (Fig 1.2*C*). CME is facilitated by the recruitment of β-arrestins, which act as clathrin adaptor proteins in this context. Specifically, binding of β-arrestin's polar core to the receptor's phosphorylated residues induces a conformational change that allows the carboxyl-terminal (C-

terminal) domain of arrestin to interact with clathrin and adaptor protein complex-2 (AP-2) (31). Clathrin-coated pits, which form as subdomains of the plasma membrane and contain receptor cargo as well as clathrin and adaptor proteins, fold inward and bud off into the cytoplasm. The GTPase dynamin facilitates scission of clathrin-coated pits from the plasma membrane-generated clathrin-coated vesicles, which subsequently shed their clathrin coat and then fuse with early endosomes.

Internalization of PAR1 has been thoroughly investigated by several studies. PAR1 undergoes two different types of internalization: constitutive and agonist-induced. PAR1 constitutive internalization serves to maintain a pool of naive receptors in the cytoplasm that are used to resensitize the cell following cleavage and activation of the surface PAR1 cohort. Constitutive internalization of PAR1 has been shown to be mediated by AP-2, which binds to a C-tail localized tyrosine (Y) based motif YXXL (39). While AP-2 is also involved in agonist-induced internalization of PAR1, this process is not completely blocked by AP-2 depletion. Rather, AP-2 works in concert with another clathrin adaptor protein, epsin-1, to facilitate internalization of activated PAR1 (40).

In addition to their role in desensitization, β-arrestins also serve as essential clathrin adaptor proteins and are necessary for the internalization of most classic GPCRs (33,41,42). Although β-arrestin is recruited to activated PAR1, it seems to only play a role in desensitization of the receptor, and is not required for internalization (37,43). In contrast, PAR2 internalization is β-arrestin-

dependent, and the receptor remains strongly associated with β-arrestin after internalization (44,45). This is primarily due to the number and pattern of juxtaposed phosphorylation sites within the C-tail domain of PAR2, which serve as a high affinity binding site for β-arrestins (38). At the onset of this dissertation research project, the mechanisms that control PAR4 trafficking had not been previously investigated. The studies in Chapter 2 of this dissertation represent the first published characterization of PAR4 agonist-induced internalization.

1.3.3 Down-regulation

Once internalized, GPCRs are either recycled back to the cell surface (Fig 1.2*D*) or sorted to the lysosome for degradation (Fig 1.2*E*). Unlike the majority of classic GPCRs, activated PARs are precluded from recycling due to their irreversible mechanism of activation. Thus, most activated PARs are ultimately destined for degradation at the lysosome. Ubiquitination commonly serves as a signal for degradation, and involves modification of a target protein by the addition of poly-ubiquitin chains. Ubiquitin is a 76 amino acid protein that can be covalently attached to lysine (K) residues of target proteins. Ubiquitinated proteins traffic through the well-characterized endosomal sorting complex required for transport (ESCRT) pathway, which shuttles protein cargo from early endosomes to late endosomes/lysosomes for degradation (46-49). ESCRT-0 is recruited to endosomes through interaction of its FYVE domain with phosphatidylinositol-3-phosphate (PI3P), which is abundant on endosomal membranes. ESCRT-0 directly interacts with the ubiquitinated cargo and the

ESCRT-I complex. The two remaining ESCRT complexes, ESCRT-II and ESCRT-III, are then recruited and facilitate the sorting of ubiquitinated proteins into late endosomes which fuse with lysosomes to mediate degradation of the cargo protein (46,48).

PAR2 follows the canonical ubiquitin-dependent ESCRT sorting pathway. Studies have demonstrated that activated PAR2 is directly ubiquitinated by the E3 ubiquitin ligase c-Cbl, and that components of the ESCRT pathway are required for its proper degradation (50,51). In contrast, PAR1 is sorted to lysosomes for degradation by an ubiquitin-independent mechanism. Studies in HeLa cells and Rat1 fibroblasts have shown that a PAR1 ubiquitin-deficient “0K” mutant, in which all cytoplasmic exposed lysine (K) residues were converted to alanine (A), is degraded to a similar extent as wild-type receptor (52). These studies suggest that PAR1 is efficiently sorted to lysosomes independent of direct receptor ubiquitination. Recent work by our lab has further demonstrated that PAR1 lysosomal sorting is mediated by ALIX, an adaptor protein that links the receptor to ESCRTs (53). Although some of the same proteins are involved, ALIX-dependent lysosomal sorting is distinct from the canonical ESCRT-dependent pathway due to its ability to sort un-ubiquitinated cargo independent of key ESCRT proteins such as hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and vacuolar protein sorting-associated protein 4 (Vps4). Virtually nothing is known about the ultimate fate of PAR3 or PAR4 following agonist-induced internalization. I have conducted the first published

characterization of PAR4 endosomal-lysosomal sorting, which is described in Chapter 2 of this dissertation.

1.4 PAR cofactoring and dimerization

Many class A GPCRs are able to physically associate to form homodimers, heterodimers, or oligomers (Fig 1.3). The role of homodimerization and heterodimerization in GPCR pharmacology has become much more appreciated in recent years. Many cell types express more than one PAR, and interaction with one another can modulate signaling activity (54). An early example of PAR cofactoring was demonstrated in murine platelets, which co-express PAR3 and PAR4. Although PAR3 is cleaved by thrombin, it does not signal autonomously, but instead acts as a cofactor to facilitate more efficient cleavage and activation of PAR4 (55). A similar mechanism may allow for more efficient cleavage of PAR4 in human platelets, which co-express PAR1 and PAR4 (56). In certain cell types, the tethered ligand of thrombin-cleaved PAR1 can bind intermolecularly and transactivate an adjacent PAR2 (57,58). Thus, PARs are able to expand their signaling diversity by localizing thrombin to facilitate efficient cleavage or by providing a tethered ligand domain to an adjacent PAR. The ability of PARs to function in these ways suggests that the receptors are in close proximity and perhaps in the form of a dimer. Several published studies provide evidence that indicates PARs are capable of forming homodimers and heterodimers.

1.4.1 mPAR3-mPAR4 cofactoring

The concept of a PAR functioning as a cofactor to facilitate activation of an adjacent PAR was first described in a study of mouse platelets (55). Mouse platelets express two thrombin receptors – mPAR3 and mPAR4. Both receptors are sensitive to thrombin cleavage, but mPAR3 cleavage occurs at much lower concentrations of thrombin due to the presence of a hirudin-like domain, which mPAR4 lacks. Platelets isolated from mPAR3 knockout mice exhibited impaired and delayed responses to thrombin stimulation, suggesting a function for mPAR3 in thrombin signaling. Blocking antibodies directed against mPAR3 also inhibited thrombin-induced platelet activation, however, thrombin cleavage of mPAR3 did not promote Ca^{2+} signaling in an ectopic expression system (55). The inability of mPAR3 to signal was not consistent with its observed role in mediating robust thrombin-induced platelet activation. A possible explanation for this phenomenon is that mPAR3 brings thrombin in close proximity to mPAR4 to promote more efficient cleavage (Fig 1.4). Thus, in the absence of its own high-affinity hirudin-like domain, mPAR4 is still able to respond to low concentrations of thrombin by using mPAR3 as a cofactor. This model was supported by studies in transfected COS7 cells which showed that coexpression of mPAR3 and mPAR4 resulted in a significant increase in the efficiency of thrombin-stimulated signaling (55). Furthermore, x-ray crystallography studies revealed that cleaved mPAR3 remains bound to exosite I of thrombin, leaving the protease's active site freely accessible to other substrates such as mPAR4 (59).

1.4.2 hPAR1-hPAR2 transactivation

The first study to provide evidence for intermolecular liganding between PAR1 and PAR2 was performed in COS7 cells coexpressing PAR2 with a PAR1 mutant containing a leucine (L) to proline (P) conversion within extracellular loop 2. This mutant was designated PAR1 L258P and was sensitive to thrombin cleavage but did not signal. Cells expressing PAR1 L258P alone or PAR2 alone failed to signal in response to thrombin treatment. However, thrombin-induced signaling was restored when PAR1 L258P was co-expressed with PAR2 (58). The use of PAR1-blocking antibodies and cross-desensitization experiments in endothelial cells confirmed that PAR2 was able to be transactivated by thrombin-cleaved PAR1 (58). This transactivation model was also supported by earlier studies that investigated the specificity of PAR1 and PAR2 tethered ligands for their respective receptors. The PAR2 tethered ligand, SLIGKV, failed to induce signaling by PAR1 expressed in *Xenopus laevis* oocytes (60). However, the PAR1 peptide agonist, SFLLRN, was able to activate PAR2 at concentrations similar to those of the native PAR2 peptide agonist (60). Later work provided additional support for this model by demonstrating that PAR1 transactivated PAR2 on endothelial cells during progression of sepsis (61). This effect was dependent upon PAR2 expression, wherein increased pathologic conditions upregulated PAR2 and switched PAR1 signaling from vascular-disruptive to vascular-protective (61). Our lab has published work consistent with this model, in which cytokine-treated endothelial cells and HeLa cells exhibited increased

PAR2 expression and enhanced thrombin-mediated activation of β-arrestin-dependent ERK1/2 signaling (Fig 1.5) (57).

1.4.3 PAR dimerization

The preceding examples are only a select few of the several reported accounts of cofactoring, synergistic signaling, and other cross-talk effects exhibited by PARs. The spatial dynamics suggested by many of these interactions suggest that the receptors involved are either in very close proximity or are physically associated as a dimer or higher order oligomer. In fact, several accounts of PAR-PAR dimerization have been reported and are summarized in Table 1.1.

1.4.4 PAR4/P2Y12 signaling cross-talk

Evidence also exists for signaling cross-talk between PARs and other GPCRs. The purinergic receptor P2Y12 is coexpressed with PAR4 in platelets and is one of the two platelet receptors activated by adenosine diphosphate (ADP). P2Y12 is a clinically-significant receptor, as the P2Y12 antagonist clopidogrel (also known as Plavix) is an important antiplatelet agent used to prevent heart attack and stroke. A study in human platelets showed that blocking P2Y12 activity caused a reduction in PAR4-mediated Akt activation and impaired recruitment of PAR4 to β-arrestin signaling complexes (62). We have investigated the role of dimerization in mediating the PAR4-P2Y12 crosstalk described above. While addressing this question, a study was published by another lab that reported on some aspects of the PAR4-P2Y12 heterodimer (63).

However, our characterization of the PAR4-P2Y12 heterodimer, which is presented in Chapter 3 of this dissertation, provides additional novel insights into its trafficking and the role it plays in β-arrestin-mediated endosomal signaling.

1.5 Acknowledgements

Some figures in Chapter 1 Section 1.4, in part, are published in a review article: Lin H, Liu AP, Smith TH, Trejo J. "Cofactoring and Dimerization of Proteinase-Activated Receptors." *Pharmacological Reviews*. **65**, 1198-1213 (2013). The dissertation author designed the figures that were used herein and is a co-author of the manuscript.

1.6 Figures

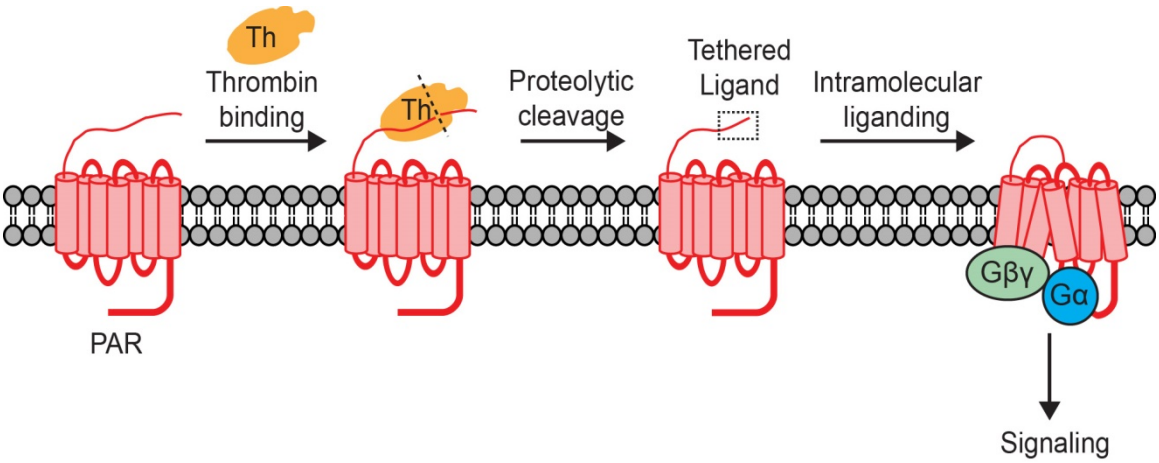

Figure 1.1: PARs are activated by proteolytic cleavage.
Protease-activated receptor activation is initiated by binding of a protease (e.g. thrombin) to the receptor's N-terminus. Thrombin cleaves the receptor at a specific site, which reveals a previously obscured tether ligand. The tethered ligand binds intramolecularly to the receptor, inducing a conformational change that facilitates G protein coupling and downstream signaling.

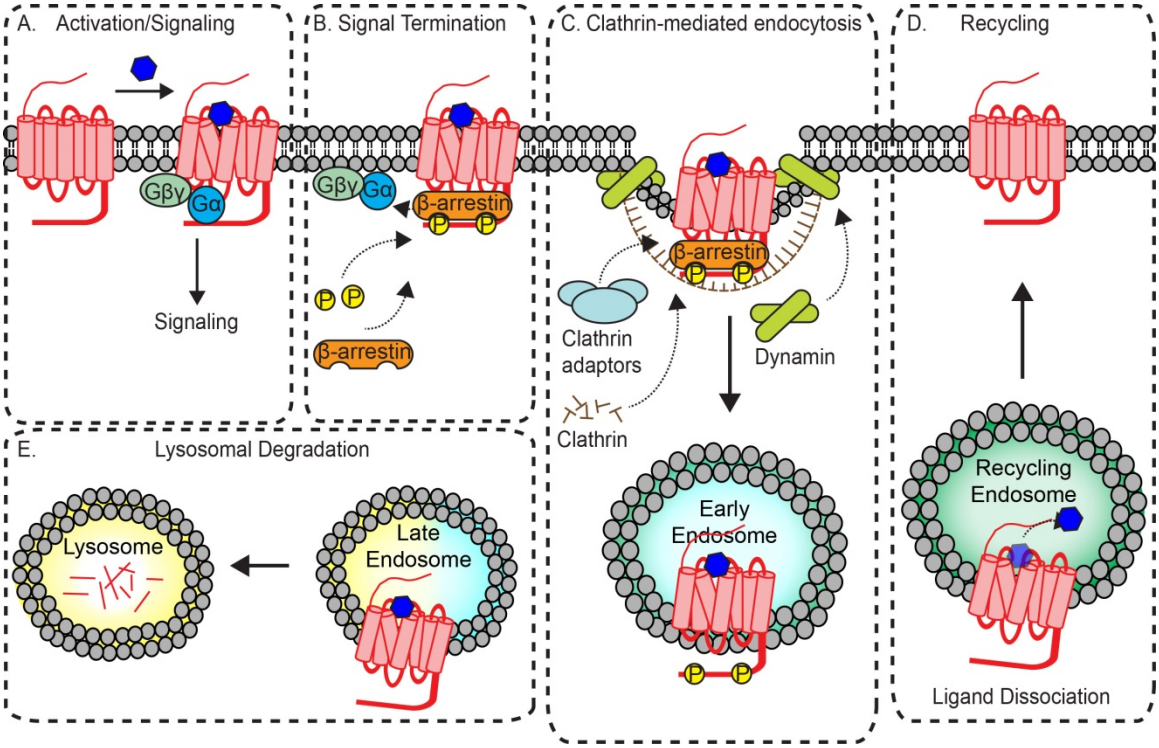

Figure 1.2: Canonical model of GPCR signal regulation.
A, A classical GPCR is activated at the plasma membrane by binding of a soluble ligand to the receptor's extracellular domain. *B,* Signal termination is initiated by phosphorylation of Ser and Thr residues present on the receptor's cytosolic domains, most often on the C-tail. β-arrestin binds to phosphorylated residues of the receptor, which causes G protein uncoupling and signal attenuation. *C*, β-arrestin and other clathrin adaptor proteins facilitate the formation of a clathrin-coated pit (CCP), which folds inward into the cytoplasm. The GTPase dynamin catalyzes scission of the CCP, allowing it to bud off from the plasma membrane. The CCP sheds its clathrin coat and fuses with the early endosome. *D*, Many GPCRs enter a recycling pathway, which involves dissociation of the ligand from the receptor and anterograde transport of the inactive receptor back to the plasma membrane. *E*, Other GPCRs do not undergo recycling, and are instead sorted from early endosomes to late endosomes/lysosomes for degradation.

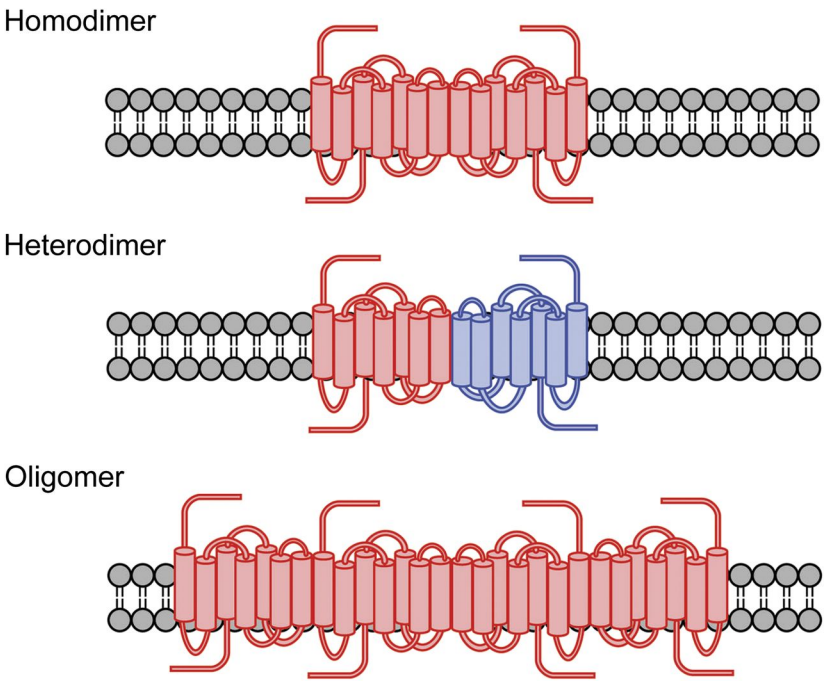

Figure 1.3: GPCR dimer formation.
Class A family of rhodopsin-like GPCRs are composed of several hundred members, and a vast literature indicates that many of these receptors can self-associate to form homodimers or interact with other GPCRs to form heterodimers or exist as higher-order oligomeric complexes. Several recent structures of GPCR dimers have been solved and indicate that distinct regions of various TMs mediate the dimer interface, depending on the receptor. Our illustration of a GPCR dimer, heterodimer, and oligomer association are not meant to reflect actual TM interactions, since these have not been definitively determined for any PAR dimers.

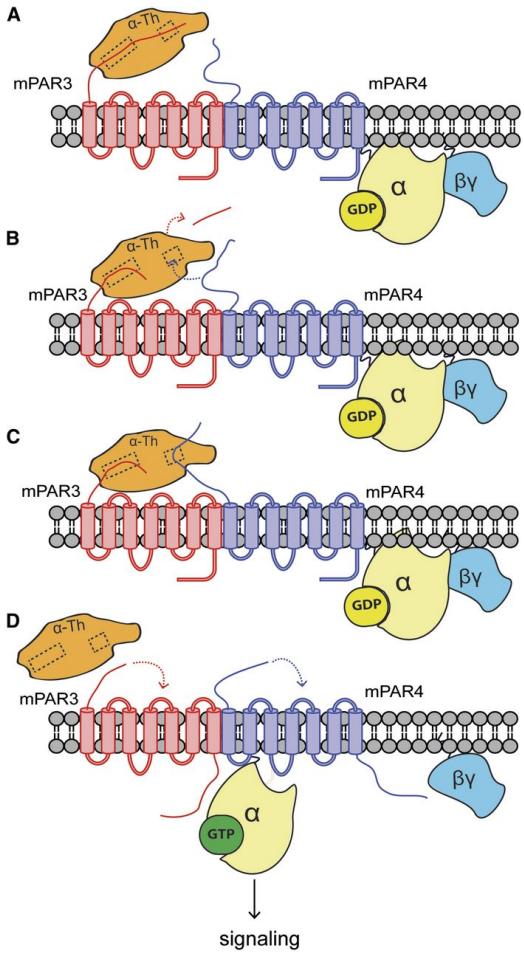

Figure 1.4: Model of mPAR3 cofactoring of mPAR4.
A, The mPAR3-thrombin complex is shown. Similar to PAR1, the mPAR3 hirudin-like sequence binds with high affinity to thrombin's (*α*-Th) exosite I (dashed rectangle), facilitating the interaction between mPAR3′s cleavage site and thrombin's active site (dashed square). *B*, After cleavage, mPAR3 remains associated with thrombin due to the high-affinity interaction at exosite I (dashed rectangle). This conformation leaves thrombin's active site unobstructed (dashed square) and accessible to the mPAR4 N-terminal region. *C*, The mPAR3-thrombin-mPAR4 complex is shown. The localization of catalytically active thrombin by mPAR3 allows the mPAR4 N-terminus to interact with thrombin's active site (dashed square) and results in efficient thrombin cleavage of mPAR4. *D*, After cleavage of mPAR4, thrombin dissociates from the complex. The newly exposed N-terminal–tethered ligand domain of mPAR4 is then able to bind intramolecularly to the second extracellular loop and triggers G protein signaling.

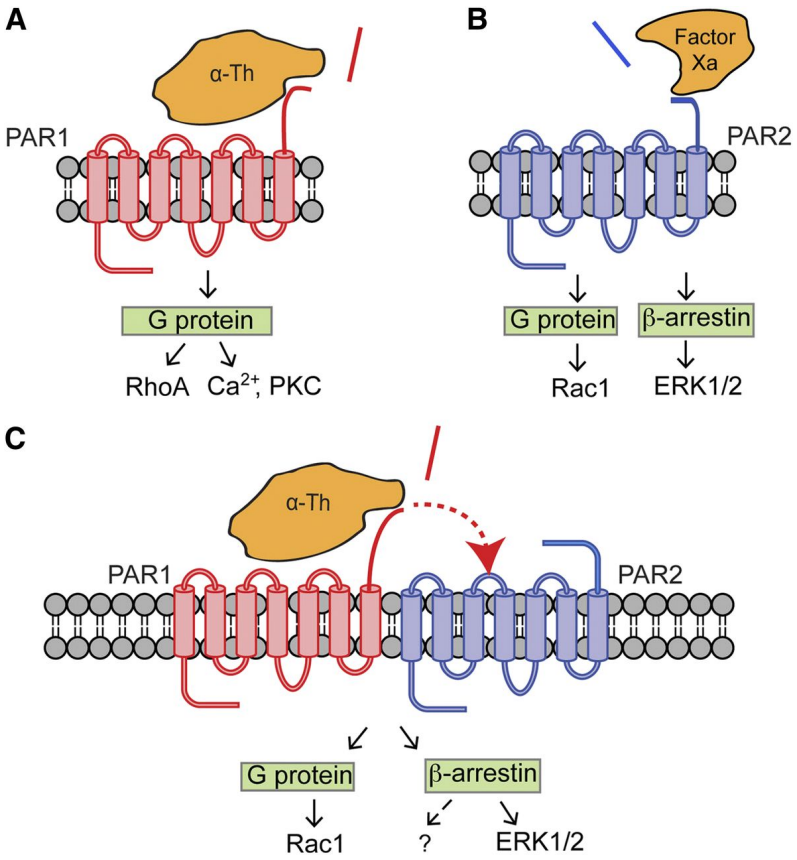

Figure 1.5: PAR1 transactivation of PAR2.
A, PAR1 is likely to exist as a monomer in naïve endothelial cells, and upon thrombin activation, efficiently couples to G proteins to promote RhoA activation, Ca^{2+} mobilization, and protein kinase C (PKC) activation, important mediators of endothelial barrier disruption. *B*, In contrast, PAR2 activation by factor Xa is known to stimulate barrier protection through G protein coupling to Rac1 activation. However, activation of PAR2 is also known to result in recruitment of β-arrestins, which function as a scaffold to facilitate ERK1/2 activation. *C*, In inflammatory conditions, PAR2 expression is increased in endothelial cells and forms dimers with PAR1. Thrombin cleavage of PAR1 results in the generation of a tethered ligand that can bind to an adjacent PAR2 *in trans* to promote a distinct signaling response that results in Rac1 activation and β-arrestin–mediated ERK1/2 activation and vascular protective responses.

