UNIVERSITY OF CALIFORNIA SAN DIEGO

The alpha-arrestin ARRDC3 functions as a metastasis suppressor by regulating GPCR trafficking and differential signaling to the Hippo pathway in breast cancer

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by

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The dissertation of Aleena Kehaulani Sachiko Arakaki is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

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DEDICATION

I would like to dedicate this work to all of my ancestors before me. I feel honored to represent my entire family well by being the first to obtain a PhD. I have only gotten this far with the love and support of my entire family, particularly from my dad Daniel, cousin Courtney, Uncle Randy and Aunty Darla, Uncle Henry and Aunty Christie, and Uncle Peter. Thank you for always being there for me.

I dedicate my work to the memories of my mother, Takane Louise Arakaki, my grandmothers, Beulah May Arakaki and Fumiko Bohannan, and my aunty Beth-Ann U'ilani Arakaki. My mom and grandmas, who I have lost to cancer, have truly been my driving force in continuing in my education and in making it my life goal to help those affected by this disease. Without them, I wouldn't be the person I am today, and I hope that I have made them proud.

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LIST OF ABBREVIATIONS

A	alanine
ALIX	ALG-interacting protein X
ANKRD1	ankyrin repeat domain-containing protein 1
ARRDC3	arrestin domain containing protein 3
β2AR	β2-adrenergic receptor
β-TrCP	β-transducin repeats-containing protein
BCSC	breast cancer stem-like cell
BLBC	basal-like breast carcinoma
CTGF	connective tissue growth factor
DOX	doxycycline
ELISA	enzyme linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
ESCRT	endosomal-sorting complex required for transport
FBS	fetal bovine serum
GEPIA	Gene Expression Profiling Interactive Analysis
GPCR	G protein-coupled receptor
GTEx	Genotype-Tissue Expression
HA	hemagglutinin
ILV	intraluminal vesicles
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
LATS1/2	large tumor suppressor kinase 1/2
LPAR	lysophosphatidic acid receptor
MMP-1	matrix metalloproteinase-1

MOB1	MOB kinase activator 1A
MST1/2	mammalian sterile 20-like kinase
MVB	multivesicular body
P	proline
PAR	protease-activated receptor
pSLIK	single lentivector for inducible knockdown
RhoA	Ras homolog gene family, member A
SAV1	Salvador family WW domain containing protein 1
S1PR	shingosine-1-phosphate receptor
TAZ	transcriptional coactivator with PDZ-binding motif
TCGA	The Cancer Genome Atlas
TEAD	transcription factor TEA domain family proteins
α-Th	thrombin
TNBC	triple negative breast cancer
WWP2	WW domain-containing protein 2
Y	tyrosine
YAP	Yes-associated protein
Yki	Yorkie

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ABSTRACT OF THE DISSERTATION

The alpha-arrestin ARRDC3 functions as a metastasis suppressor by regulating GPCR trafficking and differential signaling to the Hippo pathway in breast cancer

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2020

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G protein-coupled receptor (GPCR) signaling regulates cancer cell proliferation, invasion, migration and survival at metastatic sites. However, despite the success and promise of GPCRs as therapeutic targets, there are currently no FDA-approved drugs targeting GPCRs for cancer. Protease-activated receptor-1 (PAR1) is a GPCR that promotes breast cancer progression. PAR1 is overexpressed in breast cancer patient tissue biopsies and in breast carcinoma cell lines, and correlates with increased rates of metastasis and poor prognosis and increased invasion and metastatic potential, respectively. One mechanism that leads to PAR1 aberrant over-expression is defective lysosomal trafficking and degradation of the receptor, leading to persistent G protein signaling. Our lab recently showed that arrestin domain containing protein-3 (ARRDC3), an adaptor protein for E3 ubiquitin ligases, functions in regulating proper lysosomal trafficking and degradation. ARRDC3 has been identified as a

tumor suppressor in aggressive breast cancer and I further examined the role of ARRDC3 in PAR1 trafficking in invasive breast carcinoma cells. The Hippo pathway, which converges on the transcriptional co-activators YAP and TAZ, is a well-established mediator of tumorigenesis and cancer progression and is also activated by GPCR stimulation, including through PAR1, in breast carcinoma. However, the mechanisms by which ARRDC3 regulates GPCR-stimulated Hippo signaling to promote breast cancer metastasis remains unknown. In the work described in this thesis, I discovered that ARRDC3 displays a multifunctional role in suppressing breast cancer growth and invasion: 1) by controlling proper PAR1 trafficking and degradation, thus inhibiting persistent G protein signaling and 2) by interacting with TAZ, thus sequestering TAZ in the cytoplasm and blocking downstream Hippo pathway gene transcription to occur. I used a tetracycline-inducible pSLIK lentiviral vector to restore expression of ARRDC3 in highly invasive, basal-like MDA-MB-231 cells, which exhibit high PAR1 and low ARRDC3 expression. Re-expression of ARRDC3 restored agonist-induced PAR1 degradation, attenuated JNK and Hippo-YAP signaling, and further inhibited PAR1-mediated breast carcinoma cell invasion. Thus, the dysregulation of PAR1 trafficking due to loss of ARRDC3 expression leads to persistent JNK signaling and promotes breast cancer invasion. ARRDC3 re-expression in invasive breast carcinoma cells also attenuates GPCR-stimulated Hippo signaling and invasion that is mediated by activation of TAZ but not YAP. Furthermore, siRNA-targeted depletion of TAZ, but not YAP, inhibits GPCR-induced Hippo signaling and invasion. Our studies suggest a crucial role for ARRDC3 and TAZ in GPCR-Hippo pathway signaling in breast carcinoma invasion and metastasis. An understanding of the mechanisms by which the Hippo pathway is regulated by GPCRs may lead to new potential therapeutic targets for the treatment or prevention of metastatic breast cancer.

INTRODUCTION

G protein-coupled receptors (GPCRs) are a large and diverse family of signaling receptors that function in cancer growth and development by regulating cellular proliferation, invasion, migration, immune cell-mediated functions, angiogenesis and survival at metastatic sites [1-3]. In addition, GPCRs are known to function in metastasis [2,3], and represent a potential drug target in an area with limited targeted treatment options for patients. GPCRs are cell surface receptors with highly druggable sites and the largest class of drug targets, with over 30% of current FDA-approved drugs targeting GPCRs [4,5]. However, despite the success and promise of GPCRs as therapeutic targets, there are currently only eight drugs in the clinic used for the treatment of cancer that specifically target GPCRs [6]. It is now well recognized that GPCR activity can be altered in cancer through aberrant overexpression, gain-of-function activating mutations, mutations in downstream G protein signaling effectors, and increased production and secretion of GPCR activating ligands by both tumor cells and surrounding stromal cells [7-10]. Given the broad and diverse functions of GPCRs in cancer, understanding the mechanisms that lead to aberrant GPCR expression and function in tumor progression is important for the development of new effective treatment strategies for metastatic cancer.

Several GPCRs have been implicated in metastatic cancer, including the unique family of protease-activated receptors (PARs). PARs transmit signals to extracellular proteases and respond to coagulant serine proteases such as thrombin. There are four PARs encoded in the mammalian genome. PAR1, the prototype for this family, transmits cellular responses to thrombin, the main effector protease of the coagulation cascade. PARs also respond to proteases released by epithelial cells and various cells in the tumor microenvironment. In fact, PAR1 senses and responds to multiple proteases generated in the tumor microenvironment including thrombin, plasmin and matrix metalloproteinase-1 (MMP-1) [11-13]. The zinc-dependent MMP-1 has also been reported to promote tumor growth and invasion through activation of PAR1 [12], providing an important link between tumor-generated metalloproteases

and PAR signaling. PARs can promote tumor growth, invasion and metastasis but precisely how PARs contribute to cancer progression has yet to be fully elucidated.

The proteolytic nature of PAR activation, which results in irreversible activation, is distinct from most GPCRs. PAR1 is activated by irreversible proteolytic cleavage of the N-terminus, revealing a new N-terminal domain that acts as a tethered ligand that binds intramolecularly to the receptor to elicit transmembrane signaling [14,15]. Once activated, PAR1 signals to distinct heterotrimeric G protein subtypes including G_q, G_i and $G_{12/13}$ and triggers RhoGEF-mediated RhoA signaling, increases in intracellular Ca^{2+}, MAP kinase activation and signaling by multiple other effectors [16,17]. PAR1, along with other GPCRs, signals through G proteins to activate the Hippo pathway [18-20]. The Hippo pathway is commonly dysregulated in many cancers and plays an important role in tumorigenesis, metastasis and drug resistance, but has yet to be targeted as an FDA-approved cancer therapeutic [6,21]. $G\alpha_{12/13}$-, $G\alpha_i$-, and $G\alpha_{q/11}$-coupled receptors inhibit the Hippo pathway component LATS1/2 kinases [20]. LATS kinases phosphorylate and inhibit nuclear translocation of the transcriptional coactivators YAP and TAZ, leading to cytoplasmic retention and degradation [22]. By inhibiting LATS1/2, GPCRs facilitate nuclear translocation of YAP and TAZ, which bind to the TEAD family of transcription factors to induce expression of downstream effectors such as connective tissue growth factor (CTGF) and ankyrin repeat domain-1 (ANKRD1) to promote cell growth and invasion. Though we know RhoA activation and F-actin polymerization are required for GPCR activation of the Hippo pathway by inhibiting LATS1/2, further regulation of GPCR signaling to the Hippo pathway remains unknown [18].

Dysregulation of these signaling events triggered by GPCRs may then lead to increased tumorigenesis, invasion and metastasis. However, the defects that engender PAR1 and other GPCRs the capacity to promote cancer invasion and metastasis are not known. Signaling by PARs is directly linked to expression at the cell surface and is controlled by gene transcription as well as internalization, recycling and lysosomal degradation. Our group showed that arrestin-

domain containing protein 3 (ARRDC3), an arrestin-family member adaptor protein, regulates PAR1 lysosomal trafficking [23]. Notably, ARRDC3 was identified as a tumor suppressor in metastatic breast cancer, with low ARRDC3 expression correlating with metastasis, tumor recurrence and poor patient prognosis [24].

The work presented in this doctoral thesis focused on understanding the function of the recently discovered tumor suppressor ARRDC3 in regulating PAR1 trafficking and GPCR signaling to the Hippo pathway in the context of breast carcinoma. This work provides mechanistic insight into the multifunctional role of ARRDC3 in regulating GPCR-Hippo pathway signaling in breast carcinoma invasion and metastasis. In doing this work, I also discovered differential roles of the Hippo pathway effectors YAP and TAZ, supporting an increasing body of work suggesting TAZ plays a critical role in breast cancer metastasis and represents a promising therapeutic target for the prevention or treatment of metastatic breast cancer

0.1 Protease-activated receptor expression in human cancer

The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects have yielded RNA-Seq data for tens of thousands of cancer and non-cancer patient samples. These large data sets have provided a unique opportunity to survey expression of PARs in human cancer versus normal patient samples. Using Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) [25], we found that PAR1 and PAR3 are most often upregulated in similar human cancer types including pancreatic adenocarcinoma, esophageal carcinoma, stomach adenocarcinoma, breast invasive carcinoma, head and neck squamous carcinoma and kidney renal clear cell carcinoma, see Figure 0.1 and Table 0.1. Interestingly, PAR1 and PAR3 can form heterodimers and PAR3 has been shown to modulate the activity of PAR1 by potentiating its signaling to thrombin [26]. In addition, PAR1-PAR3 heterodimer preferentially interacts with $G\alpha_{13}$ more than monomeric PAR1 [26], and $G\alpha_{13}$ signaling is known to be important for progression of certain types of cancers [27]. Interestingly,

PAR1 and PAR2 also have a high incidence of co-expression either together with other PARs as observed in pancreatic adenocarcinoma, esophageal carcinoma, stomach adenocarcinoma, and kidney renal clear cell carcinoma, see Figure 0.1 and Table 0.1. However, in certain cancer types such as colon adenocarcinoma, glioblastoma multiforme, ovarian serous cystadenocarcinoma and rectum adenocarcinoma PAR1 and PAR2 are the only PARs expressed (Figure 0.2 and Table 0.1). Unlike other GPCR heterodimer formation, there is substantial evidence that both endogenous and exogenous PAR1 and PAR2 form a functional heterodimer [28]. There is also substantial evidence to suggest that the PAR1 tethered ligand can bind intermolecularly to transactivate PAR2 in COS7 cells and endothelial cells [29]. Thrombin-induced melanoma cell motility and metastasis also appears to require PAR1 transactivation of PAR2 [30], suggesting that PAR1-PAR2 may function together in multiple cancer types to promote tumor progression.

PAR1 has also been established as a driver of metastasis as well as a promising therapeutic target in breast cancer through *in vitro* and *in vivo* studies [17]. PAR1 is overexpressed in breast cancer patient biopsies, and high PAR1 expression correlates with metastasis and poor prognosis [31-33]. PAR1 is also selectively overexpressed in highly invasive basal-like breast carcinoma cell lines, but not in non-invasive luminal breast carcinoma cells [31,34]. Depletion of PAR1 with shRNA or blocking PAR1 with cell penetrating peptide pepducin in invasive breast cancer cells inhibits invasion *in vitro* and tumor growth and metastasis *in vivo* [35-37]. Additionally, ectopic overexpression of PAR1 in non-invasive MCF7 breast carcinoma cells lacking endogenous PAR1 is sufficient to drive invasion *in vitro* and tumor growth *in vivo* [12], suggesting that PAR1 expression is both necessary and sufficient to promote breast cancer progression.

0.2 Endocytic trafficking and lysosomal sorting of PARs and implications in cancer

In addition to gene transcription, trafficking of PARs is critical for maintaining an appropriate amount of receptor at the cell surface. Once internalized, agonist activated GPCRs are sorted at endosomal membranes by adaptor proteins and are either recycled back to the cell surface or targeted to lysosomes for degradation. Intracellular trafficking of GPCRs has important roles in signal termination, signal propagation from internal compartments and resensitization. Many GPCRs require posttranslational modification with ubiquitin and interaction with ubiquitin-binding domains of the endosomal-sorting complex required for transport (ESCRT) machinery for lysosomal sorting. However, not all GPCRs including PARs require direct ubiquitination or all components of the ESCRT machinery for degradation in the lysosome, suggesting that alternate sorting pathways exist.

Endocytic trafficking of GPCRs is important for controlling the spatial and temporal dynamics of signaling, and this is particularly relevant for PARs. PAR1 displays two modes of endocytosis; constitutive and agonist-induced internalization and both are critical for controlling the fidelity of signaling. Uncleaved, unactivated PAR1 is constitutively internalized from the cell surface to early endosomes and then recycled back to the cell surface (Figure 0.3) [38]. PAR1 constitutive internalization serves to generate an intracellular pool of uncleaved receptor that can replenish the cell surface with naïve PAR1 to allow for rapid resensitization to thrombin stimulation independent of *de novo* protein synthesis [39-41].

Due to the irreversible proteolytic cleavage of PARs, which results in the generation of a tethered ligand that cannot diffuse way (Figure 0.3), signaling by protease-activated receptors is tightly regulated. Once activated, PARs are internalized from the plasma membrane and sorted to lysosomes for degradation, a process critical for ultimately terminating signaling [42,43]. However, studies indicate that perturbation of the endocytic trafficking machinery in cancer results in slowed PAR degradation and/or recycling of activated receptors back to the cell surface that signal persistently [34-36].

Early studies of GPCR lysosomal sorting revealed a classic role for ubiquitination and canonical ESCRTs [44]. However, not all GPCRs require direct ubiquitination and canonical ESCRTs for lysosomal sorting [45]. We showed that activation of a ubiquitin-deficient PAR1 mutant sorted directly to intraluminal vesicles (ILVs) of multivesicular bodies (MVBs)/lysosomes and degraded similar to wildtype receptor (Figure 0.3) [46,47], and raised the question of how a GPCR can be targeted to lysosomes for degradation independent of ubiquitination.