Table 1.1: Protease-activated receptor cofactoring and dimerization.
This table summarizes publications that provide evidence of PAR-PAR cofactoring and dimerization.

Receptors	Function	Technique	Reference
PAR1-PAR1	Intermolecular liganding Ca^{2+} signaling	Functional reconstitution, *X. laevis* oocytes	Chen et al., 1994
PAR1-PAR1	Not studied	BRET, HEK293 cells	McLaughlin et al., 2007
PAR1-PAR2	Intermolecular liganding, Ca^{2+} signaling	Blocking antibodies, functional reconstitution, endothelial cells and COS7 cells	O'Brien et al., 2000
PAR1-PAR2	Intermolecular liganding, Cytoprotective signaling	Blocking and activating agents FRET, co-IP, in vitro and in vivo studies, endothelial cells	Kaneider et al., 2007
PAR1-PAR2	Intermolecular liganding, smooth muscle hyperplasia	Blocking and activating agents, co-IP, in vitro and in vivo studies, smooth muscle cells and COS7 cells	Sevigny et al., 2011
PAR1-PAR2	Intermolecular liganding, β-arrestin signaling	Functional reconstitution, BRET, co-IP, microscopy, COS7 cells, HeLa cells, endothelial cells	Lin and Trejo, 2013
PAR1-PAR3	Allosteric modulation of G protein signaling	BRET, fluorescence microscopy, HEK293 and endothelial cells	McLaughlin et al., 2007
mPAR1-mPAR3	Cytoprotective signaling	Blocking agents, co-IP in vitro and in vivo studies, podocytes and mesangial cells	Madhusudhan et al., 2012
PAR1-PAR4	Cofactoring platelet activation	Blocking agents, co-IP, FRET, in vitro and in vivo studies, platelets and COS7 cells	Leger et al., 2006
PAR2-PAR2	Intermolecular liganding, inflammatory signaling	Functional reconstitution, co-IP, pepducins, in vitro and in vivo studies, COS7 cells and inflammatory cells	Sevigny et al., 2011
PAR2-PAR3	Cytoprotective signaling	Blocking agents, co-IP in vitro studies, human podocytes and mesangial cells	Madhusudhan et al., 2012
PAR2-PAR4	Anterograde trafficking	FRET, co-IP, microscopy HEK293 cells	Cunningham et al., 2012
mPAR3-mPAR4	Cofactoring platelet activation	Functional reconstitution, COS7 cells	Nakanishi-Matsui et al., 2000
PAR4-PAR4	Ca^{2+} signaling	BRET, BiFC HEK293 cells	De La Fuente et al., 2012
mPAR4-mPAR3	Not studied	BRET, HEK 293 cells	Arachiche et al., 2013

1.7 References

1. Heng, B. C., Aubel, D., and Fussenegger, M. (2013) An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases. *Biotechnol Adv* **31**, 1676-1694

2. Lappano, R., and Maggiolini, M. (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov* **10**, 47-60

3. Jean-Alphonse, F., and Hanyaloglu, A. C. (2011) Regulation of GPCR signal networks via membrane trafficking. *Mol Cell Endocrinol* **331**, 205-214

4. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of thrombosis and haemostasis : JTH* **3**, 1800-1814

5. Hermans, J., and McDonagh, J. (1982) Fibrin: structure and interactions. *Semin Thromb Hemost* **8**, 11-24

6. Licari, L. G., and Kovacic, J. P. (2009) Thrombin physiology and pathophysiology. *J Vet Emerg Crit Care (San Antonio)* **19**, 11-22

7. Machlus, K. R., and Italiano, J. E., Jr. (2013) The incredible journey: From megakaryocyte development to platelet formation. *The Journal of cell biology* **201**, 785-796

8. Ghoshal, K., and Bhattacharyya, M. (2014) Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *ScientificWorldJournal* **2014**, 781857

9. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068

10. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Molecular cloning of a potential proteinase activated receptor. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9208-9212

11. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386**, 502-506

12. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr., and Coughlin, S. R. (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**, 516-519

13. Xu, W. F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6642-6646

14. Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C., and Coughlin, S. R. (1998) A dual thrombin receptor system for platelet activation. *Nature* **394**, 690-694

15. Vergnolle, N., Derian, C. K., D'Andrea, M. R., Steinhoff, M., and Andrade-Gordon, P. (2002) Characterization of thrombin-induced leukocyte rolling and adherence: a potential proinflammatory role for proteinase-activated receptor-4. *J Immunol* **169**, 1467-1473

16. Kataoka, H., Hamilton, J. R., McKemy, D. D., Camerer, E., Zheng, Y. W., Cheng, A., Griffin, C., and Coughlin, S. R. (2003) Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* **102**, 3224-3231

17. Hollenberg, M. D., Saifeddine, M., Al-Ani, B., and Gui, Y. (1999) Proteinase-activated receptor 4 (PAR4): action of PAR4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR1 and PAR2. *Can J Physiol Pharmacol* **77**, 458-464

18. Vidwan, P., Pathak, A., Sheth, S., Huang, J., Monroe, D. M., and Stouffer, G. A. (2010) Activation of protease-activated receptors 3 and 4 accelerates tissue factor-induced thrombin generation on the surface of vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 2587-2596

19. McDougall, J. J., Zhang, C., Cellars, L., Joubert, E., Dixon, C. M., and Vergnolle, N. (2009) Triggering of proteinase-activated receptor 4 leads to joint pain and inflammation in mice. *Arthritis Rheum* **60**, 728-737

20. Asfaha, S., Cenac, N., Houle, S., Altier, C., Papez, M. D., Nguyen, C., Steinhoff, M., Chapman, K., Zamponi, G. W., and Vergnolle, N. (2007) Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. *British journal of pharmacology* **150**, 176-185

21. Hamilton, J. R., Moffatt, J. D., Frauman, A. G., and Cocks, T. M. (2001) Protease-activated receptor (PAR) 1 but not PAR2 or PAR4 mediates endothelium-dependent relaxation to thrombin and trypsin in human pulmonary arteries. *J Cardiovasc Pharmacol* **38**, 108-119

22. Holinstat, M., Voss, B., Bilodeau, M. L., McLaughlin, J. N., Cleator, J., and Hamm, H. E. (2006) PAR4, but not PAR1, signals human platelet aggregation via Ca2+ mobilization and synergistic P2Y12 receptor activation. *The Journal of biological chemistry* **281**, 26665-26674

23. Coughlin, S. R., Vu, T. K., Hung, D. T., and Wheaton, V. I. (1992) Expression cloning and characterization of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Semin Thromb Hemost* **18**, 161-166

24. Ayala, Y. M., Cantwell, A. M., Rose, T., Bush, L. A., Arosio, D., and Di Cera, E. (2001) Molecular mapping of thrombin-receptor interactions. *Proteins* **45**, 107-116

25. Jacques, S. L., and Kuliopulos, A. (2003) Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem J* **376**, 733-740

26. Soh, U. J., Dores, M. R., Chen, B., and Trejo, J. (2010) Signal transduction by protease-activated receptors. *British journal of pharmacology* **160**, 191-203

27. Dabek, M., Ferrier, L., Roka, R., Gecse, K., Annahazi, A., Moreau, J., Escourrou, J., Cartier, C., Chaumaz, G., Leveque, M., Ait-Belgnaoui, A., Wittmann, T., Theodorou, V., and Bueno, L. (2009) Luminal cathepsin g and protease-activated receptor 4: a duet involved in alterations of the colonic epithelial barrier in ulcerative colitis. *The American journal of pathology* **175**, 207-214

28. Vaidyula, V. R., and Rao, A. K. (2003) Role of Galphaq and phospholipase C-beta2 in human platelets activation by thrombin receptors PAR1 and PAR4: studies in human platelets deficient in Galphaq and phospholipase C-beta2. *British journal of haematology* **121**, 491-496

29. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407**, 258-264

30. Arora, P., Ricks, T. K., and Trejo, J. (2007) Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *Journal of cell science* **120**, 921-928

31. Wolfe, B. L., and Trejo, J. (2007) Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic* **8**, 462-470

32. Zhang, J., Ferguson, S. S., Barak, L. S., Menard, L., and Caron, M. G. (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *The Journal of biological chemistry* **271**, 18302-18305

33. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**, 447-450

34. Shapiro, M. J., Weiss, E. J., Faruqi, T. R., and Coughlin, S. R. (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *The Journal of biological chemistry* **275**, 25216-25221

35. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *The Journal of biological chemistry* **271**, 32874-32880

36. Tiruppathi, C., Yan, W., Sandoval, R., Naqvi, T., Pronin, A. N., Benovic, J. L., and Malik, A. B. (2000) G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7440-7445

37. Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *The Journal of biological chemistry* **277**, 1292-1300

38. Ricks, T. K., and Trejo, J. (2009) Phosphorylation of protease-activated receptor-2 differentially regulates desensitization and internalization. *The Journal of biological chemistry* **284**, 34444-34457

39. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Molecular and cellular biology* **26**, 3231-3242

40. Chen, B., Dores, M. R., Grimsey, N., Canto, I., Barker, B. L., and Trejo, J. (2011) Adaptor protein complex-2 (AP-2) and epsin-1 mediate protease-activated

receptor-1 internalization via phosphorylation- and ubiquitination-dependent sorting signals. *The Journal of biological chemistry* **286**, 40760-40770

41. Kang, D. S., Tian, X., and Benovic, J. L. (2013) beta-Arrestins and G protein-coupled receptor trafficking. *Methods in enzymology* **521**, 91-108

42. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3712-3717

43. Chen, C. H., Paing, M. M., and Trejo, J. (2004) Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. *The Journal of biological chemistry* **279**, 10020-10031

44. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (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**, 1267-1281

45. Stalheim, L., Ding, Y., Gullapalli, A., Paing, M. M., Wolfe, B. L., Morris, D. R., and Trejo, J. (2005) Multiple independent functions of arrestins in the regulation of protease-activated receptor-2 signaling and trafficking. *Molecular pharmacology* **67**, 78-87

46. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annual review of pharmacology and toxicology* **48**, 601-629

47. Dores, M. R., and Trejo, J. (2012) Ubiquitination of G protein-coupled receptors: functional implications and drug discovery. *Molecular pharmacology* **82**, 563-570

48. Marchese, A., and Trejo, J. (2013) Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cellular signalling* **25**, 707-716

49. Dores, M. R., and Trejo, J. (2014) Atypical regulation of G protein-coupled receptor intracellular trafficking by ubiquitination. *Current opinion in cell biology* **27**, 44-50

50. Hasdemir, B., Murphy, J. E., Cottrell, G. S., and Bunnett, N. W. (2009) Endosomal deubiquitinating enzymes control ubiquitination and down-regulation of protease-activated receptor 2. *The Journal of biological chemistry* **284**, 28453-28466

51. Jacob, C., Cottrell, G. S., Gehringer, D., Schmidlin, F., Grady, E. F., and Bunnett, N. W. (2005) c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. *The Journal of biological chemistry* **280**, 16076-16087

52. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *The Journal of cell biology* **177**, 905-916

53. Dores, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T., and Trejo, J. (2012) ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *The Journal of cell biology* **197**, 407-419

54. Lin, H., Liu, A. P., Smith, T. H., and Trejo, J. (2013) Cofactoring and dimerization of proteinase-activated receptors. *Pharmacological reviews* **65**, 1198-1213

55. Nakanishi-Matsui, M., Zheng, Y. W., Sulciner, D. J., Weiss, E. J., Ludeman, M. J., and Coughlin, S. R. (2000) PAR3 is a cofactor for PAR4 activation by thrombin. *Nature* **404**, 609-613

56. Leger, A. J., Jacques, S. L., Badar, J., Kaneider, N. C., Derian, C. K., Andrade-Gordon, P., Covic, L., and Kuliopulos, A. (2006) Blocking the protease-activated receptor 1-4 heterodimer in platelet-mediated thrombosis. *Circulation* **113**, 1244-1254

57. Lin, H., and Trejo, J. (2013) Transactivation of the PAR1-PAR2 heterodimer by thrombin elicits beta-arrestin-mediated endosomal signaling. *The Journal of biological chemistry* **288**, 11203-11215

58. O'Brien, P. J., Prevost, N., Molino, M., Hollinger, M. K., Woolkalis, M. J., Woulfe, D. S., and Brass, L. F. (2000) Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. *The Journal of biological chemistry* **275**, 13502-13509

59. Bah, A., Chen, Z., Bush-Pelc, L. A., Mathews, F. S., and Di Cera, E. (2007) Crystal structures of murine thrombin in complex with the extracellular fragments of murine protease-activated receptors PAR3 and PAR4. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11603-11608

60. Blackhart, B. D., Emilsson, K., Nguyen, D., Teng, W., Martelli, A. J., Nystedt, S., Sundelin, J., and Scarborough, R. M. (1996) Ligand cross-reactivity within the protease-activated receptor family. *The Journal of biological chemistry* **271**, 16466-16471

61. Kaneider, N. C., Leger, A. J., Agarwal, A., Nguyen, N., Perides, G., Derian, C., Covic, L., and Kuliopulos, A. (2007) 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. *Nat Immunol* **8**, 1303-1312

62. Li, D., D'Angelo, L., Chavez, M., and Woulfe, D. S. (2011) Arrestin-2 differentially regulates PAR4 and ADP receptor signaling in platelets. *The Journal of biological chemistry* **286**, 3805-3814

63. Khan, A., Li, D., Ibrahim, S., Smyth, E., and Woulfe, D. S. (2014) The Physical Association of the P2Y12 Receptor with PAR4 Regulates Arrestin-mediated Akt Activation. *Molecular pharmacology*

CHAPTER 2: CHARACTERIZATION OF PAR4 TRAFFICKING

2.1 Abstract

Protease-activated receptor-4 (PAR4) is a G protein-coupled receptor (GPCR) for thrombin and is proteolytically activated, similar to the prototypical PAR1. Due to the irreversible activation of PAR1, receptor trafficking is intimately linked to signal regulation. However, unlike PAR1, the mechanisms that control PAR4 trafficking are not known. Here, we sought to define the mechanisms that control PAR4 trafficking and signaling. In HeLa cells depleted of clathrin by siRNA, activated PAR4 failed to internalize. Consistent with clathrin-mediated endocytosis, expression of a dynamin dominant-negative K44A mutant also blocked activated PAR4 internalization. However, unlike most GPCRs, PAR4 internalization occurred independently of β-arrestins and the receptor's C-tail domain. Rather, we discovered a highly conserved tyrosine-based motif in the third intracellular loop of PAR4 and found that the clathrin adaptor protein complex-2 (AP-2) is important for internalization. Depletion of AP-2 inhibited PAR4 internalization induced by agonist. In addition, mutation of the critical residues of the tyrosine-based motif disrupted agonist-induced PAR4 internalization. Using Dami megakaryocytic cells, we confirmed that AP-2 is required for agonist-induced internalization of endogenous PAR4. Moreover, inhibition of activated PAR4 internalization enhanced ERK1/2 signaling, whereas Akt signaling was markedly diminished. These findings indicate that activated PAR4 internalization requires AP-2 and a tyrosine-based motif and occurs

independent of β-arrestins, unlike most classical GPCRs. Moreover, these findings are the first to show that internalization of activated PAR4 is linked to proper ERK1/2 and Akt activation.

2.2 Introduction

Thrombin is a coagulant protease that plays an essential role in hemostasis and thrombosis in response to tissue injury (1). Thrombin is locally generated at sites of vascular injury, where it initiates responses in various cell types including platelets, endothelial, and smooth muscle cells. Thrombin elicits cellular responses through members of the protease-activated receptor (PAR) family of G protein-coupled receptors (GPCRs). Unlike classic GPCRs, which are activated by a diffusible ligand, PARs are activated by the proteolytic cleavage of the receptor's N-terminal domain (2,3). The newly exposed N-terminus, termed the "tethered ligand," then binds intramolecularly to the receptor's second extracellular loop, inducing a conformational change that facilitates coupling to heterotrimeric G proteins and downstream signaling. Studies have shown that a six amino acid synthetic peptide mimic of a PAR's tethered ligand is sufficient to activate the receptor and evoke cellular responses similar to those resulting from proteolytic cleavage of the receptor (4). Because of the irreversible mechanism of PAR1 activation with the generation of a tethered ligand that cannot diffuse away, the mechanisms that regulate PAR1

trafficking are important for maintaining proper signaling and appropriate cellular responses (5).

After activation, most GPCRs are rapidly internalized from the cell surface via clathrin-mediated endocytosis (6). This process is often initiated by the recruitment of β-arrestin, a clathrin adaptor protein, which recognizes both the activated and phosphorylated form of GPCRs (7,8). β-arrestin and other clathrin adaptor proteins coordinate the formation of clathrin-coated pits at the plasma membrane. The GTPase dynamin facilitates scission of clathrin-coated pits, which bud off from the plasma membrane and fuse with early endosomes. Internalized GPCRs are then either recycled back to the cell surface or sorted to lysosomes and degraded (9).

The majority of proteolytically activated PAR1 and PAR2 are precluded from recycling, unlike most other GPCRs. Rather, the ultimate fate of an activated PAR is degradation at the lysosome, and PAR1 and PAR2 trafficking has been linked to either signal propagation or termination (5,10,11). Several studies have characterized the regulatory mechanisms that control signaling and trafficking of the prototypical PAR1. After activation, PAR1 undergoes rapid phosphorylation and internalization through clathrin-coated pits. However, PAR1 internalization does not require β-arrestins (12), but instead requires phosphorylation and a cytoplasmic tail (C-tail) tyrosine-based motif that is recognized by the clathrin adaptor protein complex-2 (AP-2) (13,14). Internalized PAR1 is then sorted from early endosomes to lysosomes, where the receptor is

degraded (15-17). The processes that mediate PAR1 internalization and intracellular sorting are tightly regulated, and disruption of these processes has been shown to result in dysregulated signaling (5). Thus, PAR1 trafficking is important for proper signaling and appropriate cellular responses. In contrast to PAR1, the mechanisms that regulate PAR4 trafficking and signaling remain to be determined.

PAR4 is often coexpressed with PAR1 and is also an important receptor for thrombin-induced cellular responses. The activation of human platelets by thrombin is mediated by both PAR1 and PAR4 (18). PAR4 has a much lower affinity for thrombin than PAR1, and as a result PAR4 was initially hypothesized to be a “back-up” receptor. However, recent studies have demonstrated that PAR1 and PAR4 play distinct roles in platelet activation. PAR1 regulates early stages of platelet activation, whereas PAR4 function appears to be more important for the later stages (19). The signaling kinetics exhibited by the two receptors support this model (20), wherein PAR1 signaling is rapid and transient compared to PAR4, which has a slower onset but a prolonged duration.

Thrombin signaling mediated by PARs is linked to cardiovascular pathophysiologies (21). Thus, modulating thrombin signaling via PAR1 is attractive for drug development. In fact, the FDA recently approved Zontivity® (also known as vorapaxar) as the First-in-Class PAR1 antagonist, indicated for reducing the risk of heart attack, stroke, and cardiovascular death in patients with a history of adverse cardiac events (22,23). However, patients exhibit varying

levels of responsiveness to this PAR1 antagonist. Moreover, recent work has shown that platelet PAR4 reactivity varies greatly between different patients (24-26). Specifically, platelets from African American subjects exhibit increased responsiveness to PAR4-specific agonists when compared to other populations. Thus, targeting PAR4 could prove beneficial in treating patients for whom PAR1-directed therapy is not effective.

In this study, we sought to characterize the intracellular trafficking of activated PAR4 and to determine if trafficking is linked to PAR4 signaling. In contrast to most classic GPCRs, we demonstrate that, like PAR1, activated PAR4 undergoes clathrin-mediated endocytosis independent of β-arrestins. Internalized PAR4 is initially sorted to early endosomes and then to lysosomes, similar to PAR1. However, unlike PAR1, we found that the C-tail region of PAR4 is not required for internalization. Rather, we identified a highly conserved tyrosine-based sorting motif in the third intracellular loop (ICL3) of PAR4 that regulates agonist-induced internalization. The clathrin adaptor AP-2 binds to tyrosine-based motifs, and siRNA-mediated depletion of AP-2 blocked activated PAR4 internalization in HeLa cells. Moreover, internalization of activated PAR4 expressed endogenously in Dami megakaryocytic cells was similarly regulated by AP-2. Additionally, inhibition of endogenous PAR4 internalization in Dami cells by AP-2 knockdown resulted in enhanced and prolonged extracellular signal regulated kinase-1/2 (ERK1/2) signaling, consistent with defects in signal termination. In contrast, Akt activation in response to PAR4 stimulation was

significantly attenuated, suggesting that internalization is important for coupling to this pathway. This study is the first to demonstrate that activated PAR4 internalization is distinctly regulated by AP-2 via a tyrosine-based motif and that intracellular trafficking is intimately linked to the regulation of the magnitude and duration of PAR4-induced ERK1/2 and Akt signaling.

2.3 Materials and Methods

Reagents and Antibodies. The PAR4 activating peptide AYPGKF was synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography by the Tufts University Core Facility (Boston, MA). Isoproterenol was purchased from Sigma-Aldrich (St. Lois, MO). Anti-FLAG polyclonal rabbit antibody (#600-401-383) was purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse EEA1 (#610457) and anti-μ2 adaptin (anti-AP50) (#611350) antibodies were purchased from BD Biosciences. LAMP1 antibody was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA). Mouse monoclonal anti-α-adaptin (AP.6) antibody was generously provided by Dr. Linton Traub (University of Pittsburgh, Pittsburgh, PA). The mouse monoclonal anti-clathrin (X22) heavy chain antibody (GTX22731) and the anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) (GTX627408) were purchased from GeneTex (Irvine, CA). The mouse anti-β-actin antibody (AC-74) (A-5316) was purchased

from Sigma-Aldrich. The mouse anti-PAR4 antibody was provided by Dr. Marvin Nieman (Case Western Reserve University, Cleveland, OH). The rabbit β-arrestin antibody (A1CT) was a generous gift from Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). The rabbit polyclonal anti-Akt (#9272), rabbit monoclonal anti-phospho-Akt (Ser473) (D9E) XP, rabbit polyclonal anti-p44/42 MAPK (#9102), and the mouse anti-phospho-p44/42 MAPK (#9106) were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 (#A-11001), Alexa Fluor 594 (A-11005), and Alexa Fluor 647 (A-21235) and goat anti-rabbit secondary antibodies conjugated to Alex Fluor 488 (A-11008), Alex Fluor 594 (A-11012), and Alex Fluor 647 (A-21244) were purchased from ThermoFisher Scientific (Waltham, MA). FluorSave reagent was purchased from Calbiochem. Goat anti-mouse (#170-6516) and goat anti-rabbit (#170-6515) secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Bio-Rad (Hercules, CA). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Thermo Fisher Scientific.