Similar to cargo lysosomal sorting through the canonical ESCRT pathway, PAR1 is sorted to lysosomes through an atypical pathway that requires sequential interactions with distinct endocytic adaptor proteins. After sorting by AP-3 and SNX1, activated PAR1 directly interacts with the adaptor protein ALG-2-interacting protein X (ALIX) [47,48]. In fact, AP-3 is required for facilitating PAR1 interaction with ALIX, as knockdown of AP-3 and a PAR1 tyrosine-based motif mutant with impaired AP-3 binding fails to bind to ALIX following agonist stimulation [48]. These findings indicate that PAR1 is targeted to a distinct lysosomal pathway mediated by AP-3 (Figure 0.3). ALIX expression is also essential for agonist-induced PAR1 lysosomal degradation [47]. PAR1 contains a highly conserved YPX_3L motif localized within intracellular loop 2 that directly interacts with the central V domain of ALIX (Figure 0.3) [47]. Besides PAR1, seven other mammalian GPCRs were found to contain conserved YPX_nL motifs within their second intracellular loop, including the adrenoreceptor $\alpha 1_B$, angiotensin receptor AT2, galanin receptor GAL_2, histamine receptor H_2, neuropeptide FF receptor NPFF2, neuropeptide S receptor NPS, and purinergic receptor $P2Y_1$ [47]. Of this subset of GPCRs, only the $P2Y_1$ receptor has been studied and shown to use a ubiquitin-independent and ALIX-dependent lysosomal sorting pathway like PAR1 [49], suggesting that this pathway is broadly applicable to multiple GPCRs.

While it is known that intracellular trafficking of cell surface receptors is important for regulating the magnitude, duration and spatial aspects of cell signaling, emerging studies also suggest that signaling by the receptors themselves function in a reciprocal manner to modulate

the endocytic machinery [50]. Consistent with this idea, we found that ALIX activity is regulated through agonist-activated PAR1 stimulated signaling that leads to WWP2-mediated ubiquitination of ALIX, dimerization and enhanced activity at sorting PAR1 to MVBs/lysosomes (Figure 0.3) [23]. Importantly, ARRDC3 is responsible for recruitment of the WWP2 HECT-domain containing E3 ubiquitin ligase to ALIX and subsequent ubiquitination [23]. ARRDC3 is a member of the mammalian α-arrestin family that shares similar domain homology with mammalian β-arrestins, which have important and diverse roles in GPCR trafficking [51]. However, unlike β-arrestins, ARRDC3 lacks a polar core, essential for β-arrestin binding to activated and phosphorylated GPCRs and additionally contains a C-terminal PPxY motif that binds to WW domains of HECT-domain containing E3 ubiquitin ligases [52-54]. These findings suggest that β-arrestins and α-arrestins likely serve distinct functions. We showed that ARRDC3 co-associates and colocalizes with activated PAR1 [23]. In addition, ARRDC3 expression is required for agonist-induced PAR1 interaction with ALIX and lysosomal degradation [23]. Together, these studies provide substantial evidence for the existence of an atypical ALIX and ARRDC3-dependent lysosomal sorting pathway for a subset of mammalian GPCRs.

0.3 Role of ARRDC3 in breast cancer

ARRDC3 has been identified as a tumor suppressor in breast and prostate cancer [24,55-57] (Table 0.2). ARRDC3 expression is low or absent in the highly aggressive basal-like breast cancer [24], and associated with tumor grade, metastasis and recurrence. Moreover, ARRDC3 localizes to a gene cluster on chromosome 5 deleted in 17% of basal-like breast cancers compared to 0% deletion in luminal breast cancers [24,55]. ARRDC3 expression has also been shown to be suppressed through epigenetic silencing and small non-coding micro-RNAs [56,58,59]. Additionally, ARRDC3 overexpression in TNBC cells reverses epithelial-to-

mesenchymal transition (EMT) and chemo-resistance [60]. However, little is known on how ARRDC3 impacts cell signaling in breast carcinoma.

In invasive breast cancer, a target of ARRDC3 is the integral membrane protein integrin β4 [55,61], which is enriched in triple-negative breast cancer and a marker of poor prognosis [55]. In more recent work, we found that loss of ARRDC3 expression is also responsible for defective PAR1 trafficking in invasive breast cancer [34], suggesting that ARRDC3 tumor suppressor function is linked to both integrin β4 and GPCR trafficking. We previously showed that invasive breast carcinoma cells exhibit dysregulated PAR1 lysosomal degradation and persistent signaling, which promotes cellular invasion and tumor growth [35,36]. Since ARRDC3 expression is either lost or suppressed in invasive breast cancer, we employed a lentiviral induction system to restore ARRDC3 expression in MDA-MB-231 cells. Strikingly, we found that re-expression of ARRDC3 is sufficient to restore normal activated PAR1 lysosomal sorting [34]. In contrast to ARRDC3, ALIX expression in invasive *versus* non-invasive breast cancer is variable and is consistent with human cancers that exhibit both upregulated and downregulated ALIX expression (Table 0.2). In human cancers, ARRDC3 expression is also suppressed in breast, kidney, ovarian and pheochromocytoma, while other cancers clearly show increased expression. The effect of increased ARRDC3 expression in these specific cancer types is not known. Similar to studies in HeLa and endothelial cells, ALIX was shown to be required for ARRDC3-mediated degradation of activated PAR1 in invasive breast carcinoma (Figure 0.3) [34]. We showed previously that defective PAR1 lysosomal trafficking results in recycling of "activated" receptor back to the cell surface and persistent signaling and increases cellular invasion and tumor growth [35,36]. As expected, ARRDC3 re-expression attenuated persistent signaling by activated PAR1 as well as PAR1-mediated cellular invasion [34]. This study is the first to identify an important role of the ARRDC3 endocytic adaptor protein that functions as a tumor suppressor by regulating GPCR trafficking and signaling in invasive breast cancer.

0.4 Signaling to the Hippo pathway by GPCRs

The Hippo pathway was first discovered in *Drosophila* and named as such due to the role of the Hpo kinase in regulating organ size as well as cell proliferation and growth (ref). In mammals, the canonical Hippo pathway is composed of a kinase cascade, with mammalian sterile 20-like kinases (MST1/2) phosphorylating and activating the large tumor suppressor kinases (LATS1/2) which in turns phosphorylate Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). When phosphorylated, YAP and TAZ are retained in the cytoplasm through interaction with 14-3-3 protein and targeted for protein degradation. This occurs in the case of nutrient deprivation and high cell density, to inhibit cell growth. During development, low cell density, presence of growth factors and activation of other extracellular signals, the core kinase is inhibited, allowing YAP and TAZ to enter the nucleus and bind to the transcription factor TEA domain (TEAD) family proteins, leading to active gene transcription of growth-promoting factors such as connective tissue growth factor (CTGF) and ankyrin repeat domain-containing protein 1 (ANKRD1). The Hippo pathway is commonly dysregulated in many cancers and plays an important role in tumorigenesis, metastasis and drug resistance, but has yet to be targeted as an FDA-approved cancer therapeutic [21,62]. Interestingly, the core components of the Hippo pathway are rarely mutated in cancer but instead the pathway becomes dysregulated through upstream signals [63].

PAR1, along with other GPCRs such as the lysophosphatidic acid receptors (LPARs), sphingosine-1-phosphate receptors (S1PRs), and protease activated-receptor 2 (PAR2), signals through G proteins and RhoA to activate the Hippo pathway, resulting in increased cell migration and invasion in breast cancer cells [18-20]. Activation of GPCRs leads to de-phosphorylation of YAP and TAZ, facilitating nuclear translocation of YAP and TAZ, which act as transcriptional co-activators by binding to the TEAD family of transcription factors to induce expression of downstream effectors such as connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (Cyr61), ankyrin repeat domain-containing protein 1 (ANKRD1) and

amphiregulin (AREG) (Figure 0.5) [18]. Additionally, PAR1-mediated Hippo pathway activation in breast and gastric carcinoma promotes invasion, EMT, chemoresistance and cancer stem-like cell properties [64,65].

Studies in *Drosophila* suggest that Leash, the homolog of ARRDC3, can interact with Yorkie (Yki), the homolog of YAP/TAZ, resulting in Yki degradation and decreased Yki activity and proliferation [66,67]. It has also been demonstrated that ARRDC3 interacts with YAP, promoting its degradation, in renal cell carcinoma as well as colorectal cancer [68,69]. This is thought to occur by ARRDC3 binding directly to YAP via an interaction between the ARRDC3 C-terminal PPxY motifs and WW domains present on YAP [52,70]. TAZ also contains a WW domain that may facilitate interaction with ARRDC3, but has yet to be shown.