Cell Culture and Transfections. HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (v/v). The Dami human megakaryocytic cell line was purchased from ATCC and maintained in RPMI media supplemented with 10% (v/v) fetal bovine serum. Mouse embryonic fibroblasts derived from wild-type (WT) or β-arrestin knockout mice were described previously (12). HeLa cells were transiently transfected with cDNA

plasmids using Polyethylenimie (Polysciences Inc., Warrington, PA) at a ratio of 6 µl PEI (1 mg/ml solution) to 1 µg plasmid. Oligofectamine (Invitrogen) was used for siRNA transfections of HeLa cells, which were carried out according to the manufacturer's instructions. Dami cells were transfected with siRNA as previously described (27,28). Briefly, cells were plated at a density of 5.0 x 10^5 cells/ml in reduced-serum media and transfected using Oligofectamine with siRNA oligonucleotides at a final concentration of 100 nM. A second transfection was carried out 24 h following the first, and cells were assayed after 72 h of transfection. Non-specific (NS) siRNA 5'-CTACGTCCAGGAGCGCACC-'3, µ2 adaptin siRNA 5'-GTGGATGCCTTTCGGGTCA-3', and clathrin heavy chain siRNA 5'-GCAATGAGCTGTTTGAAGA-3' were previously described (29,30) and obtained from Dharmacon (Lafayette, CO).

Plasmid cDNAs. Human PAR4 wild-type containing an N-terminal FLAG epitope was cloned into the pBJ mammalian vector as previously described (18). Human FLAG-β_2AR cloned into pcDNA3.1 was generously provided by Dr. Mark von Zastrow (University of California, San Francisco, CA). Plasmids encoding wild-type or K44A dynamin-GFP were described previously (31). All mutagenesis was performed using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA) per manufacturer's protocol and confirmed by dideoxy sequencing. The PAR4 ΔK367 and ΔK350 C-tail truncation mutants were generated by site-directed mutagenesis of nucleotides a1101t and a1050t to convert lysine (K) codons at position 367 and 350, respectively, to stop

codons. The PAR4 tyrosine-based sorting motif mutant was generated using site-directed mutagenesis by mutating t792g and a793c to convert tyrosine (Y) at position 264 to alanine (A) followed by mutating c821g and t822c to convert leucine (L) at position 268 to alanine and was designated PAR4 Y264A/L268A.

Immunoblotting. Cell lysates were collected in 2X Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE, transferred to PVDF membranes, immunoblotted with appropriate antibodies, and then developed by chemiluminescence. Immunoblots were quantified by densitometry using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence Confocal Microscopy. Cells were plated at a density of 0.4 x 10^5 cells per well on fibronectin-coated glass coverslips placed in a 12-well dish and grown overnight. Cells were transfected, grown for 48 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES for 1 h at 37°C. Cells were incubated at 4°C with anti-FLAG antibody to label the cohort of receptors at the cell surface, stimulated with agonist, then fixed in 4% paraformaldehyde, permeabilized with methanol, and then immunostained with appropriate antibodies and processed as described previously (16). Coverslips were mounted with FluorSave reagent. Confocal images of 0.28 μm x-y sections were collected sequentially using an Olympus IX81 DSU spinning confocal microscope fitted with a Plan Apo 60x oil objective and a Hamamatsu ORCA-ER digital camera using SlideBook 5.0 software (Intelligent Imaging Innovations).

Pearson's correlation coefficients and line-scan analysis to assess colocalization were performed using SlideBook 5.0 software.

Internalization Assays. Cells were plated at a density of 0.5 x 10^5 cells per well on fibronectin-coated 24-well dishes and grown overnight for cell surface ELISA. Cells were transfected, grown for 48 h, and then serum starved for 1 h in DMEM containing 1 mg/ml BSA and 10 mM HEPES at 37°C. Cells were incubated at 4°C with anti-FLAG antibody to label the cell surface cohort, stimulated with agonists, and then fixed in 4% paraformaldehyde. The amount of receptor remaining on the cell surface was then detected by incubation with HRP-conjugated secondary antibody, washed and developed with ABTS for at least 20 min at room temperature. The optical density (OD) of an aliquot was determined by absorbance (A) at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Signaling Assays. Dami cells were pelleted by centrifugation at 200 *g* for 5 min then washed twice with PBS. Cells were resuspended in RPMI containing 1 mg/ml BSA then counted and diluted to 5.0 x 10^5 cells/ml. HeLa cells were plated at a density of 0.4 x 10^5 cells per well in 24-well plates and grown overnight. Cells were transfected with PEI, grown for 48 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES for 1 h at 37°C. Both Dami and HeLa cells were then stimulated with agonist at 37°C as indicated and samples were collected at specific time points by direct lysis in 2X Laemmli sample buffer containing 200 mM DTT and analyzed by immunoblotting.

Data Analysis. Statistical significance was determined by one-way ANOVA, two-way ANOVA, or student's *t*-test using Prism 4.0 software (GraphPad).

2.4 Results

2.4.1 Agonist-induced internalization of PAR4 is clathrin- and dynamin-dependent

The mechanisms that regulate PAR4 intracellular trafficking are not known. To investigate whether PAR4 is internalized through a dynamin-mediated pathway, we initially expressed PAR4 in HeLa cells, a cell model system that has been used extensively to characterize clathrin-mediated endocytosis of GPCRs, including PAR1 and the β2-adrenergic receptor (β_2AR) (16,31-33). Expression of a dominant-negative K44A mutant of dynamin, which is defective in its GTPase activity, inhibits clathrin-mediated endocytosis by preventing scission of coated pits from the plasma membrane (34). HeLa cells co-transfected with FLAG-tagged PAR4 together with dynamin wild-type or K44A mutant fused to GFP were stimulated with PAR4-specific peptide agonist AYPGKF and imaged by immunofluorescence confocal microscopy. The AYPKGF peptide was used to activate PAR4 since thrombin cleaves off the N-terminal FLAG epitope required for detection of cell surface receptor. In cells expressing wild-type dynamin, PAR4 localized to the cell surface in the absence of agonist and exhibited substantial internalization following 60 min of stimulation with PAR4 agonist peptide (Fig. 2.1*A*). PAR4 also localized to the cell surface in

cells expressing the dynamin K44A mutant (Fig. 2.1*B*). However, agonist-induced internalization of PAR4 was not detectable in cells expressing dynamin K44A mutant (Fig. 2.1*B*, *arrowhead*), whereas PAR4 internalization occurred as expected in untransfected adjacent cells that were not expressing the dynamin K44A mutant (Fig. 2.1*B)*. Thus, expression of the dynamin K44A mutant inhibits PAR4 internalization, suggesting that PAR4 undergoes dynamin-dependent endocytosis.

Dynamin-dependent GPCR internalization can occur through detachment of clathrin-coated pits or via release of caveolae from the plasma membrane (6). To determine the role of clathrin, we used siRNA targeting the clathrin heavy chain to deplete HeLa cells of endogenous clathrin. The expression of clathrin was markedly reduced in cells transiently transfected with siRNA targeting clathrin compared to non-specific siRNA transfected control cells (Fig. 2.1*E*). In cells transfected with non-specific siRNA, PAR4 localized to the cell surface in the absence of agonist, and exhibited substantial internalization after stimulation with AYPGKF (Fig. 2.1*C*). PAR4 also localized to the plasma membrane in cells transfected with clathrin siRNA (Fig. 2.1*D*). However, PAR4 failed to undergo agonist-induced internalization in clathrin-depleted cells (Fig. 2.1*D*). Together these findings suggest that activated PAR4 is internalized through a clathrin- and dynamin-dependent pathway.

2.4.2 Internalized PAR4 is trafficked through early endosomes and sorted to lysosomes

Previous studies showed that activated PAR1 and the related PAR2 are internalized and sorted predominantly to lysosomes for degradation (17,35), however the intracellular trafficking route of PAR4 has not been determined. To examine if activated and internalized PAR4 is sorted to early endosomes, HeLa cells expressing FLAG-tagged PAR4 were treated with agonist for various times at 37°C to stimulate internalization of PAR4. Trafficking to early endosomes was then measured by colocalization with early endosomal antigen-1 (EEA1), a marker of early endosomes, using immunofluorescence confocal microscopy. We observed significant accumulation of intracellular PAR4 at EEA1-positive puncta as early as 15 min following agonist stimulation compared to control cells (Fig. 2.2*A*, *top panels*), and a peak in colocalization occurs at 30 min of agonist stimulation (Fig. 2.2*A*, *middle panels*). The amount of intracellular PAR4 colocalized with EEA1 appeared to diminish after 60 and 90 min of agonist incubation (Fig. 2.2*A*, *bottom panels*). The extent of PAR4 colocalization with EEA1 was quantified by determining Pearson's correlation coefficient for PAR4 and EEA1 at different time points and is consistent with the peak in colocalization between PAR4 and EEA1 at 30 min (Fig. 2.2, *A* and *B*). A similar extent of colocalization between EEA1 and the δ-opioid and CXCR4 receptors was previously reported (36). These data indicate that internalized PAR4 is sorted into early endosomal compartments between 15 and 30 min after agonist stimulation.

To determine whether activated PAR4 is sorted to late endosomes/lysosomes, we used confocal microscopy to assess receptor colocalization with lysosomal associated membrane protein-1 (LAMP1), a specific marker of late endosomes/lysosomes. In unstimulated HeLa cells, PAR4 largely resided on the cell surface. After agonist stimulation for 15 or 30 min, PAR4 internalized to intracellular puncta and showed minimal colocalization with LAMP1 (Fig. 2.3*A, top panels*). These findings are consistent with PAR4 sorting to early endosomes within 30 min of agonist stimulation (Fig.2.2). In contrast, a significant accumulation of intracellular PAR4 was detected at LAMP1-positive late endosomes/lysosomes after 60 or 90 min of agonist stimulation (Fig. 2.3*A, bottom panels*). These findings were confirmed by calculating Pearson's correlation coefficients for PAR4 colocalization with LAMP1 at 60 min (r = 0.1957 ± 0.03360) and 90 min (r = 0.2813 ± 0.02051) (Fig. 2.3*B*). The β_2AR displayed comparable colocation with LAMP1 after prolonged agonist stimulation (37). These studies suggest that activated and internalized PAR4 sorts to early endosomes and then to late endosomes/lysosomes.

2.4.3 Internalization of PAR4 occurs through a β-arrestin-independent pathway

After agonist activation, most GPCRs interact with β-arrestins, which facilitates rapid internalization through clathrin-coated pits (38). However, some GPCRs, such as PAR1, do not require β-arrestins for clathrin-mediated internalization (6,12). To examine the role of β-arrestins in PAR4 internalization,

we expressed PAR4 in mouse embryonic fibroblasts (MEFs) derived from β-arrestin 1,2 double knockout mice and wild-type littermate control cells (39) and assessed agonist-induced internalization by immunofluorescence confocal microscopy. The loss of β-arrestin-1 and -2 expression was first confirmed in wild-type and β-arrestin knockout MEFs by immunoblotting (Fig. 2.4*A*). In these experiments, the cell surface PAR4 cohort was prelabeled with anti-FLAG antibody. In wild-type MEFs expressing both β-arrestin isoforms, we observed substantial internalization of PAR4 from the cell surface to intracellular vesicles after 60 min of agonist stimulation at 30 min (Fig. 2.4*B*, *top panels*). Interestingly, PAR4 internalization was similarly induced by agonist in cells lacking expression of both β-arrestin isoforms (Fig. 2.4*B*, *bottom panels*). To exclude the possibility that PAR4 internalization in β-arrestin-1 and 2 MEFs was due to some anomaly, we examined isoproterenol-induced internalization of β_2AR in parallel. β_2AR is a classic GPCR that requires β-arrestins for agonist-induced internalization (39). FLAG-tagged β_2AR was transiently expressed in wild-type and β-arrestin-1,2 null MEFs and internalization was examined by immunofluorescence confocal microscopy as described above. In wild-type MEFs, isoproterenol induced substantial internalization of β_2AR (Fig. 2.4*C*, *top panels*), whereas activated β_2AR internalization was completely abolished in β-arrestin-1 and -2 double knockout MEFs (Fig. 2.4*C*, *lower panels*). These findings are consistent with previously published studies demonstrating β-arrestin-dependent internalization

of β_2AR (39) and further reveal for the first time a β-arrestin-independent internalization pathway for activated PAR4.

2.4.4 The PAR4 C-tail is not necessary for agonist-stimulated internalization

Internalization of GPCRs is mediated by clathrin adaptor proteins, which recognize short linear sorting motifs or post-translational modifications including phosphorylation of serine (S) or threonine (T) residues or ubiquitination of lysine (K) residues, which often reside in the C-tail domain of the receptor (9). The PAR4 C-tail contains nine serine and threonine residues that may serve as potential phosphorylation sites and two lysines that could be targeted for ubiquitination (Fig. 2.5*A*). To delineate the mechanism responsible for internalization of PAR4, we first examined the importance of the C-tail domain by truncation mutagenesis. HeLa cells were transiently transfected with PAR4 wild-type and two C-tail truncation mutants ΔK367 and ΔK350, which were lacking the distal nineteen amino acids or thirty-six amino acids, respectively (Fig. 2.5*A*). Cells were then incubated in the absence or presence of the PAR4 specific agonist AYPGKF for 60 min at 37ºC and the amount of receptor remaining on the cell surface was quantified. In wild-type PAR4 expressing cells, agonist caused ~45% loss of surface receptor within 60 min of agonist stimulation (Fig. 2.5*B*). Remarkably, the extent of agonist induced internalization of both PAR4 C-tail truncation mutants, ΔK367 and ΔK350, was comparable to wild-type receptor, exhibiting ~45% and ~55% loss of surface receptor, respectively (Fig. 2.5*B*). To ensure that PAR4 ΔK367 and ΔK350 mutants are not globally defective, receptor

expression and signaling were further examined. Analysis of cell lysates revealed a similar pattern of mature and immature bands, indicating that both PAR4 mutants are properly post-translationally processed (Fig. 2.5*C*). In addition, both PAR4 truncation mutants express at the cell surface and activate ERK1/2 in response to agonist stimulation (Fig. 2.5, *D* and *E*), although the PAR4 ΔK350 mutant exhibits lower surface expression and consequently less ERK1/2 signaling. These findings suggest that the PAR4 C-tail is not required for agonist-induced internalization and are consistent with a previous report showing that activated PAR4 fails to undergo phosphorylation (20). These findings indicate that phosphorylation is not a critical feature of PAR4 regulation. Together these results suggest that agonist-induced internalization of PAR4 via clathrin-coated pits occurs through a non-canonical pathway that is not dependent on β-arrestins or the C-tail domain.

2.4.5 PAR4 internalization requires AP-2

To identify other clathrin adaptor proteins besides β-arrestins that could mediate internalization of PAR4, we conducted a bioinformatics search for conserved sorting motifs in the cytoplasmic loops of PAR4 and found a highly conserved tyrosine-based motif within the third intracellular loop (ICL3) that appeared to be accessible for AP-2 binding (Fig. 2.6*A*). Tyrosine-based motifs are known binding sites for AP-2, a critical adaptor protein important for clathrin-mediated endocytosis (40). To investigate the function of this motif in PAR4 internalization, we generated a mutant PAR4 in which tyrosine (Y) 264 and

leucine (L) 268 were converted to alanines (A) and designated Y264A/L268A. PAR4 Y264A/L268A mutant expressed at the cell surface like wild-type (WT) receptor when transiently expressed in HeLa cells. We next compared agonist-induced internalization between PAR4 WT and Y264A/L268A mutant. Internalization of PAR4 Y264A/L268A was markedly reduced and exhibited only ~5% loss of surface receptor following 60 min stimulation with AYPGKF, as compared to ~20% loss of surface wild-type receptor (Fig. 2.6*B*). These findings suggest that the putative AP-2 binding motif within the ICL3 domain of PAR4 is important for agonist-induced internalization.

To determine if AP-2 might regulate PAR4 internalization, we first determined whether PAR4 colocalizes with endogenous AP-2 in intact cells using immunofluorescence confocal microscopy. HeLa cells expressing FLAG-PAR4 were incubated with anti-FLAG antibody at 4°C to ensure only the cell surface receptors bound antibody. Cells were then stimulated with AYPGKF for 12.5 min, fixed and immunostained for PAR4 and the α-adaptin subunit of AP-2. PAR4 positive puncta co-stained for α-adaptin subunit as indicated by the overlapping puncta in the merge image (Fig. 2.7*A*). Line-scan analysis also revealed the extent of PAR4 and AP-2 colocalization at puncta (Fig. 2.7*A*). In contrast to wild-type PAR4, agonist-stimulation did not result in substantial colocalization between PAR4 Y264A/L268A with AP-2 (Fig. 2.7*B*). To assess AP-2 function in PAR4 internalization, we used siRNA targeting the μ2-adaptin subunit to deplete HeLa cells of endogenous AP-2 complex (13,41). The

expression of μ2-adaptin was virtually abolished in cells transiently transfected with μ2 siRNA compared to cells transfected with non-specific control siRNA (Fig. 2.7*C*). The cell surface cohort of FLAG-PAR4 was labeled with antibody in siRNA transfected HeLa cells and then incubated in the presence or absence of AYPGKF for 60 min at 37°C. In control non-specific siRNA transfected cells, exposure to agonist caused a significant ~30% loss of PAR4 from the cell surface (Fig. 2.7*D*). In contrast, agonist failed to induce PAR4 internalization in cells depleted of the μ2-adaptin subunit (Fig. 2.7*D*). These data suggest that AP-2 serves as a critical clathrin adaptor protein that facilitates agonist-induced internalization of PAR4.

2.4.6 Endogenous PAR4 internalization is regulated by AP-2

To determine if the regulation of endogenous PAR4 is also controlled by the clathrin adaptor AP-2, we utilized the human megakaryocytic Dami cell line, which endogenously expresses PAR4. We first tested whether AP-2 was required for mediating endogenous PAR4 internalization in Dami cells using μ2-adaptin specific siRNA to deplete cells of AP-2 expression. In Dami cells transfected with siRNA targeting the μ2-adaptin subunit of AP-2, we observed a significant loss of μ2 adaptin expression compared to non-specific siRNA transfected cells (Fig. 2.8*A*). Dami cells were grown on polylysine-coated glass coverslips, and then surface PAR4 was pre-labeled with an antibody specific for the N-terminus of human PAR4 and agonist-induced internalization was examined by immunofluorescence confocal microscopy (42). In the absence of

agonist, PAR4 localized predominantly to the cell surface (Fig. 2.8*B, upper panels*). Agonist-induced internalization of PAR4 and resultant formation of puncta was significantly inhibited in μ2-adaptin depleted cells compared to non-specific siRNA transfected control cells (Fig. 2.8*B, lower panels*). Quantification by automated image analysis revealed a significant blockade of agonist-induced internalization of PAR4 in cells depleted of μ2-adaptin (Fig. 2.8*C*). These data confirm that AP-2 plays an essential role in mediating internalization of endogenous PAR4.

2.4.7 PAR4 signaling is differentially regulated by AP-2 and mutation of the tyrosine-motif

To assess the role of dysregulated PAR4 trafficking caused by loss of AP-2 expression on receptor signaling, we examined ERK1/2 phosphorylation in Dami cells depleted of μ2-adaptin expression by siRNA knockdown. Activation of PAR4 elicited significantly enhanced and prolonged ERK1/2 signaling in cells depleted of μ2-adaptin relative to non-specific siRNA transfected control cells (Fig. 2.9*A*).). In addition, agonist stimulation of the PAR4 Y264A/L268A mutant expressed in HeLa cells resulted in enhanced and prolonged ERK1/2 activation (Fig. 2.9*B*). PAR1-stimulated ERK1/2 activation is known to occur though $G\alpha_q$ protein signaling (43), suggesting that internalization is critical for terminating agonist-stimulated PAR4 signaling. Unlike ERK1/2, PAR4-induced Akt signaling has been shown to occur via a β-arrestin-dependent pathway independent of G proteins and is hypothesized to occur on endosomes (44-46). Thus, we

determined if internalization of activated PAR4 was required for Akt signaling by comparing control and AP-2 depleted Dami cells. In contrast to ERK1/2, Akt signaling induced by activated PAR4 was markedly attenuated in μ2-adaptin knockdown cells compared to control cells transfected with non-specific siRNA (Fig. 2.9*C).* These findings suggest that internalization of activated PAR4 is required for Akt signaling. Together, our study suggests that activated PAR4 is internalized through a clathrin- and dynamin-dependent pathway independent of β-arrestins. Rather than β-arrestins, PAR4 internalization is mediated by AP-2 and a tyrosine-based motif localized within ICL3, and functions distinctly to regulate the magnitude and duration of ERK1/2 and Akt signaling (Fig. 2.10).

2.5 Discussion/Conclusions

In the present study, we sought to characterize the intracellular trafficking route of activated PAR4 and to determine its function on receptor signaling. We found that PAR4 internalizes via a clathrin- and dynamin-dependent pathway and then is sorted from early endosomes to late endosomes/lysosomes. We also discovered that activated PAR4 internalization requires a highly conserved YX_3L motif localized within ICL3 and is mediated by the clathrin adaptor AP-2, rather than β-arrestins and the C-tail domain of the receptor. Intriguingly, activation of PAR4 in cells in which receptor internalization is blocked resulted in enhanced ERK1/2 signaling and attenuated Akt signaling. These studies are the first to

identify the molecular determinants that mediate PAR4 internalization and demonstrate a link between PAR4 trafficking and signaling.

The majority of activated and phosphorylated GPCRs are recognized by β-arrestins, which facilitates interaction with clathrin, adaptor proteins and internalization from the plasma membrane (7,8,47). However, not all GPCRs require β-arrestins for internalization including PAR1 (12). In this study, we show that, like PAR1, β-arrestins are not required for agonist-induced PAR4 internalization in MEFs derived from β-arrestin1,2 knockout mice. However, unlike PAR1, we found that the C-tail domain of PAR4 is not essential for agonist-promoted internalization. Consistent with this observation, a previous study showed that agonist stimulation of PAR4 fails to promote its phosphorylation, which is generally a requirement for interaction with β-arrestins (20). These findings suggest that neither phosphorylation nor β-arrestins are required for activated PAR4 internalization through clathrin-coated pits.

In addition to β-arrestins, other clathrin adaptor proteins can facilitate GPCR internalization (6,9). Clathrin adaptor proteins recognize post-translational modifications or short linear peptide motifs that are typically localized within the cytoplasmic regions of the receptor. This is best characterized for PAR1 (48). The clathrin adaptor AP-2 mediates constitutive internalization of PAR1, wherein the μ2-adaptin subunit of AP-2 binds directly to a highly conserved C-tail tyrosine-based YXXL motif (48). In addition, activated PAR1 is phosphorylated and ubiquitinated within the C-tail domain, which is required for AP-2 and epsin-1

mediated internalization of the receptor (29). However, in contrast to PAR1, activated PAR4 is not phosphorylated (20) and the C-tail domain is not required for internalization, suggesting that an alternative pathway may control internalization of activated PAR4.

Given that the C-tail of PAR4 is dispensable for internalization, we examined the possibility that signals for internalization may occur within the intracellular loops of the receptor. A sequence alignment of PAR4's intracellular loops revealed a highly conserved non-canonical tyrosine-based YX_3L motif within ICL3 of the receptor and was found to be required for agonist-promoted internalization. Similarly, a non-canonical tyrosine-based $YX_3\Phi$ motif (where X is any residue and Φ is a hydrophobic residue) was identified in the thromboxane A2 TP-β isoform receptor C-tail region and shown to mediate receptor internalization (49). Although the C-tail region of most GPCRs serves as an important site for clathrin adaptor protein recognition, the seven transmembrane Wntless receptor contains a conserved $YXX\Phi$ motif within ICL3 that is required for internalization (50). However, it is not clear whether AP-2 is required for internalization of either the thromboxane TP-β or Wntless receptor. In contrast, we show that PAR4 and AP-2 colocalize and that AP-2 is required for agonist-induced internalization of both endogenous and ectopically expressed PAR4. Thus, unlike most classic GPCRs, PAR4 harbors a tyrosine-based $YX_3\Phi$ motif within ICL3 that is required together with AP-2 for internalization through clathrin-coated pits. Interestingly, a second less conserved tyrosine-based motif also

exists within ICL3 of PAR4 but may be inaccessible to AP-2 based on hydrophobicity analysis. However, it is unclear whether the second tyrosine-based motif has a function and the mechanism by which AP-2 is recruited to activated PAR4 is not known.

In addition to characterizing PAR4 trafficking, we also examined the influence of dysregulated trafficking on receptor signaling in Dami cells endogenously expressing PAR4 (18) and in HeLa cells ectopically expressing the PAR4 tyrosine-motif mutant. PAR4 couples to $G\alpha_q$ and $G\alpha_i$ subtypes and signals to ERK1/2 and Akt activation in multiple cell types (51,52). We show that the magnitude and duration of agonist-stimulated ERK1/2 signaling was enhanced in Dami cells depleted of AP-2 and in HeLa cells expressing the PAR4 Y264A/L268A mutant relative to control cells. These finding suggest that PAR4 internalization may be important for attenuation of ERK1/2 signaling. There is also evidence that PAR4 is able to act in synergy with the purinergic receptor P2Y12 to elicit β-arrestin-dependent Akt signaling (44). In striking contrast to ERK1/2, we found that Akt signaling was markedly attenuated in Dami cells depleted of AP-2 compared to control cells. Thus, blocking PAR4 internalization may disrupt Akt signaling by preventing the formation of a P2Y12-dependent endosomal β-arrestin signaling complex in Dami cells. This would be consistent with recent evidence suggesting that β-arrestin-dependent signaling occurs on endosomes, rather than at the plasma membrane (11). However, it remains to be determined if a presumptive PAR4-P2Y12 dimer signals to Akt activation via a β-

arrestin-mediated pathway from endosomes. Intriguingly, PAR4-stimulated Akt signaling was not reliably detected in HeLa cells. This may result from low or absent P2Y12 expression, as HeLa cells are not normally responsive to ADP stimulation (53).

In summary, we have shown that PAR4 internalization is clathrin and dynamin dependent and that the internalized receptor is trafficked through the endosomal-lysosomal sorting pathway, similar to a prototypical GPCR. However, we also found that PAR4 trafficking is regulated by unique determinants. We discovered that PAR4 internalization requires an intact ICL3 localized tyrosine-based motif and AP-2 rather than β-arrestins and the C-tail domain. Moreover, disruption of PAR4 trafficking increased the magnitude and duration of ERK1/2 signaling, while attenuating Akt signaling. These findings indicate that PAR4 trafficking is important for proper signaling and raise the intriguing possibility that like other GPCRs, PAR4 may elicit important signaling responses from endocytic vesicles.

2.6 Acknowledgements

The majority of Chapter 2 is published as: Smith TH, Coronel LJ, Li JG, Dores MR, Nieman MT, Trejo J. “Protease-activated Receptor-4 Signaling and Trafficking Is Regulated by the Clathrin Adaptor Protein Complex-2 Independent of β-arrestins.” *Journal of Biological Chemistry*. **291(35),** 18453-64 (2016). The

dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

2.7 Figures

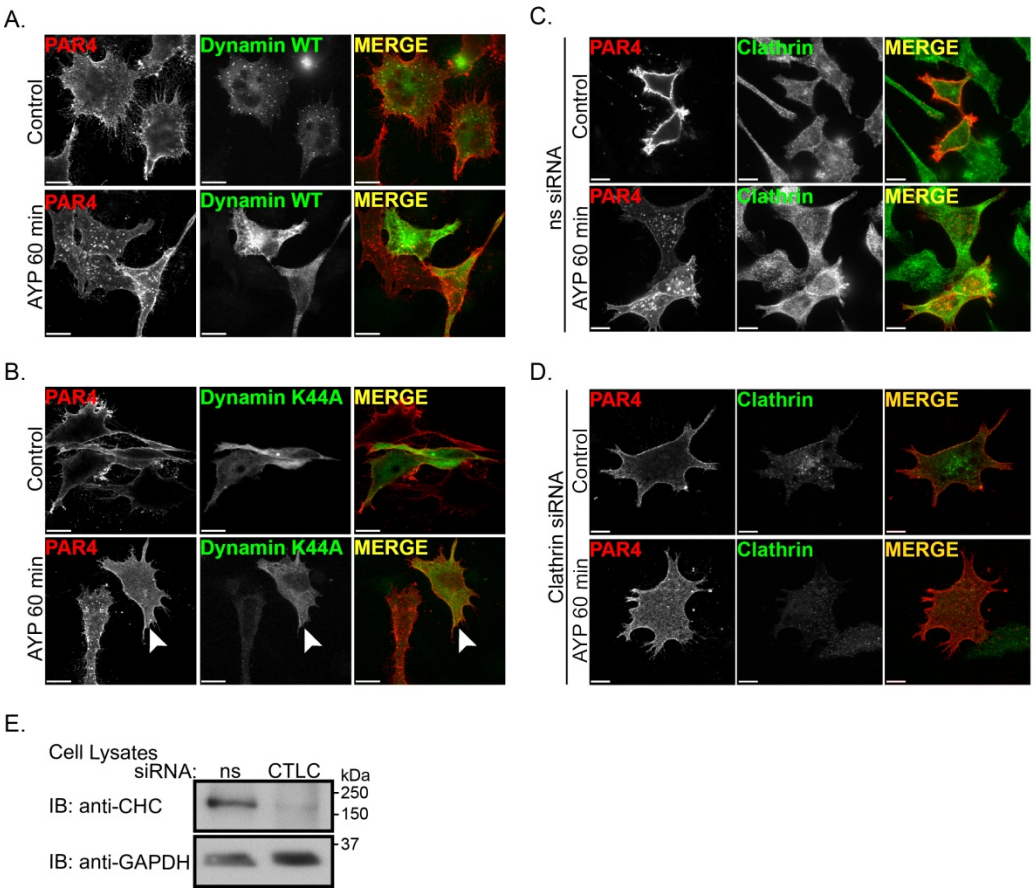

Figure 2.1: Internalization of activated PAR4 occurs through a dynamin- and clathrin-dependent pathway.

A and *B*, HeLa cells transiently transfected with FLAG-PAR4 and either dynamin wild-type (WT) fused to GFP *(A)* or the dynamin dominant-negative K44A mutant fused to GFP *(B)* were pre-labeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37°C. Cells were fixed, permeabilized, immunostained, and imaged by confocal microscopy. Arrowheads, dynamin-GFP K44A mutant expressing cells. The images are representative of three independent experiments (Scale bar, 10 μm). *C* and *D*, HeLa cells were transiently transfected with FLAG-PAR4 and either non-specific (ns) or clathrin heavy chain (CHC) specific siRNA. Cells were incubated with 500 μM AYPGKF for 60 min at 37°C, processed, and imaged by confocal microscopy. Images are representative of three independent experiments (Scale bar, 10 μm). *E*, Cell lysates were collected in parallel, resolved by SDS-PAGE and immunoblotted as indicated.

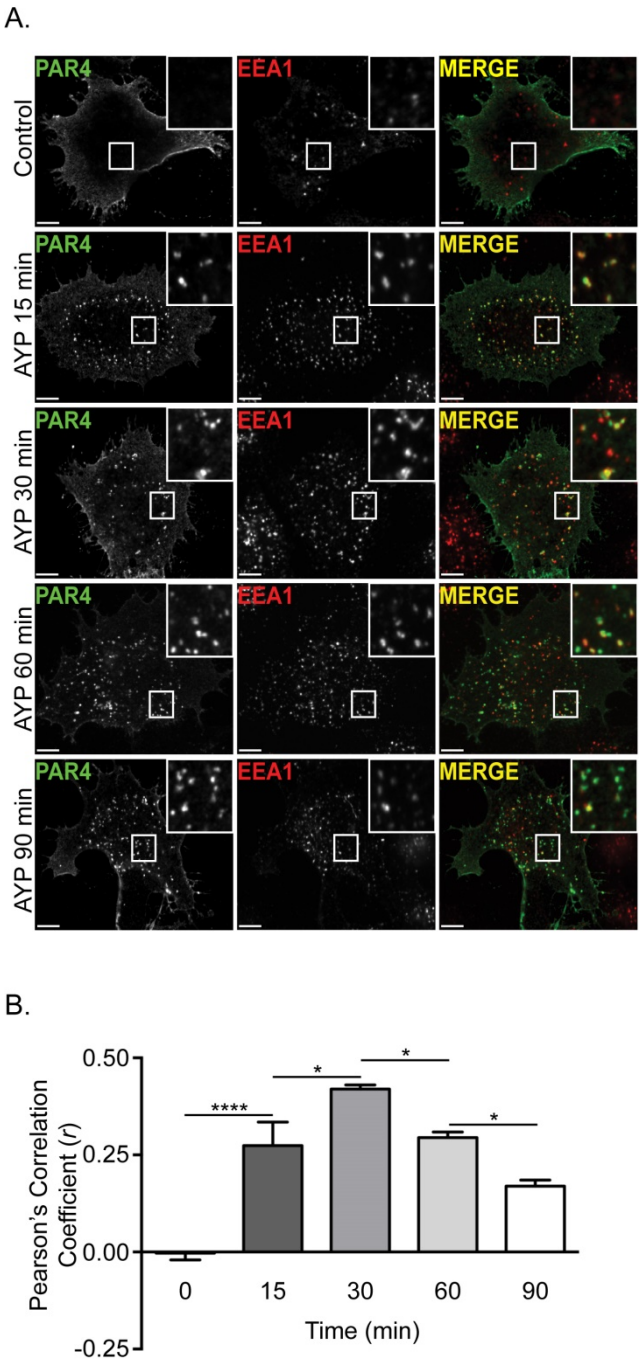

Figure 2.2: Activated PAR4 is internalized and sorted to early endosomes.
A, HeLa cells were transfected with FLAG-PAR4, pre-labeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for various times at 37°C, processed, co-stained with anti-EEA1 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (Scale bar, 10 μm). Insets, magnifications of boxed areas. *B*, Pearson's correlation coefficient was determined to quantify the degree of correlation in signal intensity between PAR4 and EEA1 at each pixel. Data (mean ± SEM; n ≥ 9 cells for each time point) shown were collected from three independent experiments and statistical significance was determined by one-way ANOVA (*, $p<0.05$; ****, $p<0.0001$).

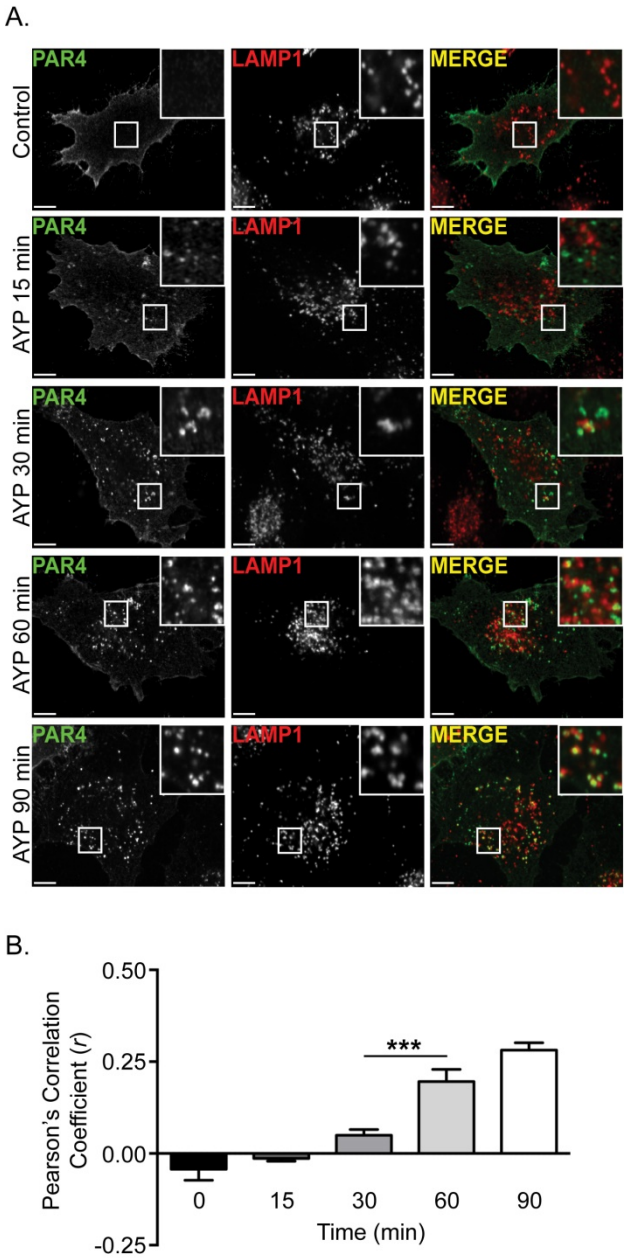

Figure 2.3: Activated internalized PAR4 is sorted to late endosomes/lysosomes.

A, HeLa cells transfected with FLAG-PAR4 were pre-labeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for various times at 37°C, processed, co-stained with anti-LAMP1 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (Scale bar, 10 μm). Insets, magnifications of boxed areas. *B*, Pearson's correlation coefficient was determined to quantify the degree of correlation in signal intensity between PAR4 and LAMP1 at each pixel. Data (mean ± SEM; n ≥ 9 cells for each time point) shown were collected from three independent experiments and statistical significance was determined by one-way ANOVA (***, $p<0.001$).

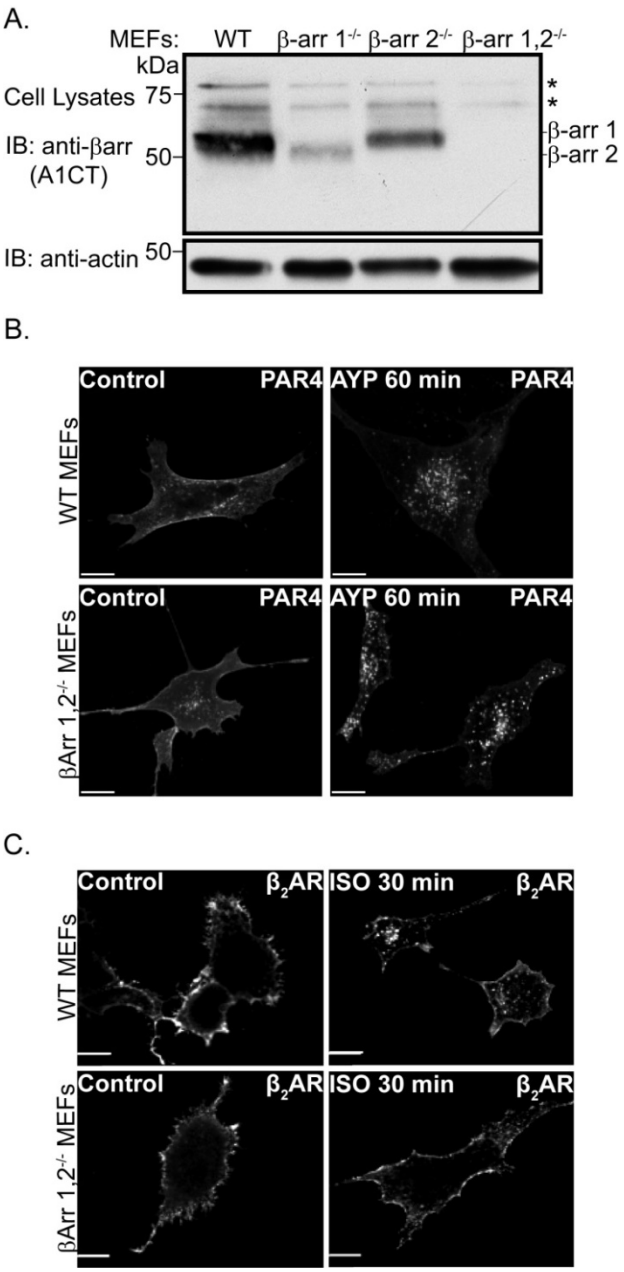

Figure 2.4: Internalization of activated PAR4 occurs independent of β-arrestins.

A, Cell lysates from wild-type (WT) and β-arrestin 1/2 double knockout (β-arr 1,2 -/-) MEFs were immunoblotted as indicated. *B* and *C*, WT or β-arr 1,2 -/- MEFs were transiently transfected with FLAG-PAR4 *(B)* or FLAG- β2AR *(C)*, pre-labeled with anti-FLAG antibody, and stimulated with 500 μM AYPGKF for 60 min at 37°C *(B)* or 10 nM isoproterenol for 30 min at 37°C *(C)*. After agonist stimulation, cells were processed, and imaged by confocal microscopy. The images are representative of several cells from three independent experiments (Scale bar, 10 μm).

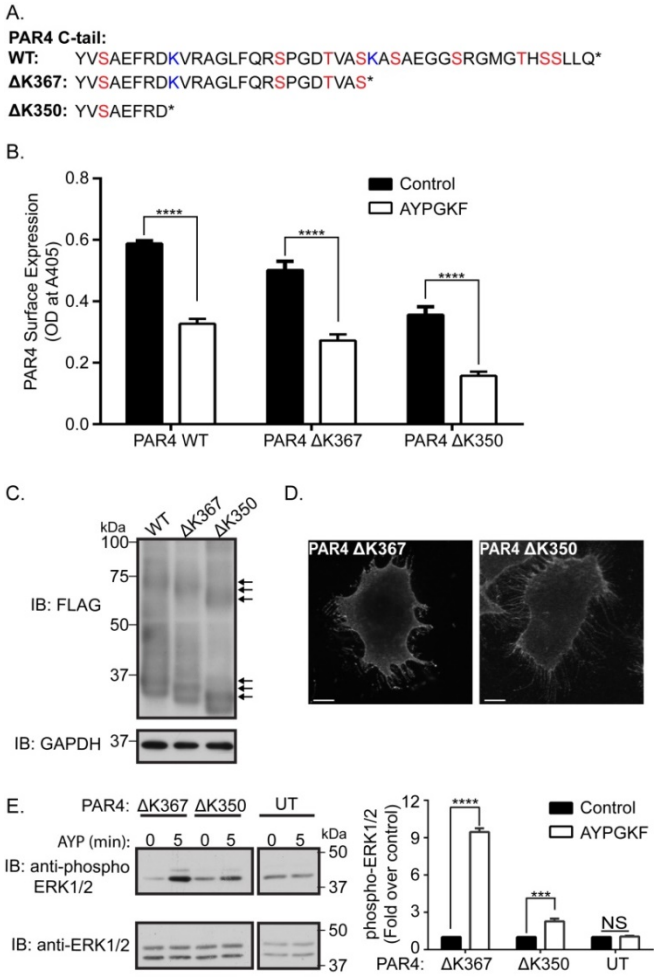

Figure 2.5: The PAR4 C-tail is not required for internalization.
A, PAR4 amino acid sequence of full length receptor and ΔK367 and ΔK350 C-tail truncation mutants. *B*, HeLa cells transfected with FLAG-PAR4 WT, ΔK367, or ΔK350 mutants were pre-labeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37°C. Cell surface ELISA was then used to quantify the amount of PAR4 remaining at the cell surface following stimulation. Data (mean ± SEM) are representative of three independent experiments, and statistical significance was determined by two-way ANOVA (****, p<0.0001). *C*, Cell lysates from HeLa cells transfected as above in *(B)* were immunoblotted as indicated. Arrows, mobility shifts representing differentially-processed PAR4 species. *D*, HeLa cells transfected as above in *(B)* were labeled with anti-FLAG antibody, processed, and imaged by confocal microscopy. The images are representative of several cells from three independent experiments (Scale bar, 10 μm). *E*, Untransfected HeLa cells and HeLa cells transfected as above in *(B)* were stimulated with 500 μM AYPGKF for 5 min at 37°C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-ERK1/2 signals were quantified, normalized to total ERK1/2, and expressed as a fraction of the untreated control. Samples were resolved on the same gel and separated for labeling. Data (mean ± SEM) are representative of three independent experiments, and statistical significance was determined by two-way ANOVA (***, p<0.001; ****, p<0.0001; NS, not significant).

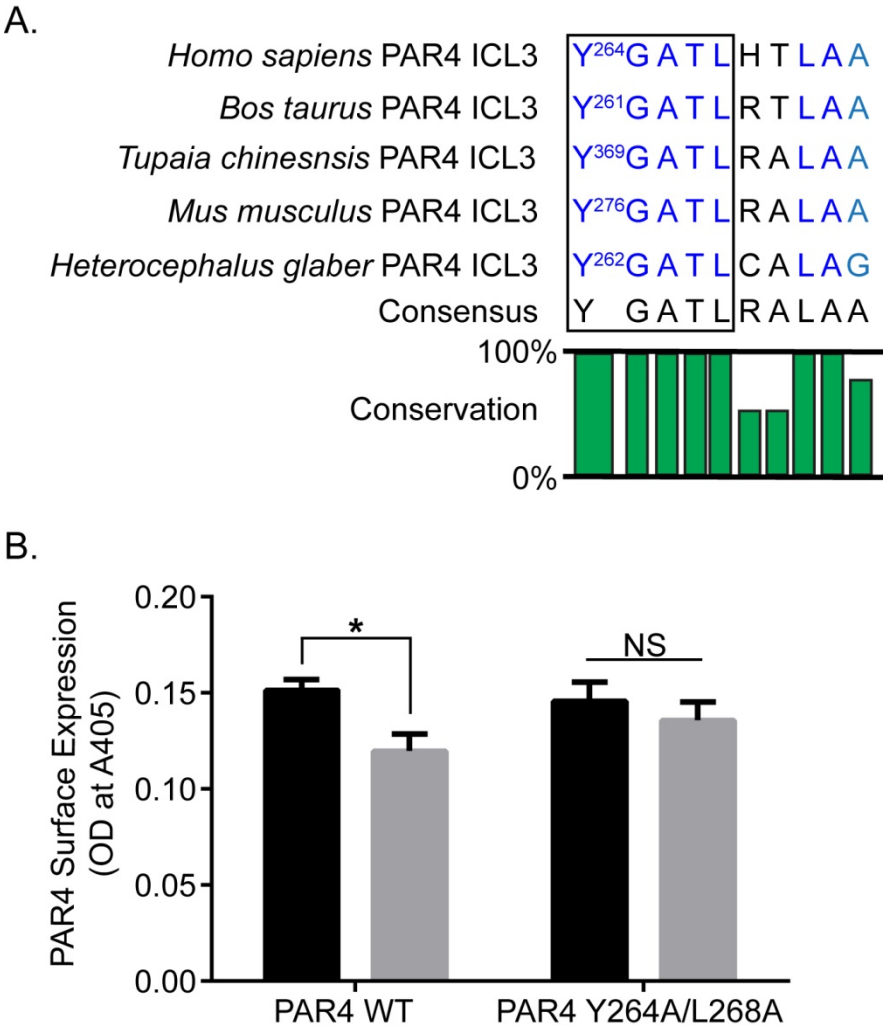

Figure 2.6: PAR4 harbors a highly conserved tyrosine-based motif that is important for internalization.

A, Alignment of human PAR4 intracellular loop 3 (ICL3) sequence with several mammalian orthologues. A highly conserved Y-(X)3-L motif is indicated by the black box. *B*, HeLa cells were transfected with FLAG-PAR4 WT or the tyrosine-motif Y264A/L268A mutant, pre-labeled with anti-FLAG antibody, and stimulated with 500 μM AYPGKF for 60 min at 37°C. Cell surface ELISA was then used to quantify the amount of PAR4 remaining at the cell surface following stimulation. Data shown (mean ± SEM) are representative of four independent experiments, and statistical significance was calculated by two-way ANOVA (*, $p < 0.05$; NS, not significant).