0.5 Rationale and significance

Here I reviewed a broad work of literature highlighting the significance of PAR1, ARRDC3 and the Hippo pathway and their interplay in breast cancer progression and metastasis. Despite the fact that GPCRs are major drug targets and the urgent need for newer, better effective therapies for treatment of metastatic cancer and other aspects of cancer progression, it is remarkable that certain GPCRs have not advanced as potential therapeutic targets. PAR1 is an attractive potential therapeutic target in metastatic breast cancer and we worked to further understand the mechanisms that regulate PAR1 trafficking as well as regulate the signaling pathways that promote cancer cell invasion. This work sought to understand how ARRDC3 is able to inhibit tumor growth and invasion in relation to its function on GPCR trafficking and signaling to the Hippo pathway. During my doctoral training, I found that loss of ARRDC3 expression in highly invasive breast carcinoma is responsible for defective ALIX-dependent lysosomal sorting of PAR1. In addition, loss of ARRDC3 expression not only perturbs PAR1 trafficking, but also is responsible for persistent signaling that drives thrombin-stimulated breast carcinoma cellular invasion. These findings provide the first evidence that

ARRDC3 functions as a tumor suppressor by modulating GPCR trafficking and signaling in invasive breast cancer. Furthermore, ARRDC3 has a critical role as a metastasis suppressor through regulation of GPCR-mediated activation of the Hippo pathway. TAZ plays a major role in mediating GPCR signaling to this pathway and subsequent cellular migration and invasion. In addition to regulating PAR1 trafficking, ARRDC3 acts as a scaffold protein, sequestering TAZ in the cytoplasm. Importantly, I also demonstrate the role of ARRDC3 in inhibiting breast cancer metastasis *in vivo.* Together, this data implicates ARRDC3 as a crucial breast cancer metastasis suppressor with multifunctional roles in inhibiting tumor growth and invasion.

0.6 Acknowledgements

The introduction, in part, is an adaptation of the material that appears in: Arakaki AKS*, Pan WA*, Trejo J. GPCRs in Cancer: Protease-activated receptors, endocytic adaptors and signaling. *Int J Mol Sci*. 2018 June 27; 19 (7):1886. The dissertation author was the primary investigator and co-first author of this paper.

0.8 Tables and figures

Table 0.1: Protease-activated receptor (PAR) expression in human cancers. Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/), a newly developed online platform using RNA sequencing expression data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression projects (GTEx) was used to compare tumor versus normal samples in numerous cancer types. The table summarizes the types of cancers that exhibit either upregulation or downregulation of PARs, compared to normal tissue.

PARs	Cancers with upregulated PARs	Cancers with downregulated PARs
PAR1	Breast invasive carcinoma Colon adenocarcinoma Lymphoid neoplasm diffuse large B-cell lymphoma Esophageal carcinoma Glioblastoma multiforme Head and neck squamous cell carcinoma Kidney renal clear cell carcinoma Brain lower grade glioma Ovarian serous cystadenocarcinoma Pancreatic adenocarcinoma Rectum adenocarcinoma Stomach adenocarcinoma Thymoma	Kidney chromophobe Kidney renal papillary cell carcinoma β
PAR2	Cervical squamous cell carcinoma and endocervical adenocarcinoma Colon adenocarcinoma Esophageal carcinoma Glioblastoma multiforme Acute myeloid leukemia Lung adenocarcinoma Lung squamous cell carcinoma Ovarian serous cystadenocarcinoma Pancreatic adenocarcinoma Prostate adenocarcinoma Rectum adenocarcinoma Stomach adenocarcinoma Testicular germ cell tumors Uterine corpus endometrial carcinoma Uterine carcinosarcoma	Kidney chromophobe Skin cutaneous melanoma
PAR3	Breast invasive carcinoma Esophageal carcinoma Head and neck squamous cell carcinoma Pancreatic adenocarcinoma Stomach adenocarcinoma	
PAR4	Kidney renal clear cell carcinoma Pancreatic adenocarcinoma	Acute myeloid leukemia Lung adenocarcinoma Lung squamous cell carcinoma Testicular germ cell tumors Thyroid carcinoma

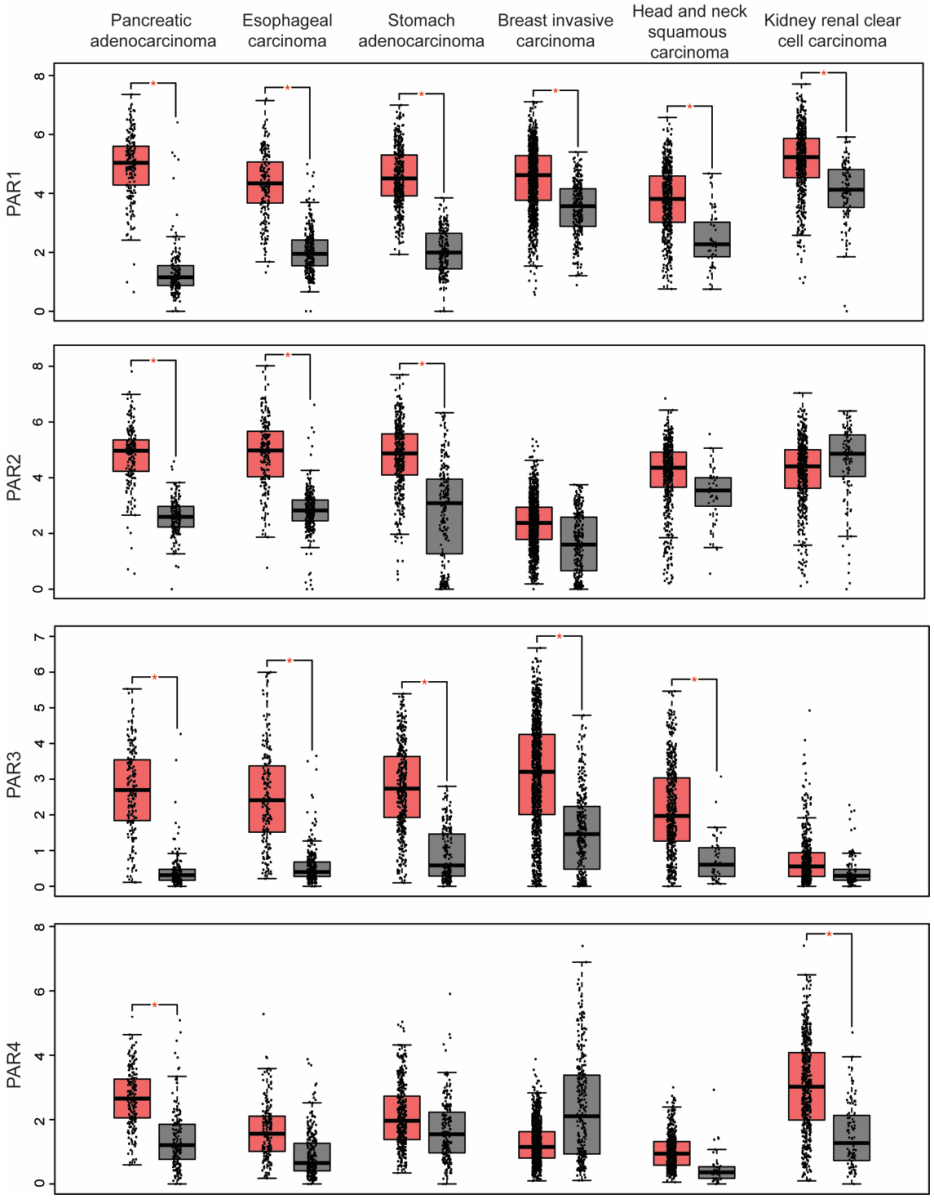

Figure 0.1: Co-expression of protease-activated receptors in human cancers. GEPIA analysis revealed that multiple PARs are significantly overexpressed in various cancer types. Pancreatic adenocarcinoma (179 tumor, 171 normal) overexpresses all PARs compared to normal tissue; esophageal carcinoma (182 tumor, 286 normal) and stomach adenocarcinoma (408 tumor, 211 normal) overexpress PAR1, PAR2, and PAR3, whereas breast invasive carcinoma (1085 tumor, 291 normal) and head and neck squamous carcinoma (519 tumor, 44 normal) overexpress PAR1 and PAR3 and kidney renal clear cell carcinoma (523 tumor, 100 normal) overexpress PAR1 and PAR4. The RNA-seq data are expressed as relative gene expression using transformed $\log_2$ (TPM+1) value (Y-axis) of tumor (red) and normal (grey) samples from different cancer types and displayed as a whisker plot. The whisker plot solid horizontal black line is the median, the box represents the upper and lower quartiles and the two lines (whiskers) outside the box extend to the highest and lowest observations of the sample population. The difference in PAR expression in tumors compared normal tissue control is significant based on one-way ANOVA *(*, $P < 0.01$)*. TPM, transcript per million.