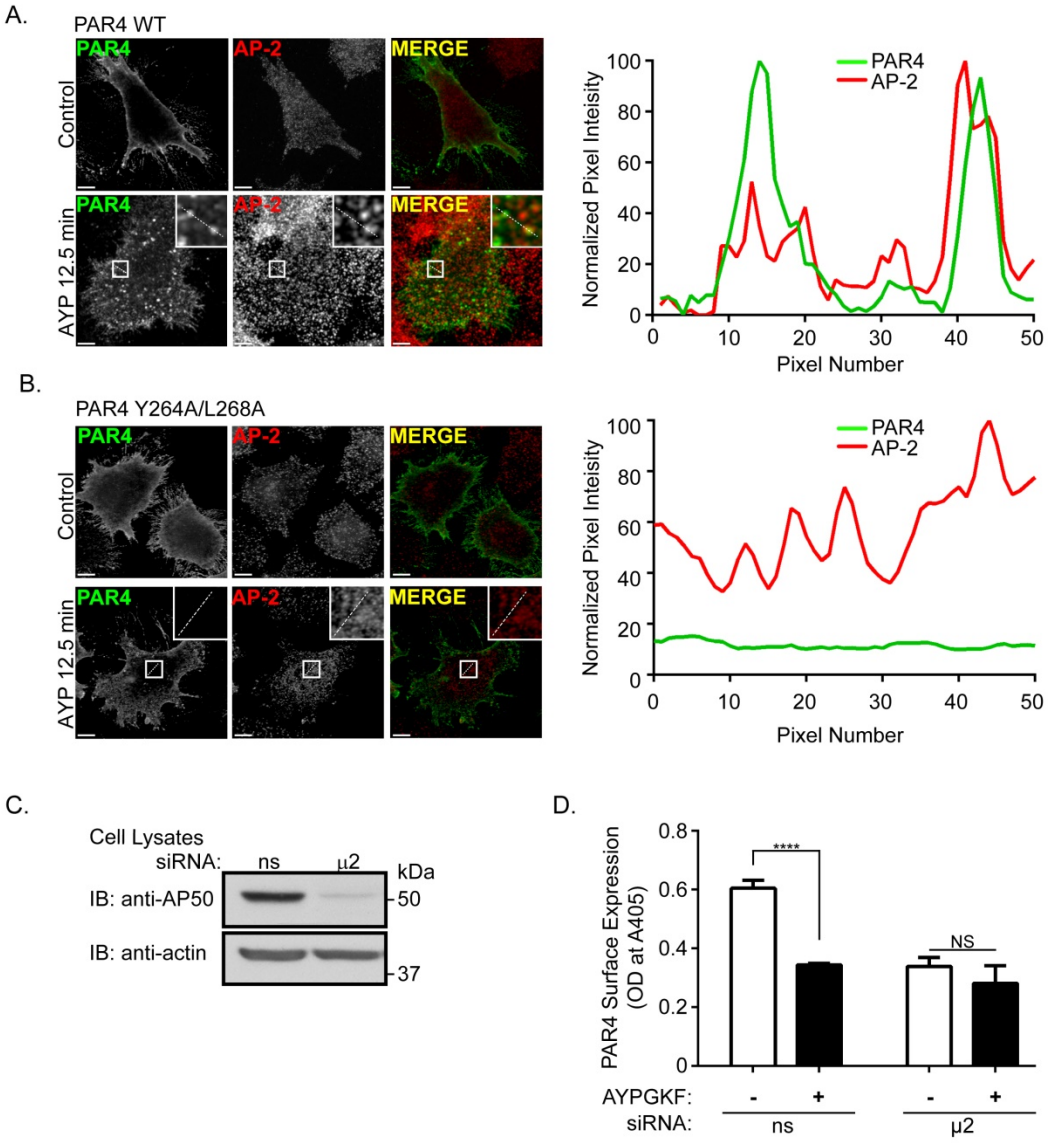

Figure 2.7: AP-2 mediates internalization of activated PAR4.
A and *B*, HeLa cells transfected with FLAG-PAR4 WT *(A)* or the Y264/L268 mutant *(B)* were pre-labeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for 12.5 min at 37°C, processed, co-stained with anti-AP-2 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (Scale bar, 10 μm). Insets, magnifications of boxed areas. The fluorescence intensity line scans were generated from the regions denoted by the white dashed line in the agonist-stimulated images (*A* and *B*, lower panels). *C*, HeLa cells expressing FLAG-PAR4 were transfected with non-specific (ns) siRNA or siRNA specific for the μ2 adaptin subunit of AP-2 and immunoblotted as indicated. *D*, HeLa cells transfected as described above in *(C)* were pre-labeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37°C. ELISA was then used to quantify the amount of receptor at the cell surface. Data shown (mean ± SEM) are representative of three independent experiments, and statistical significance was calculated by two-way ANOVA (****, $p<0.0001$; NS, not significant).

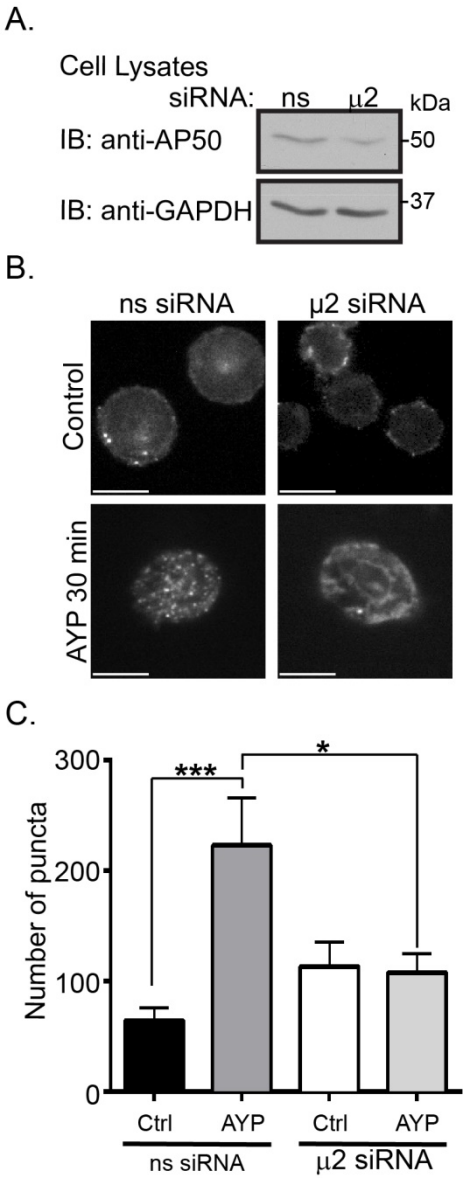

Figure 2.8: AP-2 is required for internalization of endogenous PAR4 in Dami cells.

A, Dami cells were transfected with non-specific (ns) siRNA or siRNA targeting the μ2 adaptin subunit of AP-2. Cell lysates were collected and immunoblotted as indicated. *B*, Dami cells transfected as above in *(A)* were pre-labeled with anti-PAR4 antibody, treated with 500 μM AYPGKF (AYP) or left untreated (Ctrl) for 30 min at 37°C, processed, and imaged by confocal microscopy. The images are representative of three independent experiments (Scale bar, 10 μm). *C*, PAR4 internalization was quantified using Slidebook 5.0 software's automated object counting. At least 10 cells were analyzed for each condition for each of three independent experiments. Data shown are combined from three independent experiments and were analyzed using a Student's t-test (*, $p<0.05$; ***, $p<0.001$).

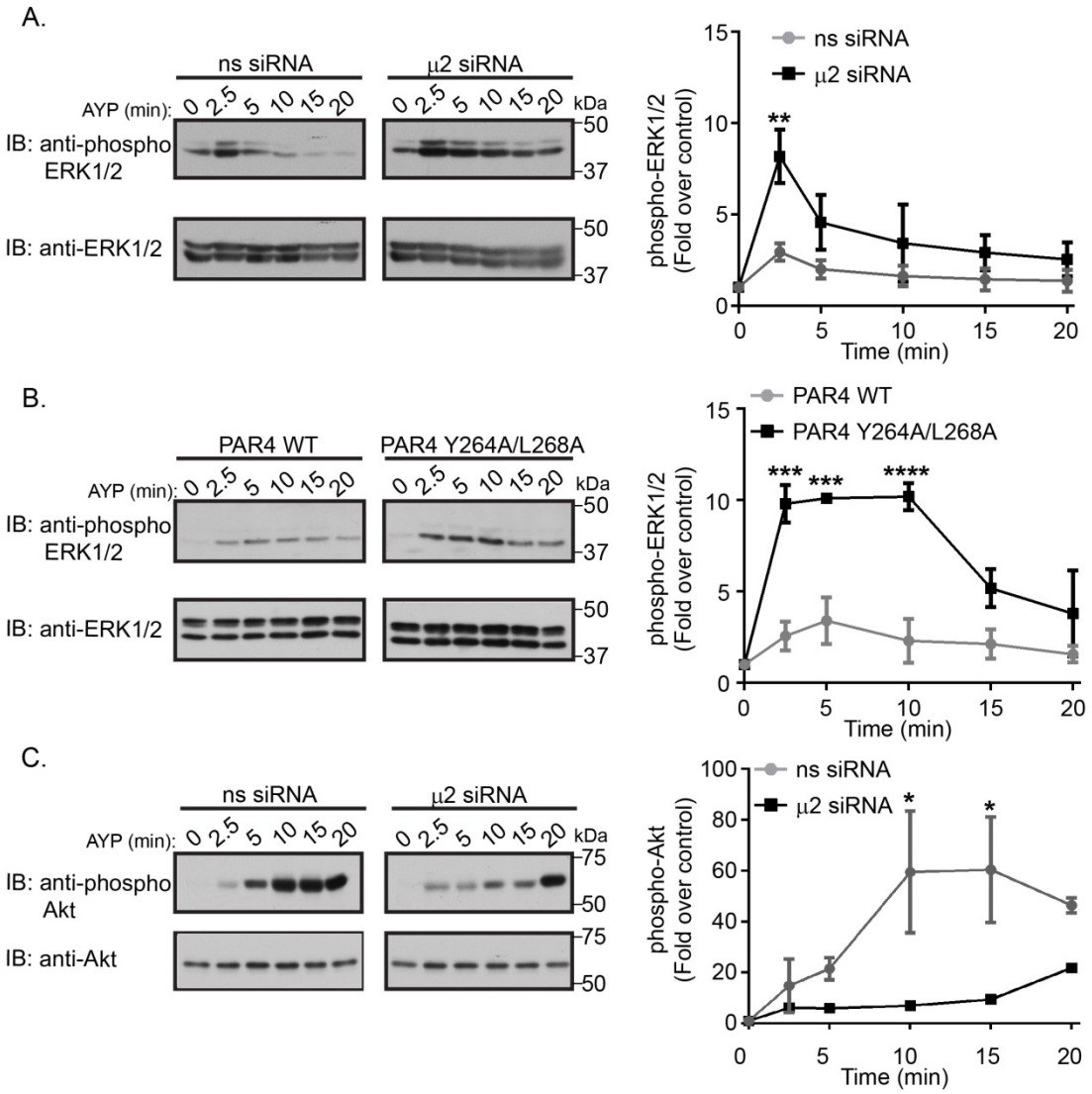

Figure 2.9: PAR4 trafficking is linked to proper ERK1/2 and Akt signaling.
A and *C*, Dami cells transfected with non-specific (ns) siRNA or siRNA targeting the μ2 adaptin subunit of AP-2 were stimulated with 500 μM AYPGKF for various times at 37°C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-ERK1/2 *(A)* and phospho-Akt *(C)* signals were quantified, normalized to total ERK1/2 and total Akt, respectively, and expressed as a fraction of the untreated controls. *B*, HeLa cells transfected with FLAG-PAR4 WT or Y264A/L268A mutant were stimulated with 500 μM AYPGKF for various times at 37°C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-ERK1/2 signals were quantified, normalized to total ERK1/2, and expressed as a fraction of the untreated controls. Samples were resolved on the same gel and separated for labeling. Data shown (mean ± SEM) are representative of three independent experiments, and statistical significance was calculated by two-way ANOVA (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

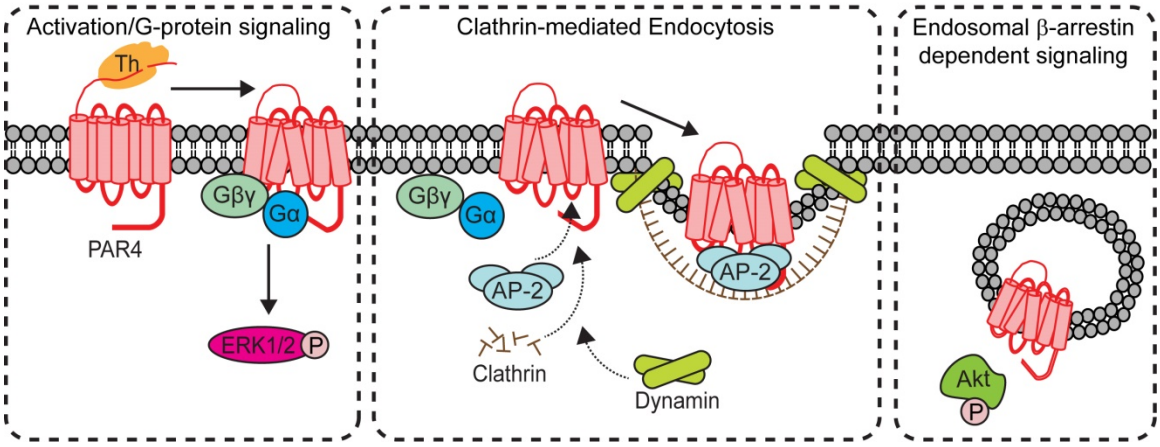

Figure 2.10: Model of PAR4 trafficking and signaling.
PAR4 is a seven transmembrane GPCR that is cleaved and activated by thrombin. Thrombin cleavage generates a tethered ligand that binds intramolecularly to the receptor, facilitating coupling to heterotrimeric G proteins, which promotes ERK1/2 signaling. After activation, PAR4 is recruited to clathrin-coated pits and requires both an intact tyrosine-based motif and AP-2 for internalization. Once internalized, PAR4 is sorted to early endosomes, where it appears to stimulate Akt signaling.

2.8 References

1. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of thrombosis and haemostasis : JTH* **3**, 1800-1814

2. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068

3. Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) Domains specifying thrombin-receptor interaction. *Nature* **353**, 674-677

4. Scarborough, R. M., Naughton, M. A., Teng, W., Hung, D. T., Rose, J., Vu, T. K., Wheaton, V. I., Turck, C. W., and Coughlin, S. R. (1992) Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *The Journal of biological chemistry* **267**, 13146-13149

5. Trejo, J., Hammes, S. R., and Coughlin, S. R. (1998) Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13698-13702

6. Wolfe, B. L., and Trejo, J. (2007) Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic* **8**, 462-470

7. Zhang, J., Ferguson, S. S., Barak, L. S., Menard, L., and Caron, M. G. (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *The Journal of biological chemistry* **271**, 18302-18305

8. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**, 447-450

9. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annual review of pharmacology and toxicology* **48**, 601-629

10. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (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**, 1267-1281

11. Grimsey, N. J., Aguilar, B., Smith, T. H., Le, P., Soohoo, A. L., Puthenveedu, M. A., Nizet, V., and Trejo, J. (2015) Ubiquitin plays an atypical role in GPCR-induced p38 MAP kinase activation on endosomes. *The Journal of cell biology* **210**, 1117-1131

12. Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *The Journal of biological chemistry* **277**, 1292-1300

13. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Molecular and cellular biology* **26**, 3231-3242

14. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *The Journal of biological chemistry* **271**, 32874-32880

15. Dores, M. R., Paing, M. M., Lin, H., Montagne, W. A., Marchese, A., and Trejo, J. (2012) AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Molecular biology of the cell* **23**, 3612-3623

16. Dores, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T., and Trejo, J. (2012) ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *The Journal of cell biology* **197**, 407-419

17. Trejo, J., and Coughlin, S. R. (1999) The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. *The Journal of biological chemistry* **274**, 2216-2224

18. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation* **103**, 879-887

19. Holinstat, M., Voss, B., Bilodeau, M. L., McLaughlin, J. N., Cleator, J., and Hamm, H. E. (2006) PAR4, but not PAR1, signals human platelet aggregation via Ca2+ mobilization and synergistic P2Y12 receptor activation. *The Journal of biological chemistry* **281**, 26665-26674

20. Shapiro, M. J., Weiss, E. J., Faruqi, T. R., and Coughlin, S. R. (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *The Journal of biological chemistry* **275**, 25216-25221

21. Leger, A. J., Covic, L., and Kuliopulos, A. (2006) Protease-activated receptors in cardiovascular diseases. *Circulation* **114**, 1070-1077

22. French, S. L., Arthur, J. F., Tran, H. A., and Hamilton, J. R. (2015) Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Rev* **29**, 179-189

23. Morrow, D. A., Braunwald, E., Bonaca, M. P., Ameriso, S. F., Dalby, A. J., Fish, M. P., Fox, K. A., Lipka, L. J., Liu, X., Nicolau, J. C., Ophuis, A. J., Paolasso, E., Scirica, B. M., Spinar, J., Theroux, P., Wiviott, S. D., Strony, J., Murphy, S. A., Committee, T. P. T. S., and Investigators. (2012) Vorapaxar in the secondary prevention of atherothrombotic events. *The New England journal of medicine* **366**, 1404-1413

24. Edelstein, L. C., Simon, L. M., Lindsay, C. R., Kong, X., Teruel-Montoya, R., Tourdot, B. E., Chen, E. S., Ma, L., Coughlin, S., Nieman, M., Holinstat, M., Shaw, C. A., and Bray, P. F. (2014) Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood* **124**, 3450-3458

25. Tourdot, B. E., Conaway, S., Niisuke, K., Edelstein, L. C., Bray, P. F., and Holinstat, M. (2014) Mechanism of race-dependent platelet activation through the protease-activated receptor-4 and Gq signaling axis. *Arteriosclerosis, thrombosis, and vascular biology* **34**, 2644-2650

26. Edelstein, L. C., Simon, L. M., Montoya, R. T., Holinstat, M., Chen, E. S., Bergeron, A., Kong, X., Nagalla, S., Mohandas, N., Cohen, D. E., Dong, J. F., Shaw, C., and Bray, P. F. (2013) Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nature medicine* **19**, 1609-1616

27. Withey, J. M., Marley, S. B., Kaeda, J., Harvey, A. J., Crompton, M. R., and Gordon, M. Y. (2005) Targeting primary human leukaemia cells with RNA interference: Bcr-Abl targeting inhibits myeloid progenitor self-renewal in chronic myeloid leukaemia cells. *British journal of haematology* **129**, 377-380

28. Khandoga, A. L., Pandey, D., Welsch, U., Brandl, R., and Siess, W. (2011) GPR92/LPA(5) lysophosphatidate receptor mediates megakaryocytic cell shape change induced by human atherosclerotic plaques. *Cardiovasc Res* **90**, 157-164

29. Chen, B., Dores, M. R., Grimsey, N., Canto, I., Barker, B. L., and Trejo, J. (2011) Adaptor protein complex-2 (AP-2) and epsin-1 mediate protease-activated receptor-1 internalization via phosphorylation- and ubiquitination-dependent sorting signals. *The Journal of biological chemistry* **286**, 40760-40770

30. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *The Journal of cell biology* **177**, 905-916

31. Trejo, J., Altschuler, Y., Fu, H. W., Mostov, K. E., and Coughlin, S. R. (2000) Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *The Journal of biological chemistry* **275**, 31255-31265

32. Conner, S. D., and Schmid, S. L. (2003) Differential requirements for AP-2 in clathrin-mediated endocytosis. *The Journal of cell biology* **162**, 773-779

33. Scarselli, M., and Donaldson, J. G. (2009) Constitutive internalization of G protein-coupled receptors and G proteins via clathrin-independent endocytosis. *The Journal of biological chemistry* **284**, 3577-3585

34. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *The Journal of cell biology* **127**, 915-934

35. Ricks, T. K., and Trejo, J. (2009) Phosphorylation of protease-activated receptor-2 differentially regulates desensitization and internalization. *The Journal of biological chemistry* **284**, 34444-34457

36. Rosciglione, S., Theriault, C., Boily, M. O., Paquette, M., and Lavoie, C. (2014) Galphas regulates the post-endocytic sorting of G protein-coupled receptors. *Nature communications* **5**, 4556

37. Tian, X., Irannejad, R., Bowman, S. L., Du, Y., Puthenveedu, M. A., von Zastrow, M., and Benovic, J. L. (2016) The alpha-Arrestin ARRDC3 Regulates the Endosomal Residence Time and Intracellular Signaling of the beta2-Adrenergic Receptor. *The Journal of biological chemistry*

38. Shenoy, S. K., and Lefkowitz, R. J. (2011) beta-Arrestin-mediated receptor trafficking and signal transduction. *Trends in pharmacological sciences* **32**, 521-533

39. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1601-1606

40. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269**, 1872-1875

41. Motley, A., Bright, N. A., Seaman, M. N., and Robinson, M. S. (2003) Clathrin-mediated endocytosis in AP-2-depleted cells. *The Journal of cell biology* **162**, 909-918

42. Mumaw, M. M., de la Fuente, M., Arachiche, A., Wahl, J. K., 3rd, and Nieman, M. T. (2015) Development and characterization of monoclonal antibodies against Protease Activated Receptor 4 (PAR4). *Thrombosis research* **135**, 1165-1171

43. Marinissen, M. J., Servitja, J. M., Offermanns, S., Simon, M. I., and Gutkind, J. S. (2003) Thrombin protease-activated receptor-1 signals through Gq- and G13-initiated MAPK cascades regulating c-Jun expression to induce cell transformation. *The Journal of biological chemistry* **278**, 46814-46825

44. Li, D., D'Angelo, L., Chavez, M., and Woulfe, D. S. (2011) Arrestin-2 differentially regulates PAR4 and ADP receptor signaling in platelets. *The Journal of biological chemistry* **286**, 3805-3814

45. Grimsey, N., Lin, H., and Trejo, J. (2014) Endosomal signaling by protease-activated receptors. *Methods in enzymology* **535**, 389-401

46. Khan, A., Li, D., Ibrahim, S., Smyth, E., and Woulfe, D. S. (2014) The Physical Association of the P2Y12 Receptor with PAR4 Regulates Arrestin-mediated Akt Activation. *Molecular pharmacology*

47. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3712-3717

48. Paing, M. M., Temple, B. R., and Trejo, J. (2004) A tyrosine-based sorting signal regulates intracellular trafficking of protease-activated receptor-1: multiple regulatory mechanisms for agonist-induced G protein-coupled receptor internalization. *The Journal of biological chemistry* **279**, 21938-21947

49. Parent, J. L., Labrecque, P., Driss Rochdi, M., and Benovic, J. L. (2001) Role of the differentially spliced carboxyl terminus in thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization. *The Journal of biological chemistry* **276**, 7079-7085

50. Gasnereau, I., Herr, P., Chia, P. Z., Basler, K., and Gleeson, P. A. (2011) Identification of an endocytosis motif in an intracellular loop of Wntless protein, essential for its recycling and the control of Wnt protein signaling. *The Journal of biological chemistry* **286**, 43324-43333

51. Woulfe, D. S. (2005) Platelet G protein-coupled receptors in hemostasis and thrombosis. *Journal of thrombosis and haemostasis : JTH* **3**, 2193-2200

52. Brass, L. F. (2003) Thrombin and platelet activation. *Chest* **124**, 18S-25S

53. Magnone, M., Basile, G., Bruzzese, D., Guida, L., Signorello, M. G., Chothi, M. P., Bruzzone, S., Millo, E., Qi, A. D., Nicholas, R. A., Kassack, M. U., Leoncini, G., and Zocchi, E. (2008) Adenylic dinucleotides produced by CD38 are negative endogenous modulators of platelet aggregation. *The Journal of biological chemistry* **283**, 24460-24468

CHAPTER 3: CHARACTERIZATION OF THE PAR4-P2Y12 HETERODIMER

3.1 Abstract

Published studies provide evidence for signaling cross-talk between PAR4 and the purinergic receptor P2Y12, a GPCR that is activated by the nucleotide ADP. A hallmark of platelet aggregation is the activation of integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb/IIIa), which mediates platelet-platelet and platelet-extracellular matrix (ECM) adhesion (1). A study in human platelets demonstrated that full integrin activation involves both G protein- and β-arrestin-mediated activation of Akt (2). The latter signaling pathway was shown to involve P2Y12-dependent recruitment of β-arrestin to PAR4. PARs are known to form heterodimers with other PAR family members and other GPCRs (3). Thus, we hypothesized that PAR4 physically associates with P2Y12 to mediate β-arrestin-dependent Akt activation. In this study, we show that PAR4 and P2Y12 form a heterodimer or oligomer that is trafficked as a complex and recruits β-arrestin on endosomes.