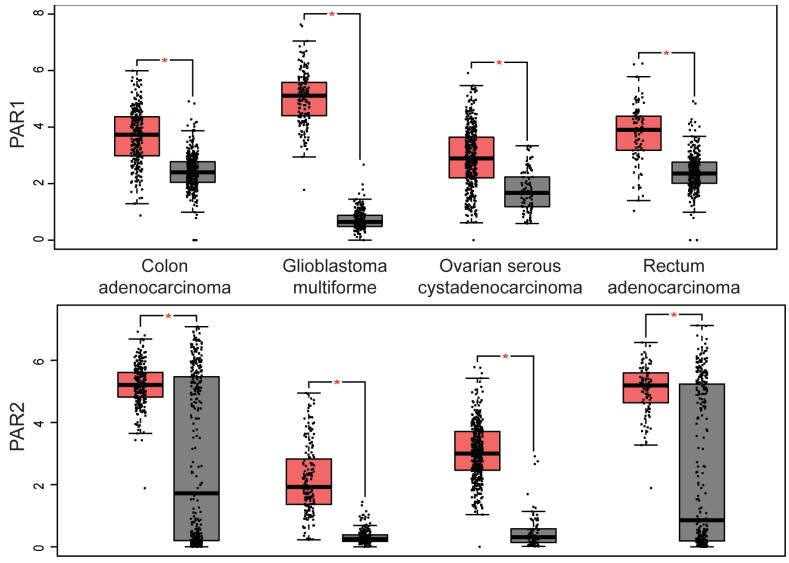

Figure 0.2: Human cancers with significant PAR1 and PAR2 overexpression. A survey of cancers using GEPIA analysis revealed several cancer types with only PAR1 and PAR2 overexpression compared to normal tissue, including colon adenocarcinoma (275 tumor, 349 normal), glioblastoma multiforme (163 tumor, 207 normal), ovarian serous cystadenocarcinoma (426 tumor, 88 normal), and rectum adenocarcinoma (92 tumor, 318 normal). The RNA-seq data are expressed as the relative gene expression using transformed $\log_2$ (TPM+1) value (Y-axis) of tumor (red) and normal (grey) in different cancer types and displayed as whisker plots as described in Figure 1. The data showed a significant difference in PAR1 and PAR2 expression in tumors compared to normal tissue using one-way ANOVA *(*, P < 0.01)*.

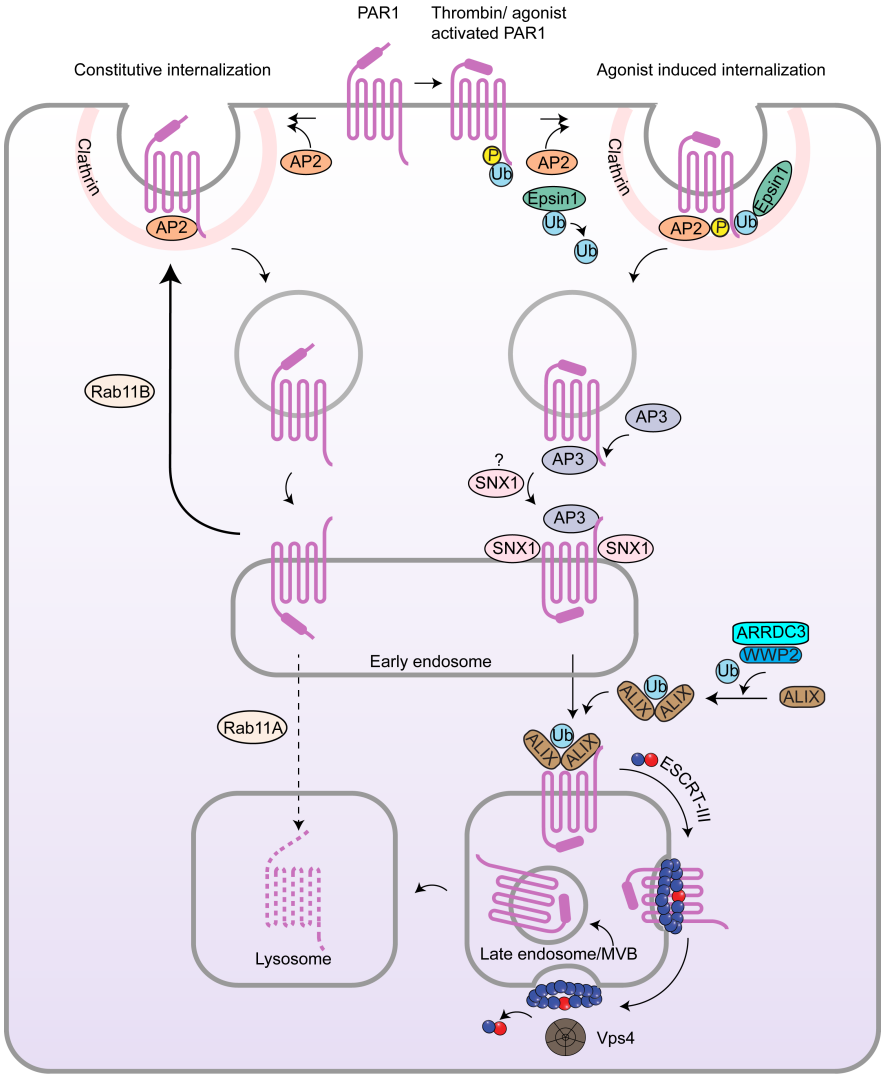

Figure 0.3: Endocytic trafficking of PAR1. PAR1 undergoes constitutive and agonist-activated internalization induced by thrombin cleavage of the N-terminus or by the peptide agonist SFLLRN. Unactivated PAR1 is constitutively internalized by AP-2 recognition of a distal tyrosine-based motif within the cytoplasmic C-terminus of PAR1. Internalized PAR1 is then sorted to early endosomes and then recycled back to the cell surface via a Rab11B-dependent pathway, whereas a small pool of receptor escapes recycling and is sorted by Rab11A to lysosomes and degraded. In contrast, agonist activation of PAR1 results in rapid phosphorylation and ubiquitination and internalization through a dynamin- and clathrin-dependent pathway mediated by AP-2 and epsin-1. AP-2 binds the phosphorylated distal C-terminus of activated PAR1 rather than the tyrosine-based motif to regulate activated PAR1 internalization. PAR1 activation also promotes epsin-1 deubiquitination, facilitating the ability of epsin-1 to bind activated PAR1 to facilitate internalization. Internalized PAR1 is then sorted sequentially at early endosomes by engaging AP-3 and SNX1 followed by ALIX, which requires ARRDC3 and WWP2-mediated ALIX ubiquitination and dimerization. ALIX, ARRDC3 and WWP2 are essential for targeting PAR1 to intraluminal vesicles of multivesicular bodies (MVBs)/late endosomes via ECSRT-III charged MVB protein 4 (CHMP4) and AAA-ATPase vacuolar protein sorting 4 (Vps4). Degradation of PAR1 in lysosomes is ultimately required for signal termination.

Table 0.2: Expression of endocytic adaptors associated with PAR trafficking in human cancers. The expression of the key endocytic adaptors implicated in PAR trafficking was analyzed using the GEPIA database tool. The table is a summary of the different types of cancers that display an upregulation or downregulation of various endocytic adaptors compared to normal tissue.

Adaptors	Cancers with upregulated adaptors	Cancers with downregulated adaptors
PDCD6IP (ALIX)	Glioblastoma multiforme Brain lower grade glioma Pancreatic adenocarcinoma	Adrenocortical carcinoma Uterine corpus endometrial carcinoma Uterine carcinosarcoma
ARRDC3	Glioblastoma multiforme Brain lower grade glioma Pancreatic adenocarcinoma	Breast invasive carcinoma Kidney chromophobe Ovarian serous cystadenocarcinoma Pheochromocytoma and paraganglioma

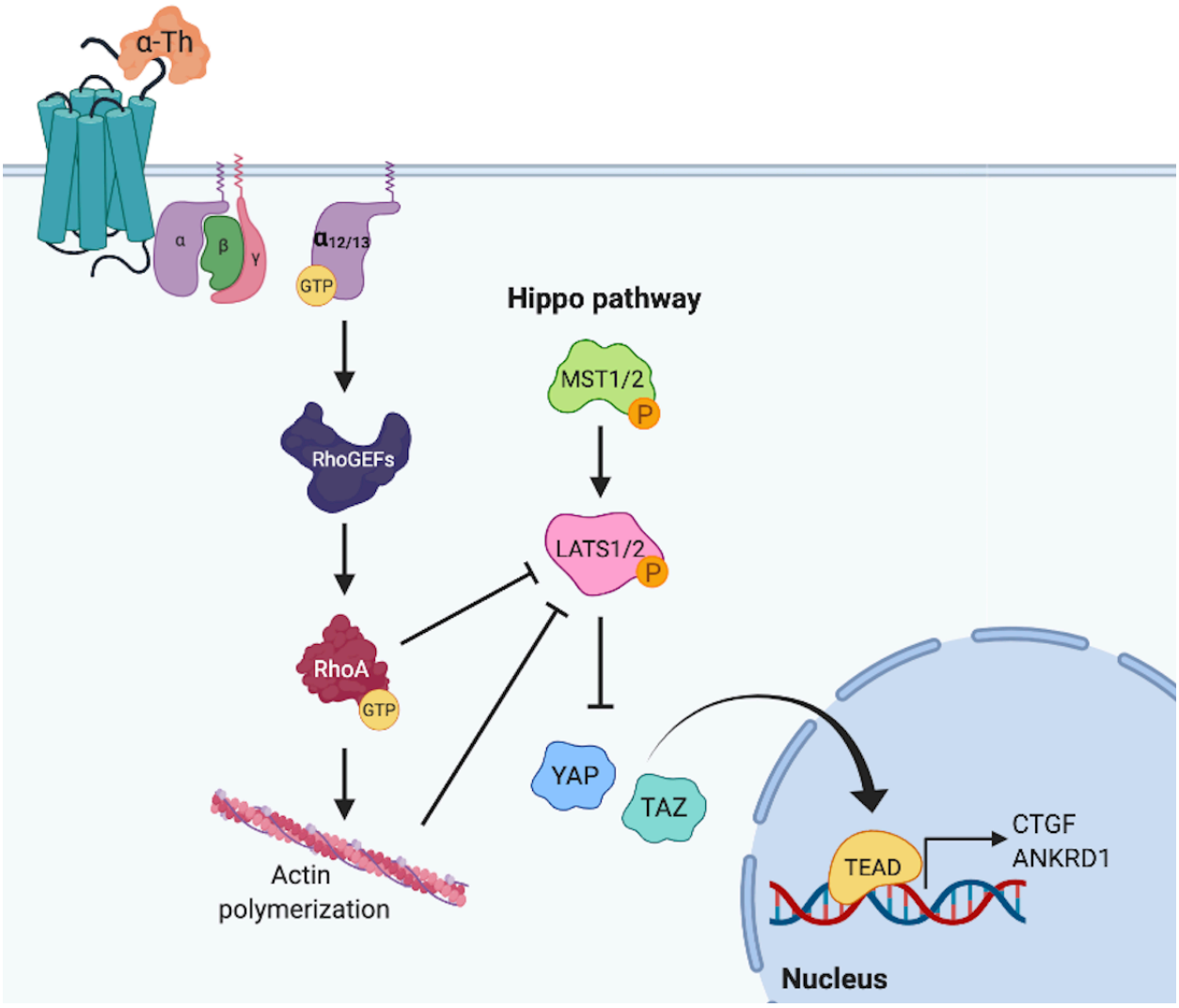

Figure 0.4: GPCR activation of the Hippo pathway. Upon agonist treatment of GPCRs, including PAR1 (pictured here), PAR2, LPARs, and S1PRs, G protein signaling is activated. In relation to the Hippo pathway, $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_{i}$ activate RhoGEFs and RhoA as well as promote F-actin polymerization. Active RhoA and actin polymerization then inhibit LATS1/2 kinase, thus blocking phosphorylation of YAP and TAZ. When YAP and TAZ are de-phosphorylated, they translocate into the nucleus, bind to TEAD family transcription factors and promote gene transcription of downstream targets such as CTGF and ANKRD1 which contribute to cell proliferation and tumor cell invasion.

0.9 References

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CHAPTER 1: The α-arrestin ARRDC3 suppresses breast carcinoma invasion by regulating G protein-coupled receptor lysosomal sorting and signaling

1.1 Abstract

Aberrant G protein-coupled receptor (GPCR) expression and activation has been linked to tumor initiation, progression, invasion and metastasis. However, compared with other cancer drivers, the exploitation of GPCRs as potential therapeutic targets has been largely ignored, despite the fact that GPCRs are highly druggable. Therefore, to advance the potential status of GPCRs as therapeutic targets, it is important to understand how GPCRs function together with other cancer drivers during tumor progression. We now report that the α-arrestin domain-containing protein-3 (ARRDC3) acts as a tumor suppressor in part by controlling signaling and trafficking of the GPCR, protease-activated receptor-1 (PAR1). In a series of highly invasive basal-like breast carcinomas, we found that expression of ARRDC3 is suppressed while PAR1 is aberrantly overexpressed because of defective lysosomal sorting that results in persistent signaling. Using a lentiviral doxycycline-inducible system, we demonstrate that re-expression of ARRDC3 in invasive breast carcinoma is sufficient to restore normal PAR1 trafficking through the ALG-interacting protein X (ALIX)-dependent lysosomal degradative pathway. We also show that ARRDC3 re-expression attenuates PAR1-stimulated persistent signaling of c-Jun NH2-terminal kinase (JNK) in invasive breast cancer. Remarkably, restoration of ARRDC3 expression significantly reduced activated PAR1-induced breast carcinoma invasion, which was also dependent on JNK signaling. These findings are the first to identify a critical link between the tumor suppressor ARRDC3 and regulation of GPCR trafficking and signaling in breast cancer.

1.2 Introduction

G protein-coupled receptors (GPCRs) are a large family of cell surface signaling receptors that play a critical role in cancer growth and development by regulating cellular proliferation, invasion, migration, immune cell-mediated functions, angiogenesis and survival at metastatic sites [1-3]. GPCR function can be altered in cancer through aberrant overexpression, gain-of-function activating mutations, mutations in downstream G protein signaling effectors, and increased production and secretion of GPCR activating ligands by both tumor cells and surrounding stromal cells [4-7]. As cell surface receptors with highly druggable sites, GPCRs are the largest class of drug targets, with over 30% of current FDA-approved drugs targeting GPCRs [8,9]. Despite the success and promise of GPCRs as therapeutic targets, there are currently no drugs in the clinic used for the treatment of cancer that specifically target GPCRs. In addition, GPCRs are known to function in metastasis [2,3], and there are limited targeted treatment options for patients with metastatic cancer. Thus, understanding how GPCRs function together with other drivers of cancer in tumor progression is important for the development of new effective treatment strategies for metastatic cancer.