3.2 Introduction

Thrombin-mediated signaling via PARs is necessary, but not sufficient, for platelet activation. In fact, platelet activation requires the concerted actions of several different GPCRs that engage a variety of signaling effectors. The purinergic receptor P2Y12 is one such receptor, and has been linked to important

aspects of platelet activation, such as enhancement of dense granule secretion, recruitment of additional platelets to the site of vascular injury, and enhancement of the efficacy of other pro-coagulant agonists (4). P2Y12 couples to $G_{\alpha i}$ and has been shown to mediate PI3K-dependent Akt activation, an important contributor to integrin activation in platelets (5). Integrins are heterodimeric transmembrane receptors that mediate cell-cell adhesion and cell adhesion to extracellular matrix components. In agonist-stimulated platelets, the integrin subunits glycoprotein IIb (GPIIb) and glycoprotein IIIa (GPIIIa) heterodimerize to yield their active form, integrin $\alpha_{IIb}\beta_3$ (also called GPIIb/IIIa), which binds fibrinogen with high affinity. Platelet aggregation occurs by multiple platelets binding to the same molecule of fibrinogen (1).

A 2011 study of human platelets provided evidence that signaling cross-talk between PAR4 and P2Y12 is important for proper Akt-mediated integrin activation (2). Platelets treated with a P2Y12 antagonist exhibited diminished PAR4-mediated Akt activation. PAR4-induced Akt activation was shown to occur via a G protein-independent β-arrestin-mediated signaling pathway. However, in Chapter 2 of this dissertation, we provided evidence that PAR4 internalization does not require β-arrestin. Furthermore, previously published work has demonstrated that activated PAR4 is not phosphorylated, which is generally a requirement for β-arrestin recruitment (6). In contrast, activated P2Y12 has been shown to associate with β-arrestins shortly after activation, and this interaction is necessary for the receptor to undergo clathrin-mediated endocytosis (7). Thus,

we hypothesized that physical association with P2Y12 allows PAR4 to induce β-arrestin-dependent Akt activation.

In this Chapter, we employ several different approaches to evaluate PAR4 and P2Y12 physical association. We demonstrate that the interaction occurs under basal conditions and that the complex is present at the cell surface and responsive to agonist stimulation. Intriguingly, we show that P2Y12 co-internalizes with PAR4, and that β-arrestin is recruited to the internalized complex only in the presence of P2Y12. Biochemical experiments confirm that β-arrestin associates with PAR4 only when coexpressed with P2Y12 and that an internalization-defective PAR4 mutant fails to recruit β-arrestin even in the presence of P2Y12. In cells expressing both receptors, we visualized β-arrestin association with Akt on intracellular puncta. Taken together, these findings suggest that PAR4 and P2Y12 physically associate to mediate β-arrestin-dependent Akt signaling on endosomes.

3.3 Materials and Methods

Reagents and Antibodies. The PAR4 activating peptide AYPGKF was synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography by the Tufts University Core Facility (Boston, MA). Human α-thrombin was obtained from Enzyme Research Technologies (South Bend, IN). Protein A-Sepharose CL-4B beads were from GE Healthcare. Adenosine diphosphate (ADP) and anti-FLAG monoclonal mouse antibody M2

was purchased from Sigma-Aldrich. Anti-FLAG polyclonal rabbit antibody (#600-401-383) was purchased from Rockland Immunochemicals (Gilbertsville, PA). Anti-HA monoclonal mouse antibody HA.11 was purchased from Covance (Princeton, New Jersey). The mouse anti-PAR4 antibody was provided by Dr. Marvin Nieman (Case Western Reserve University, Cleveland, OH). Goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 (#A-11001), Alexa Fluor 594 (A-11005), and Alexa Fluor 647 (A-21235) and goat anti-rabbit secondary antibodies conjugated to Alex Fluor 488 (A-11008), Alex Fluor 594 (A-11012), and Alex Fluor 647 (A-21244) were purchased from ThermoFisher Scientific (Waltham, MA). FluorSave reagent was purchased from Calbiochem. Goat anti-mouse (#170-6516) and goat anti-rabbit (#170-6515) secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Bio-Rad (Hercules, CA). HRP-conjugated mouse anti-HA antibody was obtained from Roche Applied Science. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Thermo Fisher Scientific.

Cell Culture and Transfections. COS-7 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (v/v). The Dami human megakaryocytic cell line was purchased from ATCC and maintained in RPMI media supplemented with 10% (v/v) fetal bovine serum. HeLa cells were transiently transfected with cDNA plasmids using Polyethylenimine (Polysciences Inc., Warrington, PA) at a ratio of 6 µl PEI (1 mg/ml solution) to 1 µg plasmid.

Plasmid cDNAs. Human PAR4 wild-type containing an N-terminal FLAG epitope was cloned into the pBJ mammalian vector as previously described (8). All mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) per manufacturer's protocol and confirmed by dideoxy sequencing. Generation of the PAR4 tyrosine-based sorting motif mutant, PAR4 Y264A/L268A, was described in Chapter 2. The PAR4 and P2Y12 cDNA plasmids used for BRET contain a flexible linker region and either yellow fluorescent protein (YFP) or *Renilla* luciferase (Rluc), respectively, fused to the C-terminus of the receptor.

Immunoblotting. Cell lysates were collected in 2X Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE, transferred to PVDF membranes, immunoblotted with appropriate antibodies, and then developed by chemiluminescence. Immunoblots were quantified by densitometry using ImageJ software (NIH, Bethesda, MD).

Immunoprecipitations. Cells were plated in 6-well dishes at a density of 2.5 x 10^5 cells per well and transfected the following day. After 48 h, cells were placed on ice, washed with PBS, and lysed with Triton X-100 lysis buffer (50mMTris-HCl (pH 7.4), 100 mM NaCl, 5 mMEDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1% Triton X-100) supplemented with a cocktail of protease inhibitors. Cell lysates were cleared by centrifugation, BCA assay (Thermo Fisher Scientific) was performed to determine protein concentrations, and equal amounts of lysates were immunoprecipitated with appropriate antibodies

overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer and proteins were eluted in 50 µl 2X Laemmli sample buffer containing 200 mM - DTT. Cell lysates and immunoprecipitates were analyzed my immunoblotting.

Bioluminescence Resonance Energy Transfer (BRET) Assays. COS-7 cells were transfected for 48 h, lifted with Cellstripper^{TM} (Mediatech), washed with PBS, and resuspended in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose at a density of 5.0 x 10^5 cells/ml. Cells were added (80 µl per well) in triplicate to a 96-well microplate followed by 10 µl of coelenterazine *h* to obtain a final concentration of 5 mM. Rluc luminescence and YFP fluorescence signals were measured after an 8 minute delay using a TriStar LB 941 plate reader running MicroWIN 2000 software (Berthold Biotechnologies) using two filter settings (480 nm for Rluc and 530 nm for YFP). The BRET ratio was calculated as (emission at 530 nm)/(emission at 480 nm), and $BRET_{net}$ was obtained by subtracting the background BRET ratio (cells expressing the Rluc-tagged protein alone). The YFP signal was determined by excitation at 485 nm, and emission was detected at 535 nm. Total luminescence was measured by integrating the signal for 1 s/well without filter selection.

Immunofluorescence Confocal Microscopy. Cells were plated at a density of 0.4 x 10^5 cells per well on fibronectin-coated glass coverslips placed in a 12-well dish and grown overnight. Cells were transfected, grown for 24 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES overnight. Cells were incubated at 4°C with appropriate antibodies to label the cohort of

receptors at the cell surface, stimulated with agonist, then fixed in 4% paraformaldehyde, permeabilized with methanol, and then immunostained with appropriate antibodies and processed as described previously (9). Coverslips were mounted with FluorSave reagent. Confocal images of 0.28 μm x-y sections were collected sequentially using an Olympus IX81 DSU spinning confocal microscope fitted with a Plan Apo 60x oil objective and a Hamamatsu ORCA-ER digital camera using SlideBook 5.0 software (Intelligent Imaging Innovations).

Internalization Assays. Cells were plated at a density of 0.5 x 10^5 cells per well on fibronectin-coated 24-well dishes and grown overnight for cell surface ELISA. Cells were transfected, grown for 48 h, and then serum starved for 1 h in DMEM containing 1 mg/ml BSA and 10 mM HEPES at 37°C. Cells were incubated at 4°C with primary antibody to label the cell surface cohort, stimulated with agonists, and then fixed in 4% paraformaldehyde. The amount of receptor remaining on the cell surface was then detected by incubation with HRP-conjugated secondary antibody, washed and developed with ABTS for at least 20 min at room temperature. The optical density (OD) of an aliquot was determined by absorbance (A) at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Data Analysis. Statistical significance was determined by one-way ANOVA, two-way ANOVA, or student's *t*-test using Prism 4.0 software (GraphPad).

3.4 Results

3.4.1 PAR4 and P2Y12 physically associate under basal conditions

PAR4 and P2Y12 exhibit cross-talk to mediate arrestin-dependent Akt signaling in human platelets, but whether they co-associate in various cell types was not known. We performed co-immunoprecipitation experiments to determine whether PAR4 and P2Y12 physically associate. HeLa cells were transfected with FLAG-tagged PAR4 alone, HA-tagged P2Y12 alone, or both receptors together. In cells coexpressing both receptors, we observed co-immunoprecipitation of P2Y12 with PAR4 isolated by the anti-FLAG antibody, but not with the IgG istotype control (Fig 3.1*A*). The association occurred in untreated cells, and was not affected by 15 min treatment with the PAR4 peptide agonist AYPGKF or the P2Y12 agonist ADP. These findings suggest that PAR4 and P2Y12 have the ability to physically associate basally when co-expressed in cells.

We used bioluminescence resonance energy transfer (BRET) titration experiments to assess whether the PAR4-P2Y12 interaction occurs in living cells. BRET was measured in COS-7 cells transfected with a fixed amount of P2Y12-Rluc and varying doses of PAR4-YFP cDNA plasmid. When plotted as a function of the ratio of PAR4-YFP expression to P2Y12-Rluc expression, we observed a hyperbolic, saturable $BRET_{net}$ curve (Fig 3.1*B*). The hyperbolic relationship between YFP/Rluc expression ratio and $BRET_{net}$ is indicative of a specific protein-protein interaction, rather than non-specific “bystander BRET,” wherein

the relationship would be linear (10). These findings are consistent with the results of the co-immunoprecipitation experiments and provide further evidence of dimerization or oligomerization between PAR4 and P2Y12.

3.4.2 The PAR4-P2Y12 complex is present at the plasma membrane

Generally, GPCRs must be present at the plasma membrane in order to function properly. We used immunofluorescence confocal microscopy to visualize localization of co-expressed PAR4 and P2Y12 in COS-7 cells and observed a high degree of PAR4-P2Y12 colocalization at the cell surface (Fig 3.1*C*). To confirm this finding, we performed sequential co-immunoprecipitation experiments to segregate the internal and surface populations of PAR4. These experiments provide additional biochemical evidence that the PAR4-P2Y12 interaction occurs at the cell surface (Fig 3.2, *Surface*). PAR4-P2Y12 was also present in the internal population, and we observed differences in the P2Y12 species that co-immunoprecipitated with surface and internal PAR4. A low molecular weight form of P2Y12 was the predominant species associated with surface PAR4, whereas low and high molecular weight P2Y12 was associated with internal PAR4 (Fig 3.2, *Surface vs. Intracellular).* Taken together, the above findings indicate that PAR4 and P2Y12 physically associate under basal conditions and that the interaction occurs at the plasma membrane and inside the cell.

3.4.3 The PAR4-P2Y12 complex is sensitive to PAR4 agonist stimulation

Plasma membrane localization of the PAR4-P2Y12 complex is important, but not necessarily sufficient, for the receptors to properly function. To further assess the activity of the surface PAR4-P2Y12 complex, we used BRET to determine whether the complex was responsive to agonist treatment. BRET saturation curves can be used to derive quantitative data such as the association affinity of a protein-protein interaction ($BRET_{50}$) or the conformation of the interaction ($BRET_{max}$). In cells coexpressing PAR4-YFP and P2Y12-Rluc, activation of PAR4 by treatment with either thrombin or AYPGKF caused a statistically significant change in $BRET_{max}$ with no accompanying change in $BRET_{50}$ (Fig 3.3*B*). These data indicate that activation of PAR4 induces a conformational change in the PAR4-P2Y12 complex, suggesting that PAR4 protomers incorporated into these complexes are functional. Furthermore, the ability of thrombin to mediate a conformational change provides further evidence that the interaction is present at the cell surface, since thrombin is not membrane permeable. Interestingly, stimulation with the P2Y12 agonist ADP did not cause a measurable change in association affinity or conformation (Fig 3.3*D*).

3.4.4 P2Y12 is co-internalized with activated PAR4

Previous work demonstrated that P2Y12 trafficking is β-arrestin dependent and involves rapid internalization of the activated receptor followed by recycling back to the cell surface. Conversely, our work presented in Chapter 2 demonstrates that PAR4 trafficking is β-arrestin independent and follows a

slower lysosomal sorting pathway. Given these two contrasting trafficking phenotypes, we were interested in determining the trafficking fate of the PAR4-P2Y12 complex. We performed immunofluorescence confocal microscopy experiments using COS-7 cells as a model system because they natively express low levels of β-arrestins. When expressed alone in COS-7 cells, P2Y12 did not undergo agonist-induced internalization after 15 min of stimulation with ADP (Fig 3.4*A*). Co-transfection with β-arrestin2-GFP rescued P2Y12 trafficking, which we observed as P2Y12 puncta formation after 15 min ADP treatment (Figure 3.4*B*). These experiments confirmed that P2Y12 internalization was β-arrestin-dependent and that COS-7 cells are an appropriate model system to observe β-arrestin-independent endocytosis. Consistent with our previously published work, PAR4 was still able to undergo agonist-induced internalization when expressed alone in COS-7 cells (Fig 3.4*C*). Remarkably, when PAR4 and P2Y12 were coexpressed in COS-7 cells, we observed colocalization of both receptors in cytosolic puncta after 15 min AYPGKF treatment (Fig 3.4*D*). PAR4 did not colocalize with internalized P2Y12 after 15 min ADP treatment of cells expressing both receptors with β-arrestin2-GFP (Fig 3.4*E*). Thus, activation of PAR4, but not P2Y12, elicits co-internalization of the PAR4-P2Y12 complex. To rule out the possibility that this observation was an artifact of the exogenous expression system used, we assessed internalization of endogenous receptors. Immunofluorescence experiments performed in Dami cells, which natively express PAR4 and P2Y12, confirmed that the endogenous PAR4-P2Y12 complex also undergoes PAR4-mediated co-internalization (Fig 3.5*A*).

3.4.5 β-arrestin is recruited to internalized PAR4-P2Y12

We used immunofluorescence confocal microscopy to determine whether association with P2Y12 affected β-arrestin2 recruitment to PAR4. In cells coexpressing PAR4 and β-arrestin2-GFP, AYPGKF treatment did not affect the localization of β-arrestin2 (Fig 3.6*A*). However, when PAR4 was coexpressed with P2Y12, AYPGKF treatment caused robust recruitment of β-arrestin2-GFP to colocalized PAR4-P2Y12 on internal puncta (Fig 3.6*B*). We confirmed this finding by performing co-immunoprecipitation of PAR4 or P2Y12 in cells cotransfected with a myc-tagged β-arrestin2. In cells expressing PAR4 alone, treatment with AYPGKF did not cause recruitment of myc-β–arrestin2 to PAR4 (Fig 3.7*A*). This finding is consistent with a previously published study performed in Rat1 fibroblasts which showed that activated PAR4 does not undergo phosphorylation, which is generally required for β-arrestin recruitment. However, in cells coexpressing PAR4 and P2Y12, we observed a statistically significant increase in myc-β-arrestin2 association with PAR4 after treatment with AYPGKF (Fig 3.7*A*). This finding is consistent with the results of the immunofluorescence experiments described above, and suggests that interaction with P2Y12 may allow β-arrestin to associate with activated PAR4. We also assessed recruitment of β-arrestin to the PAR4 Y264A/L268A mutant, which is deficient in agonist-induced internalization (Fig 2.6). Remarkably, agonist-induced recruitment of myc-β-arrestin2 was abolished in cells expressing PAR4 Y264A/L268A mutant, suggesting that internalization of PAR4 is important for recruitment of β-arrestin2.

In similar experiments, we immunoprecipitated P2Y12 following treatment with ADP. Interestingly, we did not observe ADP-induced recruitment of myc-β-arrestin2 to P2Y12 when expressed alone or when coexpressed with PAR4 (Fig 3.7*B*). Taken together with the immunofluorescence data, these experiments suggest that association with P2Y12 allows for agonist-induced recruitment of β-arrestin to internalized PAR4.

3.4.6 PAR4 activation induces colocalization of β-arrestin2 and Akt in internal puncta

Generally, G protein signaling occurs at the plasma membrane and is characterized by a rapid onset and short-lived duration. The delayed onset and prolonged duration of PAR4-induced Akt activation suggest that this response occurs via a non-G protein signaling pathway (Fig 2.9*C*). Furthermore, we have shown that PAR4, P2Y12, and β-arrestin2 are colocalized at internal puncta when Akt signaling is at its peak, between 10 and 15 min. Our characterization of PAR4 trafficking revealed that the receptor is present at early endosomes within 15 min of activation (Fig 2.2*B*). Thus, we reasoned that β-arrestin2 is recruited to internalized PAR4-P2Y12 at early endosomes. To determine if Akt is also recruited to these endosomal signaling complexes, we performed immunofluorescence microscopy experiments using a myc-tagged Akt fusion protein and β-arrestin2-GFP. In cells coexpressing PAR4 and P2Y12, we observed colocalization of Akt and β-arrestin2 at internal puncta after 15 min treatment with AYPGKF (Fig 3.8*A*). This supports our hypothesis that PAR4 and

P2Y12 coordinate β-arrestin2-mediated Akt signaling at endosomes. In cells expressing PAR4 alone, we did not observe colocalization of β-arrestin2 and Akt, suggesting that this event requires the PAR4/P2Y12 heterodimer (Fig 3.8*B*).

3.5 Discussion/Conclusions

For several years, GPCRs have been known to have the capacity to form dimers and oligomers. However, the consequences of these interactions on cell signaling are varied. There are several reports of GPCR dimers affecting the ligand binding properties of the participating receptor protomers. A study of the δ- and μ-opioid receptors (δOR/ μOR) showed that these receptors exist as heterodimers in native cells, and that treatment with δOR antagonists increased the binding affinity of μOR ligands (11). Dimerization has also been shown to alter the signaling properties of GPCRs. In one study, in which μOR was coexpressed with the α_{2A}-adrenergic receptor (α_{2A}-AR), morphine treatment was shown to suppress α_{2A}-AR-mediated $G\alpha_i$ activity and ERK1/2 phosphorylation (12). Heterodimerization has also been implicated in G protein coupling specificity. The dopamine D_1R and D_2R receptors normally couple to $G\alpha_s$ and $G\alpha_i$, respectively, however, the D_1R-D_2R heterodimer is able to elicit $G\alpha_{q/11}$ signaling (13,14).

Dimerization has also been shown to play a role in GPCR trafficking. There are several reports of GPCRs oligomerization playing important roles in biogenesis and anterograde trafficking from the endoplasmic reticulum (ER) to

the cell surface (15-18). Although limited, there are also some reports of agonist-induced internalization of GPCR dimers. For example, stimulation with a δOR-specific agonist induced internalization of both members of the δOR- μOR heterodimer. This co-trafficking is thought to be a mechanism by which the cell can link activation of one receptor to desensitization of another (13).

We provide evidence by co-immunoprecipitation, BRET, and immunofluorescence microscopy in multiple cell types that PAR4 and P2Y12 exist as a stable complex. These methods do not provide information regarding the stoichiometry of this interaction, thus we cannot definitively say whether this interaction is a 1:1 heterodimer or some higher-order oligomer. One important consideration in performing these studies was whether this relationship occurred at the cell surface. Our sequential co-IP experiments provide compelling evidence that the PAR4-P2Y12 complex is present at the cell surface. BRET experiments revealed that the complex undergoes a conformational change after treatment with either thrombin of the PAR4 peptide agonist. The ability of the PAR4-P2Y12 complex to respond to thrombin provides strong evidence that the PAR4 protomer is functional in this context. The BRET experiments also provide further support that the interaction occurs as the cell surface, since thrombin, which is not cell permeable, was able to elicit a conformational change.

Upon finding that PAR4 and P2Y12 physically interact, we were very interested in determining the trafficking behavior of the dimer. P2Y12 had previously been shown to follow the canonical model of GPCR clathrin-mediated

endocytosis involving β-arrestin-dependent endocytosis and subsequent rapid recycling back to the cell surface (4,7,19). This was in striking contrast to the trafficking behavior of PAR4, which undergoes β-arrestin-independent internalization and sorting to the lysosome. Thus, we examined the trafficking of the PAR4-P2Y12 complex to determine how these two different sorting phenotypes were reconciled in the context of the dimer. We found that P2Y12 co-internalizes with PAR4 following agonist stimulation. Interestingly, P2Y12 agonist-induced internalization did not result in reciprocal co-internalization of PAR4.

The original motivation for studying the PAR4-P2Y12 interaction was the reported cross-talk observed between these receptors in mediating β-arrestin-dependent Akt signaling. Thus, we probed the role of P2Y12 in mediating β-arrestin recruitment to PAR4. Biochemical experiments demonstrated that β-arrestin co-immunoprecipitated with PAR4 in cells coexpressing P2Y12, but not in cells expressing PAR4 alone. However, in cells coexpressing the internalization-deficient PAR4 Y264A/L268A mutant with P2Y12, β-arrestin failed to co-immunoprecipitate with PAR4. This suggests that internalization of PAR4 is necessary for its association with β-arrestin, and raises the possibility that the β-arrestin-mediated Akt signaling may occur on endosomes. Consistent with this model, we showed by immunofluorescence microscopy that β-arrestin-GFP colocalizes with co-internalized PAR4-P2Y12 on internal structures. In cells lacking P2Y12, however, β-arrestin-GFP does not colocalize with internalized

PAR4, indicating a necessity for both receptors. Finally, we show that β-arrestin and Akt colocalize on distinct puncta in AYP-stimulated cells co-expressing PAR4 and P2Y12, but not in cells expressing PAR4 alone.

While conducting our investigation of the PAR4-P2Y12 dimer, another research group published a study characterizing the interaction (20). Their study characterizes the interaction mostly using BRET in HEK293T cells, and strongly focuses on identification of the PAR4-P2Y12 interaction domain. Thus, our work is distinct in that we utilized multiple techniques in different cell types to explore how trafficking on the PAR4-P2Y12 complex influences β-arrestin recruitment.

3.6 Acknowledgements

The majority of the data in Chapter 3 is being prepared for publication: Smith TH, Li JG, Dores MD, Trejo J. "Functional consequences of PAR4 heterodimerization with P2Y12 on endocytic sorting and β-arrestin recruitment." *Journal TBD.* The dissertation author is the primary author who directed experiments and analyzed the data.

3.7 Figures

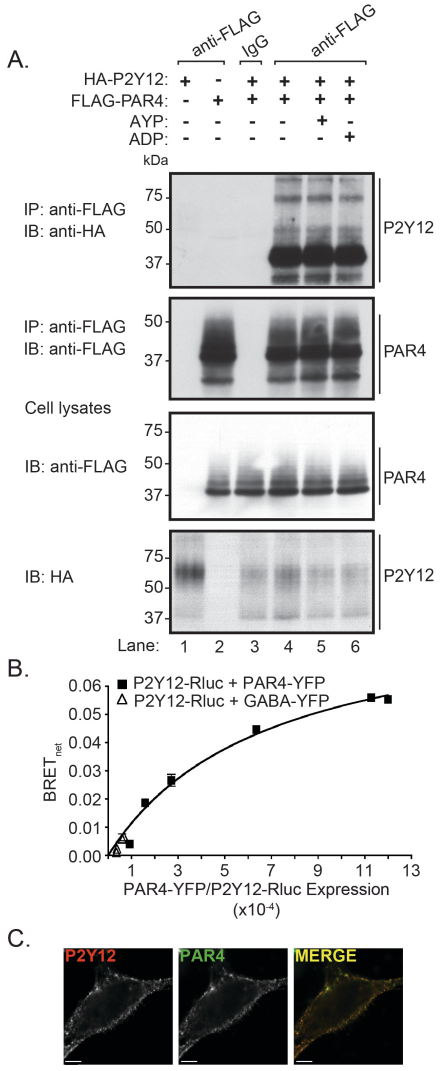

Figure 3.1: PAR4 and P2Y12 form a stable heterodimer.
A, HeLa cells expressing FLAG-PAR4 and/or HA-P2Y12 were treated with 500 μM AYPGKF or 10 μM ADP at 37°C then lysed and immunoprecipitated with mouse M2 anti-FLAG antibody or mouse IgG isotype control. Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. *B*, COS-7 cells transfected with a fixed amount of P2Y12-Rluc and a range of PAR4-YFP or GABA-YFP. $BRET_{net}$ is plotted as a function of the expression ratios of PAR4-YFP to P2Y12-Rluc, which were empirically determined by measuring basal YFP fluorescence and Rluc luminescence, respectively. *C*, COS-7 cells were plated on fibronectin-coated glass coverslips and transfected with FLAG-PAR4 and HA-P2Y12 for 48 h. Cells were pre-labeled on ice for 1 h with anti-FLAG and anti-HA antibodies, and then fixed, processed, immunostained, and imaged by confocal microscopy. The yellow color in the merged image is indicative of colocalization of P2Y12 (red) and PAR4 (green).

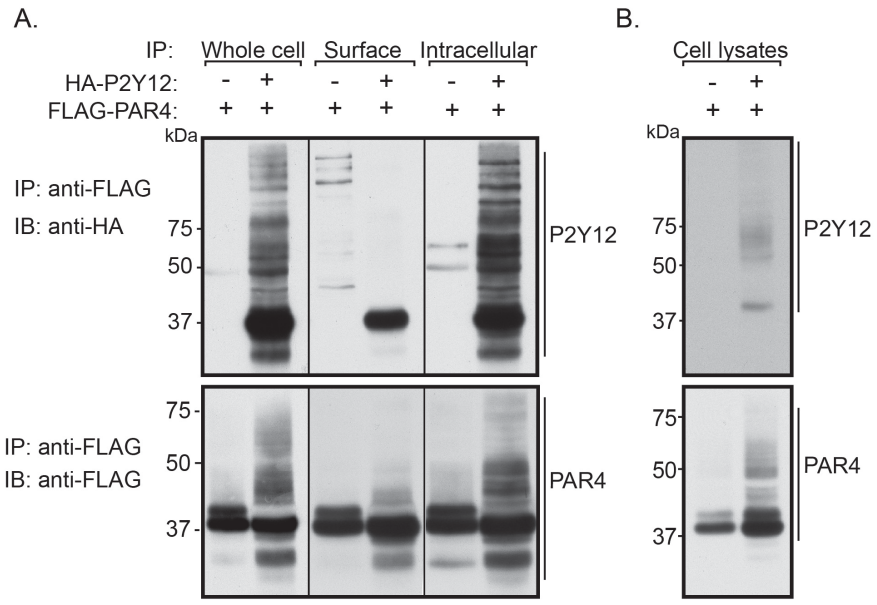

Figure 3.2: PAR4 and P2Y12 interact at the cell surface.
A, COS-7 cells were transfected with FLAG-PAR4 alone or together with HA-P2Y12. Surface PAR4 was labeled by incubating with anti-FLAG antibody on ice for 1.5 h. Cells were lysed and split into two fractions – *Whole cell* and *Surface*. ProteinA-sepharose beads were used to pull down pre-bound PAR4 from the *Surface* sample, and then the supernatant was incubated with anti-FLAG antibody to IP the *Intracellular* fraction. The *Whole cell* fraction was incubated with anti-FLAG antibody for 1.5 h to IP total PAR4 (combination of internal and surface populations). IP *(A)* and lysate *(B)* samples were resolved by SDS-PAGE and immunoblotted with indicated antibodies.

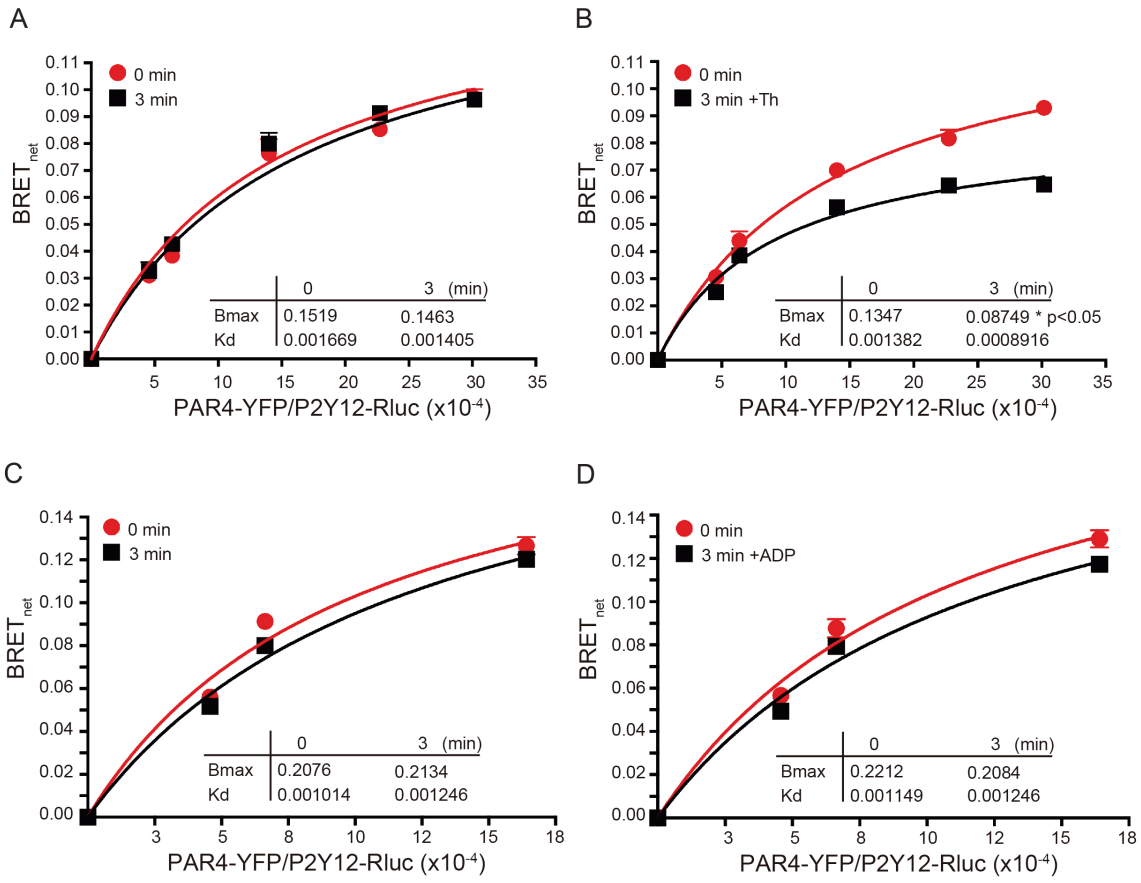

Figure 3.3: The PAR4-P2Y12 complex undergoes an agonist-induced conformational change.

A, *B*, *C*, and *D*, COS-7 cells were transfected with a fixed amount of P2Y12-Rluc and a range of PAR4-YFP. $BRET_{net}$ was measured at 37°C following addition of 30 nM Thrombin *(B)*, 10 μM ADP *(D)*, or buffer control *(A, C)*. $BRET_{net}$ is plotted as a function of the ratio of PAR4-YFP/P2Y12-Rluc expression. Data shown are representative of three independent experiments, which were used to calculate $BRET_{max}$ and $BRET_{50}$ values.

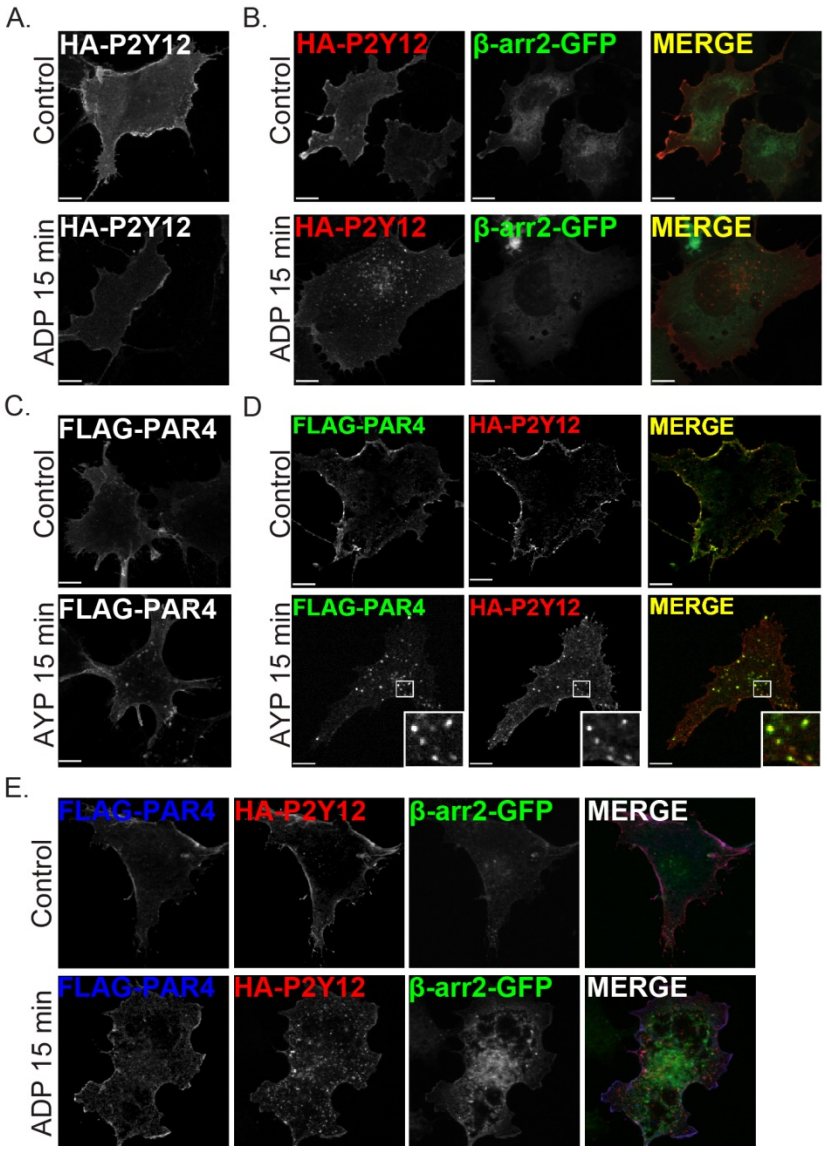

Figure 3.4: The PAR4-P2Y12 complex undergoes β-arrestin-independent co-internalization.

A and *B*, COS-7 cells expressing HA-P2Y12 alone (*A*) or together with β-arrestin2-GFP *(B)* were pre-labeled with anti-HA antibody, stimulated with 10 μM ADP for 15 min at 37°C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. *C* and *D*, COS-7 cells expressing FLAG-PAR4 alone *(C)* or co-transfected with HA-P2Y12 (*D*) were pre-labeled with anti-FLAG antibody alone *(C)* or together with anti-HA antibody (*D*), stimulated with 500 μM AYPGKF for 15 min at 37°C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. *E*, COS-7 cells coexpressing HA-P2Y12, FLAG-PAR4, and β-arrestin2-GFP were pre-labeled with anti-HA and anti-FLAG antibodies, stimulated with 10 μM ADP for 15 min at 37°C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. Yellow puncta in the merged image of (*D*) are co-internalized P2Y12 (red) and PAR4 (green). Images shown are representative of three independent experiments.

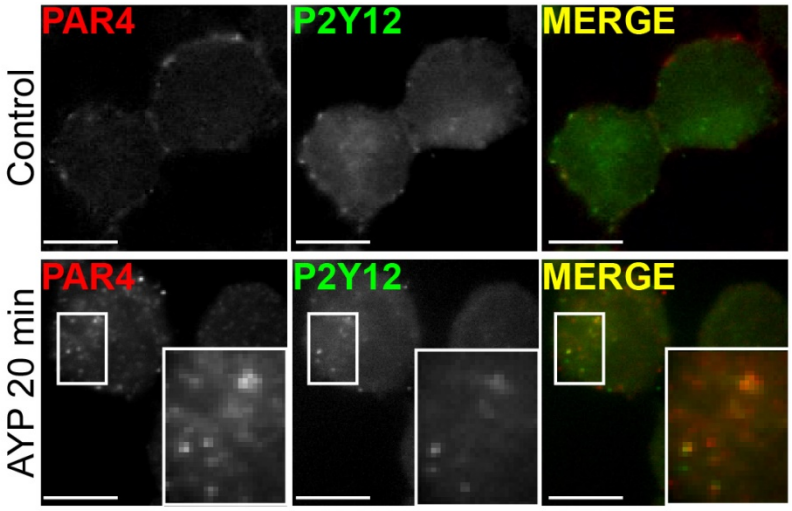

Figure 3.5: Endogenous PAR4 and P2Y12 undergo agonist-induced co-internalization.
Dami cells were plated on poly-lysine coated glass coverslips and grown for 48 h. Cells were pre-labeled on ice with anti-PAR4 and anti-P2Y12 antibody, stimulated with 500 μM AYPGKF for 15 min at 37°C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. Yellow puncta in the merged image are co-internalized P2Y12 (green) and PAR4 (red). Images shown are representative of three independent experiments.

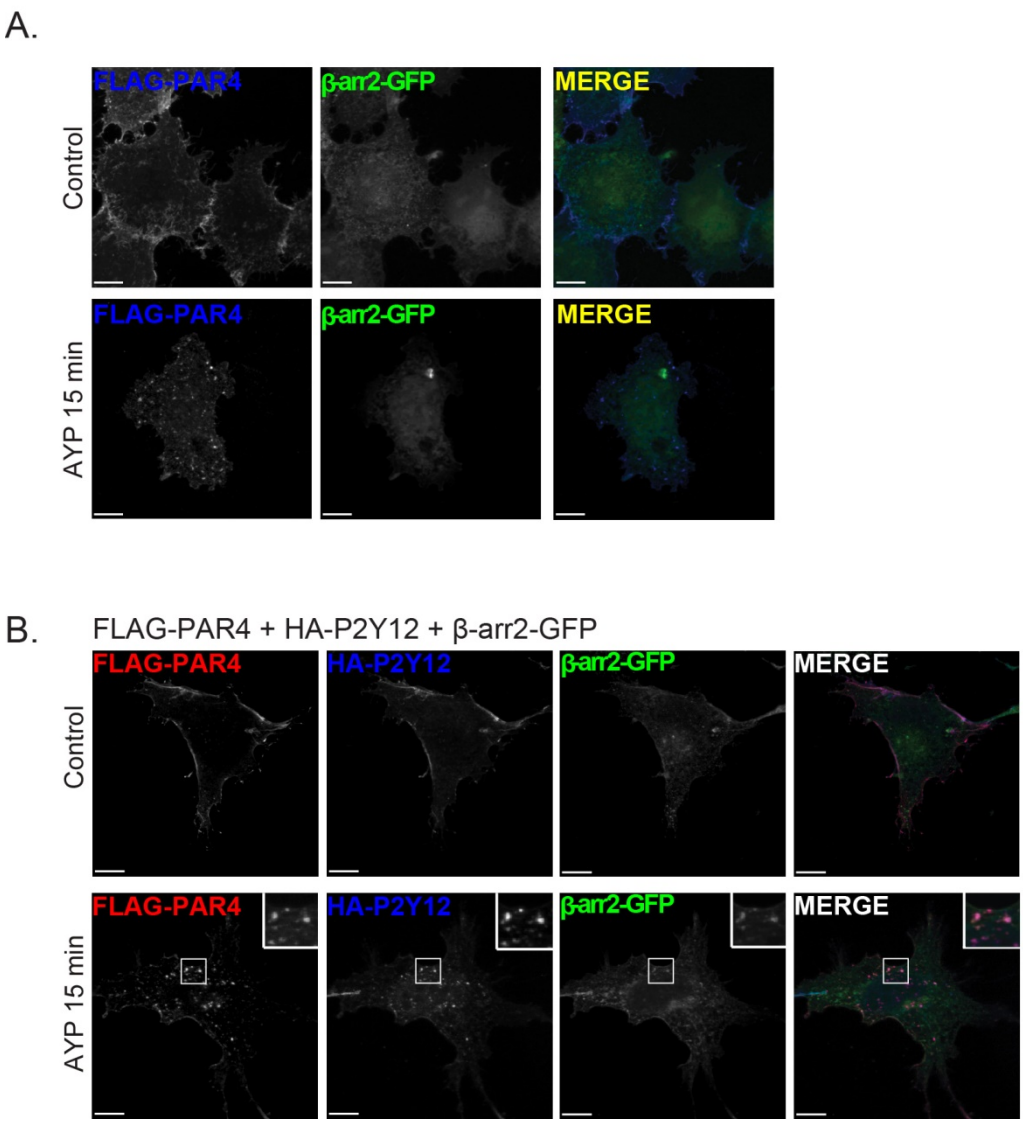

Figure 3.6: β-arrestin2 is recruited to internalized PAR4-P2Y12 complexes.
A and *B*, COS-7 cells expressing β-arrestin2-GFP and FLAG-PAR4 alone (*A*) or together with HA-P2Y12 (*B*) were pre-labeled with anti-FLAG antibody alone (*A*) or in combination with anti-HA antibody (*B*). Cells were stimulated with 500 μM AYPGKF for 15 min at 37C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. Pink puncta in the merged image of (*B*) are colocalized PAR4 (red), P2Y12 (blue) and β-arrestin2 (green). Images shown are representative of three independent experiments.

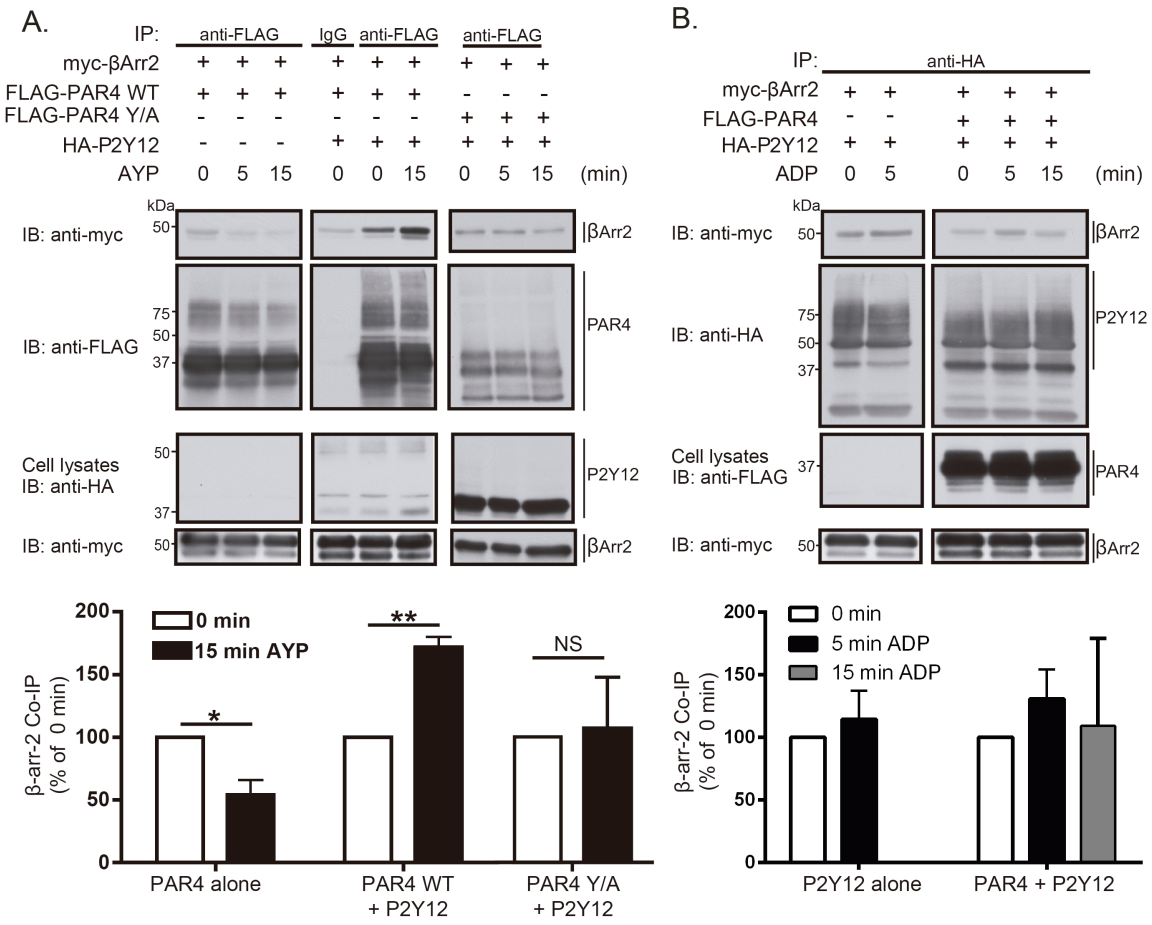

Figure 3.7: PAR4-P2Y12 association and internalization is required for agonist-induced β-arrestin2 recruitment.

A and *B*, COS-7 cells were cotransfected with myc-β-arrestin2 and FLAG-PAR4 alone, HA-P2Y12 alone, or FLAG-PAR4 (WT or Y264A/L268A mutant) together with HA-P2Y12. After 48 h, cells were treated with 500 μM AYPGKF *(A)* or 10 μM ADP *(B)* at 37°C for indicated times, after which cells were lysed and IP was performed with anti-FLAG *(A)* or anti-HA *(B)* antibody. Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Agonist-induced β-arrestin2 co-IP was quantified by densitometry analysis of three independent experiments and is expressed as the percent change relative to untreated. Statistical significance was determined by one-way ANOVA (* - $p<0.05$, ** - $p<0.01$)

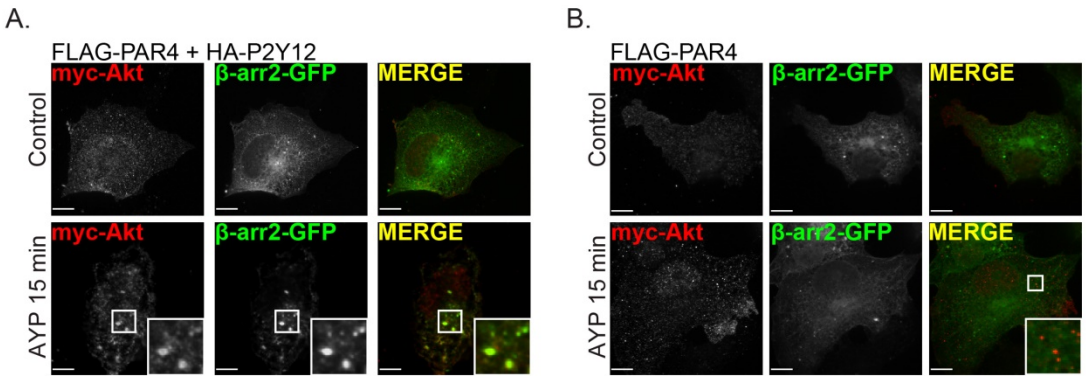

Figure 3.8: Coexpression with P2Y12 facilitates PAR4-induced recruitment of Akt to β-arrestin2 on endosomes.