Several GPCRs implicated in metastatic cancer, including protease-activated receptor-1 (PAR1), have also proven to be highly druggable [10,11]. PAR1 is overexpressed in invasive ductal breast carcinoma but not in normal mammary epithelial tissue [12,13]. In addition, overexpression of PAR1 in breast cancer patient biopsies correlates with increased rates of metastasis and poor prognosis [12,14]. PAR1 is also highly expressed in invasive breast carcinoma cell lines, but not in non-invasive breast cancer cells and high PAR1 expression correlates with increased cellular invasion *in vitro* and tumor growth *in vivo* [12,15,16]. Moreover, loss of PAR1 function by either knockdown of PAR1 or inhibition of PAR1 signaling in highly invasive breast carcinoma cells results in decreased cellular migration and invasion *in vitro* and tumor growth *in vivo* [15-17]. In contrast, ectopic overexpression of PAR1 in non-invasive MCF7 breast carcinoma is sufficient to drive cellular invasion *in vitro* and tumor growth

in vivo possibly by initiating epithelial-mesenchymal transition [18,19] suggesting that PAR1 expression is both necessary and sufficient to promote breast cancer progression. Together, these studies strongly support a role for PAR1 as a key mediator of breast cancer progression.

PAR1 is activated by irreversible proteolytic cleavage of the N-terminus, revealing a new N-terminal domain that acts as a tethered ligand that binds intramolecularly to the receptor to elicit transmembrane signaling [20,21]. Thrombin is the main effector protease for PAR1 activation in most cell types, however other proteases can cleave and activate the receptor in different cellular contexts. In fact, PAR1 senses and responds to multiple proteases generated in the tumor microenvironment including thrombin, plasmin and matrix metalloproteinase-1 (MMP-1) [18,22,23]. Once activated, PAR1 signals to distinct heterotrimeric G protein subtypes including G_q, G_i and $G_{12/13}$ and triggers RhoGEF-mediated RhoA signaling, increases in intracellular Ca^{2+}, MAP kinase activation and signaling by multiple other effectors [24,25]. However, previous studies have shown that PAR1 signaling through $G_{12/13}$ is the primary driver of breast carcinoma invasion and metastasis *in vitro* and *in vivo* [26]. In addition, PAR1-$G_{12/13}$ signaling to RhoA and activation of c-Jun NH2-terminal kinase (JNK), a downstream effector of G_{12}, is required for thrombin-induced breast carcinoma invasion [27]. However, the defects that engender PAR1 and other GPCRs the capacity to promote breast cancer invasion and metastasis are not known.

One mechanism that contributes to PAR1 dysregulation in cancer is defective endocytic trafficking. Similar to most GPCRs, activated PAR1 signaling to heterotrimeric G proteins is rapidly desensitized at the plasma membrane [28,29]. Unlike most GPCRs, however, internalization and lysosomal sorting of proteolytically activated PAR1 is critical for termination of G protein signaling [28]. In fibroblasts, a chimeric PAR1 that internalizes and recycles back to the cell surface displayed persistent signaling after activation and removal of thrombin [28,30]. PAR1 persistent signaling was caused by recycling and continued signaling by proteolytically activated PAR1 that returned to the cell surface with its tethered ligand intact.

Importantly, activated PAR1 trafficking is also severely altered in metastatic breast cancer but not in non-metastatic or normal breast epithelial cells [15]. Consequently, dysregulated PAR1 trafficking and signaling drives breast cancer invasion and tumor growth [15,16]. Thus, dysregulated GPCR trafficking is utilized as a gain-of-function mechanism to prolong GPCR signaling in invasive breast carcinomas.

Most classic GPCRs sort to lysosomes for degradation through the endosomal sorting complexes required for transport (ESCRT) pathway, which functions sequentially to sort ubiquitinated receptors to intraluminal vesicles of multivesicular bodies (MVBs)/lysosomes [31]. However, we showed previously that several GPCRs including PAR1 sort to lysosomes independent of receptor ubiquitination and components of the ubiquitin-binding ESCRT machinery [32]. Rather, activated PAR1 engages ALG-interacting protein X (ALIX), an ESCRT-III interacting protein, for lysosomal degradation [33,34]. We further identified α-arrestin domain-containing protein-3 (ARRDC3) as a key regulator of ALIX-dependent PAR1 lysosomal trafficking in HeLa cells [35]. ARRDC3 has also been show to regulate endosomal sorting of other GPCRs including the β2-adrenergic receptor (β2AR) [36-38] through mechanisms that remain poorly defined.

Importantly, ARRDC3 is a newly identified tumor suppressor in metastatic breast cancer [39,40]. ARRDC3 expression is either lost or suppressed in basal-like invasive breast cancer that results from either gene deletion or epigenetic silencing [40,41]. In addition, low ARRDC3 expression correlates with tumor recurrence and patient poor prognosis [39,42], further suggesting a role for ARRDC3 in tumor suppression. Given that ALIX and ARRDC3 are key regulators of PAR1 trafficking, we examined whether dysregulation of PAR1 trafficking in invasive breast carcinoma was due to defective ALIX or ARRDC3 function. Here, we report that loss of ARRDC3 expression in highly invasive breast carcinoma is responsible for defective ALIX-dependent lysosomal sorting of PAR1. In addition, loss of ARRDC3 expression not only

perturbs PAR1 trafficking, but also is responsible for persistent signaling that drives thrombin-stimulated breast carcinoma cellular invasion. These findings provide the first evidence that ARRDC3 functions as a tumor suppressor by modulating GPCR trafficking and signaling in invasive breast cancer.

1.3 Materials & Methods

Reagents and Antibodies – The PAR1 agonist peptide TFLLRNPNDK ("TFLLRN") 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. Mouse IgG (#010-0102) and polyclonal anti-HA antibodies (#600-401-384) were purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse monoclonal anti-HA antibody (HA.11) (#MMS-101R) was purchased from Covance (Princeton, New Jersey). Mouse monoclonal anti-PAR1 WEDE antibody (#IM2584) was purchased from Beckman Coulter (Fullerton, CA). Rabbit anti-PAR1 polyclonal antibody was described previously [43]. Rabbit polyclonal anti-ARRDC3 antibody (#ab64187) was purchased from Abcam (Cambridge, UK). Monoclonal anti-ALIX antibody (#sc-53538) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-LAMP1 (#15665), rabbit anti-phospho-JNK1/2 (#9251), and rabbit anti-JNK (#9252) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal anti-β-actin antibody (#A5316) and SP600125 (JNK inhibitor) were purchased from Sigma-Aldrich (Carlsbad, CA) and anti-GAPDH antibody (#GTX627408) was from GeneTex (Irvine, CA). Goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (#A-11001) and goat anti-rabbit secondary antibody conjugated to Alex Fluor 647 (A-21244) were purchased from ThermoFisher Scientific (Waltham, MA). 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). Doxycycline was

purchased from Clontech Takara Bio USA (Mountain View, CA). LysoTracker, ProLong Gold and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Thermo Fisher Scientific.

Cell Culture and Transfections – MDA-MB-231 cells were purchased from ATCC and maintained without CO_2 in Leibowitz-15 media supplemented with 10% fetal bovine serum (v/v). MCF7, BT549, T47D, BT474, Hs578T, and SKBR3 cells were all purchased from ATCC and grown according to ATCC instructions. siRNA transfections were performed using Oligofectamine (Life Technologies) per the manufacturer's instructions. Degradation and invasion assays described were performed 48 h after transfection. All single siRNAs were purchased from Qiagen: non-specific (ns) siRNA sequence, 5'-CUACGUCCAGGAGCGCACC-3'; ALIX #1 target sequence, 5′-AAGTACCTCAGTCTATATTGA-3′; ALIX #3 target sequence, 5′-AATCGAGACGCTCCTGAGATA-3′.

ARRDC3 Lentiviral Construct and Stable Cell Lines – The pSLIK lentiviral vectors were constructed as described previously [44,45]. pSLIK-Hygro (Addgene plasmid # 25737) and pEN_TmiRc3 (Addgene plasmid # 25748) were gifts from Dr. Iain Fraser (NIH) [45]. Briefly, N-terminal HA-tagged human ARRDC3 cDNA was cloned into the entry vector pEN_TmiRc3 using restriction sites SpeI and EcoRI downstream of tetracycline response element (TRE) promoter. TRE promoter and ARRDC3 cDNA were then transferred to pSLIK-Hygro destination vector expressing reverse tetracycline transactivator (rtTA) and hygromycin-selection marker by Gateway recombination (Invitrogen) to generate pSLIK encoding tetracycline-inducible ARRDC3 (pSLIK-ARRDC3). Lentivirus was produced by transfecting 293T cells with pSLIK-ARRDC3 vector along with packaging plasmids pMDL, pRSV and pVSV using polyethylenimine (PEI). Lentivirus-contained supernatant was harvested on day 2 post-transfection, filtered, and used to transduce MDA-MB-231 cells overnight with 8 μg/ml polybrene (EMD Millipore). MDA-MB-231

cells stably expressing pSLIK-ARRDC3 vector were selected using 200 μg/ml hygromycin (Omega Scientific).