A and *B*, COS-7 cells were cotransfected with β-arrestin2-GFP, myc-Akt, and either FLAG-PAR4 together with HA-P2Y12 (*A*) or FLAG-PAR4 alone (*B*). Cells were stimulated with 500 μM AYPGKF for 15 min at 37°C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. Yellow puncta in the merged image of (*A*) are colocalized Akt (red) and β-arrestin2 (green). Images shown are representative of three independent experiments.

3.8 References

1. Nieswandt, B., Varga-Szabo, D., and Elvers, M. (2009) Integrins in platelet activation. *Journal of thrombosis and haemostasis : JTH* **7 Suppl 1**, 206-209

2. Li, D., D'Angelo, L., Chavez, M., and Woulfe, D. S. (2011) Arrestin-2 differentially regulates PAR4 and ADP receptor signaling in platelets. *The Journal of biological chemistry* **286**, 3805-3814

3. Lin, H., Liu, A. P., Smith, T. H., and Trejo, J. (2013) Cofactoring and dimerization of proteinase-activated receptors. *Pharmacological reviews* **65**, 1198-1213

4. Cattaneo, M. (2015) P2Y12 receptors: structure and function. *Journal of thrombosis and haemostasis : JTH* **13 Suppl 1**, S10-16

5. O'Brien, K. A., Gartner, T. K., Hay, N., and Du, X. (2012) ADP-stimulated activation of Akt during integrin outside-in signaling promotes platelet spreading by inhibiting glycogen synthase kinase-3beta. *Arteriosclerosis, thrombosis, and vascular biology* **32**, 2232-2240

6. Shapiro, M. J., Weiss, E. J., Faruqi, T. R., and Coughlin, S. R. (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *The Journal of biological chemistry* **275**, 25216-25221

7. Nisar, S. P., Cunningham, M., Saxena, K., Pope, R. J., Kelly, E., and Mundell, S. J. (2012) Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalization. *The Journal of biological chemistry* **287**, 24505-24515

8. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation* **103**, 879-887

9. Dores, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T., and Trejo, J. (2012) ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *The Journal of cell biology* **197**, 407-419

10. Kufareva, I., Stephens, B., Gilliland, C. T., Wu, B., Fenalti, G., Hamel, D., Stevens, R. C., Abagyan, R., and Handel, T. M. (2013) A novel approach to quantify G-protein-coupled receptor dimerization equilibrium using bioluminescence resonance energy transfer. *Methods in molecular biology* **1013**, 93-127

11. Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E., and Devi, L. A. (2004) A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 5135-5139

12. Vilardaga, J. P., Nikolaev, V. O., Lorenz, K., Ferrandon, S., Zhuang, Z., and Lohse, M. J. (2008) Conformational cross-talk between alpha2A-adrenergic and mu-opioid receptors controls cell signaling. *Nat Chem Biol* **4**, 126-131

13. Verma, V., Hasbi, A., O'Dowd, B. F., and George, S. R. (2010) Dopamine D1-D2 receptor Heteromer-mediated calcium release is desensitized by D1 receptor occupancy with or without signal activation: dual functional regulation by G protein-coupled receptor kinase 2. *The Journal of biological chemistry* **285**, 35092-35103

14. Hasbi, A., O'Dowd, B. F., and George, S. R. (2010) Heteromerization of dopamine D2 receptors with dopamine D1 or D5 receptors generates intracellular calcium signaling by different mechanisms. *Curr Opin Pharmacol* **10**, 93-99

15. Milligan, G. (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *British journal of pharmacology* **158**, 5-14

16. Canals, M., Lopez-Gimenez, J. F., and Milligan, G. (2009) Cell surface delivery and structural re-organization by pharmacological chaperones of an oligomerization-defective alpha(1b)-adrenoceptor mutant demonstrates membrane targeting of GPCR oligomers. *Biochem J* **417**, 161-172

17. Salahpour, A., Angers, S., Mercier, J. F., Lagace, M., Marullo, S., and Bouvier, M. (2004) Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting. *The Journal of biological chemistry* **279**, 33390-33397

18. Zhu, X., and Wess, J. (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37**, 15773-15784

19. Nisar, S., Daly, M. E., Federici, A. B., Artoni, A., Mumford, A. D., Watson, S. P., and Mundell, S. J. (2011) An intact PDZ motif is essential for correct P2Y12 purinoceptor traffic in human platelets. *Blood* **118**, 5641-5651

20. Khan, A., Li, D., Ibrahim, S., Smyth, E., and Woulfe, D. S. (2014) The Physical Association of the P2Y12 Receptor with PAR4 Regulates Arrestin-mediated Akt Activation. *Molecular pharmacology*

CHAPTER 4: CONCLUSIONS

PAR4 was the last of the thrombin-responsive GPCRs to be discovered (1). PAR4's low affinity for thrombin as well as its coexpression with the high affinity thrombin receptor, PAR1, may explain the historic disinterest in studying PAR4. Today, the importance of PAR4 has become much more appreciated. The recent interest in PAR4 is encapsulated by a 2016 article published in the *Journal of Thrombosis and Haemostasis*, entitled "PAR4 is more important than PAR1 for the thrombin-induced procoagulant effect on platelets" (2).

PAR4 represents an excellent potential drug target for regulating thrombin-induced platelet activation. Currently, the PAR1 antagonist vorapaxar is the only FDA-approved drug that targets a thrombin receptor. Vorapaxar is prescribed to stroke and heart attack patients as a preventative therapy to decrease the risk of future adverse cardiac events (3,4). However, vorapaxar treatment has been linked to serious side effects that may arguably outweigh the therapeutic benefit for some patients. Furthermore, patients exhibit varying degrees of responsiveness to vorapaxar, with treatment being less efficacious than placebo for certain groups. Modulating PAR4 activity is an attractive alternative strategy that may produce less off-target effects and potentially prove more effective for some patients.

Racial difference in PAR4 activity may have important pharmacogenetic implications. A recent study reported a 3.7-fold increase in PAR4-induced platelet aggregation in platelets collected from black subjects relative to platelets

from whites (5). This difference is hypothesized to be due to a single nucleotide polymorphism (SNP) in the PAR4 gene, *F2RL3*, that is significantly more common in black subjects than white subjects. The SNP, rs773902, translates into differences in residue 120 of PAR4, which is located in transmembrane domain 2. The Thr-120 variant, which is more common in blacks, was associated with increased platelet aggregation, Ca^{2+} flux, and inositol phosphate generation than the Ala-120 variant that is more often found in whites (6). As personalized medicine becomes more feasible, these variations in PAR4 reactivity may have important clinical implications.

Epigenetic regulation of the PAR4 gene has recently been identified as a strong predictor of lung cancer risk, cardiovascular disease, and mortality (7-10). DNA methylation is an epigenetic mechanism that allows cells to regulate expression of specific genes or sets of genes. Methylated DNA is inaccessible to transcriptional machinery, thus hypermethylated genes are not expressed, whereas hypomethylated genes are. Genome-wide methylation analysis studies have shown that tobacco smoking is correlated with hypomethylation of *F2RL3*, the gene encoding PAR4 (10). A follow-up prospective analysis involving 3588 participants aged 50–75 found that lower levels of *F2RL3* methylation were strongly associated with mortality (11). The molecular mechanisms responsible for *F2RL3* hypomethylation are unknown, and the effects on PAR4 protein expression, function, and pharmacodynamics have not been investigated.

Clearly the importance of PAR4 has become much more apparent in the years following its initial discovery and characterization. The studies described in this dissertation provide several novel insights into fundamental aspects of PAR4 function. Our findings may aid in future efforts to explore the role of PAR4 in novel disease contexts or in the development of drugs that modulate PAR4 activity.

4.1 Characterization of PAR4 trafficking

Due to their irreversible mechanism of activation, internalization and subsequent intracellular trafficking of PARs is an important regulator of signaling. Prior to this work, there were no published reports describing the mechanism by which PAR4 is internalized or the fate of internalized PAR4. We demonstrate that PAR4 undergoes β-arrestin-*independent* clathrin-mediated endocytosis, similar to PAR1. However, in contrast to PAR1, the PAR4 C-tail does not play a role in its agonist-induced internalization. Instead, we show that PAR4 internalization is mediated by AP-2 binding to an ICL3-localized recognition motif. This is a departure from the norm, as the majority of GPCR regulation and binding protein interactions occur on the C-tail. This difference may help to explain the delayed kinetics of PAR4 internalization compared to PAR1. For example, it is possible that steric constraints may make the ICL3-located motif less accessible to AP-2. Thus, PAR4's delayed rate of internalization may be the result of slower recruitment of AP-2 to the activated receptor.

One aspect of PAR4 trafficking that we did not examine was constitutive recycling. For PAR1, this process has been well-studied and allows cells to maintain an intracellular pool of naïve, unactivated receptors to be used for resensitization following activation of the surface cohort. In contrast, PAR2 resensitization is thought to occur predominantly by *de novo* biosynthesis and antergrade trafficking of PAR2 stored in the Golgi (12). Whether PAR4 is resensitized in a manner similar to PAR1, PAR2, or neither may be an important question addressed in future studies.

4.2 Role of trafficking in PAR4 signal regulation

Endocytosis has long been associated with attenuation of GPCR signaling as a secondary mechanism following rapid desensitization (13). However, in the majority of cases, this relationship is correlative rather than causative. That is, events associated with signal termination are often also involved in internalization. For example, binding of β-arrestin is known to promote uncoupling of activated receptor from G proteins for many GPCRs (13,14). However, β-arrestin also acts as a clathrin adaptor protein in this context, facilitating internalization of the receptor (15). Phosphorylation of activated receptors by GRKs has also been shown to be important for signal attenuation and clathrin-mediated endocytosis (16). PAR4 internalization is somewhat unique in that it is not phosphorylated after activation and does not require β-arrestins for internalization. Rather than utilize one of these two canonical signal

termination mechanisms, we believe that PAR4 G protein signal shut-off occurs as a result of internalization itself. G proteins are anchored to the plasma membrane by lipid modifications such as palmitoylation and/or myristoylation (17). Thus, internalization of PAR4 mediates signal termination by spatially separating the receptor from heterotrimeric G proteins. This unique mechanism of signal termination may be important for PAR4's sustained signaling observed in multiple cell types and correlates with its slow rate of internalization compared to PAR1. The model of signal regulation by endocytosis we propose could also be employed by other GPCRs. It may be worthwhile to investigate other receptors that are known to internalize in the absence of phosphorylation and β-arrestins.

4.3 Characterization of the PAR4/P2Y12 heterodimer

We used several different approaches to interrogate whether PAR4 and P2Y12 physically associate. Our data is generally in agreement with a recently published study that also characterized heterodimerization between PAR4 and P2Y12 (18). However, many questions still remain with regard to the PAR4-P2Y12 interaction. Many receptor oligomers are known to form within the endoplasmic reticulum (19,20). We detected an intracellular population of PAR4-P2Y12 in our sequential co-IP experiments, suggesting that this interaction also occurs during biosynthesis, rather than spontaneously at the plasma membrane. This raises the question of what regulates dimer formation and the relative

abundance of monomeric PAR4, monomeric P2Y12, and the PAR4-P2Y12. Modulating the expression of each of these subpopulations may allow the cell to fine-tune signaling responses.

Dimerization has been reported to alter the ligand binding kinetics for some GPCRs. For example, the dopamine D_{2L} receptor exhibits 34-fold lower binding affinity for a D_2 agonist when coexpressed with the neurotensin NTS_1 receptor (21,22). Our sequential co-IP experiments suggest that a predominantly low molecular weight form of P2Y12 is associated with surface PAR4. Whether P2Y12 is still responsive to agonist in this context is not clear. We did not observe an ADP-induced conformational change in the PAR4-P2Y12 complex by BRET, and ADP treatment failed to elicit co-internalization of PAR4-P2Y12. However, in the initial report of PAR4-P2Y12 cross-talk, synergistic signaling was disrupted by treatment with a P2Y12 antagonist (23). Furthermore, the published characterization of the PAR4-P2Y12 heterodimer showed that the interaction was disrupted by treatment with a P2Y12 antagonist (18). This raises interesting questions about how the PAR4-P2Y12 heterodimer is affected by treatment with clopidogrel, a clinically used P2Y12 antagonist.

4.4 Trafficking of the PAR4/P2Y12 heterodimer and its role in endosomal signaling

Due to the vast differences in trafficking of the receptor protomers, we were very interested in the fate of the activated PAR4-P2Y12 complex.

Dimerization of other GPCRs has been shown to play varied roles in trafficking. Some receptors require dimerization to enhance anterograde transport and cell surface expression (24,25). Co-internalization of GPCR dimers has also been reported as a mechanism of heterologous desensitization. For example, treatment with δOR-selective agonists causes endocytosis of the δOR-μOR heterodimer. This effectively results in the cell being desensitized to μOR agonists in response to δOR activation, and causes down-regulation of morphine-mediated analgesia (26).

We show that the PAR4-P2Y12 complex is co-internalized in response to PAR4, but not P2Y12, agonist treatment. We also demonstrate that co-expression with P2Y12 is necessary for agonist-promoted β-arrestin recruitment to PAR4. Furthermore, β-arrestin recruitment was abolished by an internalization-defective PAR4 mutant. This suggests a model in which PAR4-P2Y12 is internalized as a complex to which β-arrestin is recruited. This model is consistent with our immunofluorescence data showing colocalization of PAR4, P2Y12, and β-arrestin at internal puncta.

Previous studies have shown that P2Y12 internalization requires GRKs, which suggests that the receptor is phosphorylated after activation (27). P2Y12 internalization is also known to require β-arrestins (28). Since activated PAR4 is not phosphorylated, it most likely does not directly recruit β-arrestins. This raises the possibility that distinct characteristics of PAR4 and P2Y12 both contribute to the ability of the complex to recruit β-arrestins as a signaling

scaffold. As a monomer, the interaction of P2Y12 with β-arrestin is most likely transient due to the receptor's rapid rate of internalization and subsequent recycling. However, co-internalization with PAR4 effectively sequesters P2Y12 away from the plasma membrane, potentially allowing for more sustained interaction with β-arrestin. It is unclear what dictates the shift in β-arrestin's role from clathrin adaptor to signaling scaffold, but intracellular localization may be one possible determinant. This would explain why the P2Y12 protomer, with its very low endosomal residence time, does not mediate arrestin-dependent Akt activation. PAR4-mediated redistribution of P2Y12-bound β-arrestin may serve to localize β-arrestin in closer proximity to signaling effectors such as PI3K and Akt.

4.5 Concluding Remarks

The work presented in this dissertation illuminates the importance of two mechanisms of PAR4 signal regulation. We show that disrupting PAR4 internalization results in prolonged ERK1/2 activation, which is mediated by coupling to $G\alpha_{12/13}$. Because G proteins are known to be membrane-anchored, we propose that PAR4 endocytosis attenuates ERK1/2 signaling by spatially separating the receptor from G proteins. It is unclear whether this mechanism of desensitization is unique to PAR4 or if it is also used by other GPCRs.

We also demonstrate the importance of physical association with P2Y12 in coordinating G protein-independent β-arrestin-mediated Akt activation. We

show that PAR4, P2Y12, and β-arrestin colocalize at internal puncta in response to PAR4 activation. We posit that characteristics of each receptor protomer are important for the unique activity of the PAR4-P2Y12 heterodimer. Unlike PAR4, activated P2Y12 is phosphorylated and associates with β-arrestin (28). However, the interaction of β-arrestin and P2Y12 is transient, as the receptor is rapidly recycled back the cell surface following internalization (29-31). We propose that co-trafficking with PAR4 allows P2Y12 to bypass its normal recycling pathway and remain associated with β-arrestin. We further postulate that colocalization of PAR4, P2Y12, and β-arrestin at early endosomes positions the complex in close proximity to signaling effectors that facilitate β-arrestin-mediated Akt activation. This model is supported by our finding that disruption of PAR4 internalization diminished Akt activation. Taken together, the studies summarized in this dissertation highlight the importance of PAR4 internalization and heterodimerization in modulating activity of functionally and spatially distinct signaling pathways.

4.6 References

1. Xu, W. F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W., and Foster, D. C. (1998) Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6642-6646

2. Lindahl, T. L., Macwan, A. S., and Ramstrom, S. (2016) Protease-activated receptor 4 is more important than protease-activated receptor 1 for the thrombin-induced procoagulant effect on platelets. *Journal of thrombosis and haemostasis : JTH* **14**, 1639-1641

3. French, S. L., Arthur, J. F., Tran, H. A., and Hamilton, J. R. (2015) Approval of the first protease-activated receptor antagonist: Rationale, development, significance, and considerations of a novel anti-platelet agent. *Blood Rev* **29**, 179-189

4. Morrow, D. A., Braunwald, E., Bonaca, M. P., Ameriso, S. F., Dalby, A. J., Fish, M. P., Fox, K. A., Lipka, L. J., Liu, X., Nicolau, J. C., Ophuis, A. J., Paolasso, E., Scirica, B. M., Spinar, J., Theroux, P., Wiviott, S. D., Strony, J., Murphy, S. A., Committee, T. P. T. S., and Investigators. (2012) Vorapaxar in the secondary prevention of atherothrombotic events. *The New England journal of medicine* **366**, 1404-1413

5. Edelstein, L. C., Simon, L. M., Montoya, R. T., Holinstat, M., Chen, E. S., Bergeron, A., Kong, X., Nagalla, S., Mohandas, N., Cohen, D. E., Dong, J. F., Shaw, C., and Bray, P. F. (2013) Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nature medicine* **19**, 1609-1616

6. Edelstein, L. C., Simon, L. M., Lindsay, C. R., Kong, X., Teruel-Montoya, R., Tourdot, B. E., Chen, E. S., Ma, L., Coughlin, S., Nieman, M., Holinstat, M., Shaw, C. A., and Bray, P. F. (2014) Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood* **124**, 3450-3458

7. Zhang, Y., Yang, R., Burwinkel, B., Breitling, L. P., Holleczek, B., Schottker, B., and Brenner, H. (2014) F2RL3 methylation in blood DNA is a strong predictor of mortality. *Int J Epidemiol* **43**, 1215-1225

8. Zhang, Y., Schottker, B., Ordonez-Mena, J., Holleczek, B., Yang, R., Burwinkel, B., Butterbach, K., and Brenner, H. (2015) F2RL3 methylation, lung cancer incidence and mortality. *Int J Cancer* **137**, 1739-1748

9. Hossain, M. B., Li, H., Hedmer, M., Tinnerberg, H., Albin, M., and Broberg, K. (2015) Exposure to welding fumes is associated with hypomethylation of the F2RL3 gene: a cardiovascular disease marker. *Occup Environ Med* **72**, 845-851

10. Breitling, L. P., Salzmann, K., Rothenbacher, D., Burwinkel, B., and Brenner, H. (2012) Smoking, F2RL3 methylation, and prognosis in stable coronary heart disease. *Eur Heart J* **33**, 2841-2848

11. Zhang, Y., Yang, R., Burwinkel, B., Breitling, L. P., and Brenner, H. (2014) F2RL3 methylation as a biomarker of current and lifetime smoking exposures. *Environ Health Perspect* **122**, 131-137

12. Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G., and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *The Journal of biological chemistry* **271**, 22003-22016

13. Luttrell, L. M., and Lefkowitz, R. J. (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of cell science* **115**, 455-465

14. Shenoy, S. K., and Lefkowitz, R. J. (2011) beta-Arrestin-mediated receptor trafficking and signal transduction. *Trends in pharmacological sciences* **32**, 521-533

15. Kang, D. S., Tian, X., and Benovic, J. L. (2013) beta-Arrestins and G protein-coupled receptor trafficking. *Methods in enzymology* **521**, 91-108

16. Ribas, C., Penela, P., Murga, C., Salcedo, A., Garcia-Hoz, C., Jurado-Pueyo, M., Aymerich, I., and Mayor, F., Jr. (2007) The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochimica et biophysica acta* **1768**, 913-922

17. Marrari, Y., Crouthamel, M., Irannejad, R., and Wedegaertner, P. B. (2007) Assembly and trafficking of heterotrimeric G proteins. *Biochemistry* **46**, 7665-7677

18. Khan, A., Li, D., Ibrahim, S., Smyth, E., and Woulfe, D. S. (2014) The Physical Association of the P2Y12 Receptor with PAR4 Regulates Arrestin-mediated Akt Activation. *Molecular pharmacology*

19. Salahpour, A., Angers, S., Mercier, J. F., Lagace, M., Marullo, S., and Bouvier, M. (2004) Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting. *The Journal of biological chemistry* **279**, 33390-33397

20. Zhu, X., and Wess, J. (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37**, 15773-15784

21. Koschatzky, S., Tschammer, N., and Gmeiner, P. (2011) Cross-receptor interactions between dopamine D2L and neurotensin NTS1 receptors modulate binding affinities of dopaminergics. *ACS Chem Neurosci* **2**, 308-316

22. Koschatzky, S., and Gmeiner, P. (2012) Selective agonists for dopamine/neurotensin receptor heterodimers. *ChemMedChem* **7**, 509-514

23. Li, D., D'Angelo, L., Chavez, M., and Woulfe, D. S. (2011) Arrestin-2 differentially regulates PAR4 and ADP receptor signaling in platelets. *The Journal of biological chemistry* **286**, 3805-3814

24. Chalothorn, D., McCune, D. F., Edelmann, S. E., Garcia-Cazarin, M. L., Tsujimoto, G., and Piascik, M. T. (2002) Differences in the cellular localization and agonist-mediated internalization properties of the alpha(1)-adrenoceptor subtypes. *Molecular pharmacology* **61**, 1008-1016

25. Uberti, M. A., Hall, R. A., and Minneman, K. P. (2003) Subtype-specific dimerization of alpha 1-adrenoceptors: effects on receptor expression and pharmacological properties. *Molecular pharmacology* **64**, 1379-1390

26. He, S. Q., Zhang, Z. N., Guan, J. S., Liu, H. R., Zhao, B., Wang, H. B., Li, Q., Yang, H., Luo, J., Li, Z. Y., Wang, Q., Lu, Y. J., Bao, L., and Zhang, X. (2011) Facilitation of mu-opioid receptor activity by preventing delta-opioid receptor-mediated codegradation. *Neuron* **69**, 120-131

27. Hardy, A. R., Conley, P. B., Luo, J., Benovic, J. L., Poole, A. W., and Mundell, S. J. (2005) P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**, 3552-3560

28. Nisar, S. P., Cunningham, M., Saxena, K., Pope, R. J., Kelly, E., and Mundell, S. J. (2012) Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalization. *The Journal of biological chemistry* **287**, 24505-24515

29. Nisar, S., Daly, M. E., Federici, A. B., Artoni, A., Mumford, A. D., Watson, S. P., and Mundell, S. J. (2011) An intact PDZ motif is essential for correct P2Y12 purinoceptor traffic in human platelets. *Blood* **118**, 5641-5651

30. Cunningham, M. R., Nisar, S. P., and Mundell, S. J. (2013) Molecular mechanisms of platelet P2Y(12) receptor regulation. *Biochemical Society transactions* **41**, 225-230

31. Cunningham, M. R., Nisar, S. P., Cooke, A. E., Emery, E. D., and Mundell, S. J. (2013) Differential endosomal sorting of a novel P2Y12 purinoreceptor mutant. *Traffic* **14**, 585-598