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). For comparison of ARRDC3 and ALIX expression, subconfluent cells in the exponential growth stage were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA and 1% Triton X-100) with freshly added protease inhibitors (2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml Pepstatin A, 1 mM PMSF) and phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate), quantified by BCA analysis (Pierce BCA Protein Assay Kit; Thermo Scientific) and equal amounts of lysates were used for immunoblotting.

Cell Surface ELISA – PAR1 surface expression and internalization assays were performed by ELISA as described previously [46]. Briefly, 2×10^5 cells were plated in triplicate on fibronectin-coated 12-well plate. After three days, confluent cells were incubated with starvation medium (Dulbecco's Modified Eagle Medium (DMEM) with 1mM HEPES and 0.1% BSA) for 1 h at 37°C, washed with chilled PBS and fixed with 4% paraformaldehyde on ice for 15 min. Cells were then incubated with mouse anti-PAR1 WEDE antibody or mouse IgG for 1 h at 4 °C and followed by secondary HRP-conjugated goat anti-mouse antibody for 1 h at room temperature. Antibody bound to cell surface was detected by incubation with one-step ABTS substrate for 10–20 min at room temperature and measured at the absorbance of 405 nm using a microplate reader (SpectraMax Plus, Molecular Devices). The amount of cell surface PAR1 was determined by subtracting the background mouse IgG from the samples labeled with anti-PAR1 antibody. For PAR1 internalization assays, 24 well plates were coated with fibronectin

and 7.5 × 10^4 MDA-MB-231 cells per well were plated. Cells treated with doxycycline for 48 h were serum starved for 1 h then incubated on ice with anti-PAR1 antibody to label surface PAR1. Cells were stimulated with PAR1 agonist for various times, fixed and incubated with HRP-conjugated secondary antibody. The amount of antibody remaining at the cell surface was detected and quantified as described above.

Immunoprecipitation – To assess PAR1 degradation, immunoprecipitation of endogenous PAR1 was performed as previously described [47]. Briefly, cells were plated in 6 cm dishes at a density of 1×10^6 cells per dish and treated with 1 μg/ml doxycyline the following day. For siRNA knockdown, cells were seeded in 6 well plates at 5×10^5 cells per well, transfected as described and treated with doxycycline the following day. After 48 h doxycycline treatment, cells were serum starved for 1 h, treated with α-thrombin then placed on ice, washed with PBS, and lysed with Triton X-100 lysis buffer supplemented with a cocktail of protease inhibitors. Cell lysates were sonicated for 10 sec at 10% amplitude (Branson Model 450 sonifier) and cleared by centrifugation. BCA assay (Thermo Fisher Scientific) was performed to determine protein concentrations. Equal amounts of normalized lysates were immunoprecipitated with appropriate antibodies and pre-blocked Protein A-Sepharose beads 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 PAR1 immunoprecipitates were analyzed by immunoblotting.

Immunofluorescence Confocal Microscopy – Cells were plated at a density of 4 ×10^5 cells per well on fibronectin-coated glass coverslips placed in a 12-well dish and grown overnight. Cells were treated with doxycycline for 48 h, and then serum-starved in DMEM media containing 1 mg/ml BSA, 2 mM leupeptin, and 100 nM LysoTracker for 1 h at 37°C. Cells were then incubated at 4°C with anti-PAR1 WEDE antibody to label the surface population of receptors, then stimulated with 100 μM TFLLRN, fixed with 4% paraformaldehyde in PBS,

permeabilized with methanol, immunostained with polyclonal anti-HA antibody, and processed as described previously [46]. Coverslips were mounted with ProLong Gold 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 ORCAER digital camera using Metamorph 7.7.4.0 software (Molecular Devices). Fluorescence intensity line-scan analysis was performed using ImageJ software.

Signaling Assays – Signaling assays were performed essentially as described previously (3). Briefly, cells were treated with or without 1 μg/ml doxycycline for 48 h and starved for 1 h at 37°C. Cells were stimulated with 10 nM α-thrombin for indicated times at 37°C and cell lysates were collected by direct lysis in 2X Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE and immunoblotted with anti-phospho-JNK antibody. PVDF membranes were stripped and reprobed with anti-JNK antibody.

Invasion Assays – For ARRDC3 re-expression experiments, cells were treated with doxycycline for 48 h and serum starved overnight. For JNK inhibitor experiments, cells were serum starved overnight then treated with DMSO or JNK inhibitor SP600125 (20 μM) for 2 h. Cells were dissociated using Cellstripper solution (Corning, NY) and seeded onto BioCoat™ Matrigel® invasion chambers (Corning, NY) with or without α-thrombin added. For JNK inhibitor experiments, DMSO or SP600125 (20 μM) were also added during seeding into invasion chambers. Cells were allowed to invade for 5 h at 37°C, fixed and stained with 0.5% crystal violet in ethanol. Membranes were dried overnight and cells that had invaded through the matrigel and membrane were imaged using Leica DMi1 inverted microscope (Leica Microsystems). Cell invasion was quantified by cell count in nine fields of view at 10X magnification for each condition, from three biological independent replicates.

Data Analysis – Statistical significance was determined by unpaired *t*-test or one-way ANOVA using Prism 4.0 software (GraphPad).

1.4 Results

1.4.1 PAR1, ARRDC3 and ALIX expression in non-invasive luminal versus invasive basal-like breast carcinoma

To determine the mechanisms responsible for dysregulated PAR1 trafficking in invasive breast carcinoma, we profiled the expression of PAR1 and two key regulators ARRDC3 and ALIX [33,35], in a series of human mammary luminal non-invasive and basal-like invasive breast carcinoma cell lines. The expression of ARRDC3 and ALIX in breast carcinoma was detected by immunoblotting and PAR1 expression was determined by cell surface ELISA. Immunoblot analysis of equivalent amounts of cell lysates revealed high ARRDC3 expression in non-invasive luminal breast carcinoma and HER2-positive SKBR3 breast cancer cells (Fig. 1.1A, lanes 1-4 and 9), whereas ARRDC3 expression was minimally detected in invasive basal-like breast carcinoma (Fig. 1.1A, lanes 5-8). These findings are consistent with loss of ARRDC3 expression previously reported in invasive breast carcinoma [39-41]. Interestingly, PAR1 expression was high in basal-like invasive breast carcinoma with low ARRDC3 expression and low in non-invasive luminal breast carcinoma with high ARRDC3 expression (Fig. 1.1A and B) including luminal ZR75-1 cells as previously reported [15]. In contrast to ARRDC3 and PAR1, ALIX expression was variable and detected in all cell lines irrespective of luminal *versus* basal subtype of breast carcinoma (Fig. 1.1A, lanes 1-9). While ALIX is expressed in BT474 cells, its size is shifted possibly due to post-translational modifications such as ubiquitination [35,48]. Nonetheless, these findings reveal an inverse correlation between PAR1 and ARRDC3 expression and raise the intriguing idea that loss of ARRDC3 expression may be responsible for aberrant PAR1 expression in invasive breast carcinoma.

1.4.2 Ectopic expression of ARRDC3 restores activated PAR1 degradation in invasive breast carcinoma

In invasive breast carcinoma, PAR1 trafficking is dysregulated and fails to sort to a lysosomal degradation pathway [15,16]. However, the mechanism responsible for defective PAR1 trafficking in breast cancer is not known. To determine if ARRDC3 expression is sufficient to restore proper trafficking of PAR1 in invasive breast carcinoma, we used a lentiviral pSLIK tetracycline-inducible expression vector system encoding ARRDC3 containing an N-terminal HA epitope tag (Fig. 1.2A). MDA-MB-231 breast carcinoma cells stably expressing HA-ARRDC3 pSLIK were generated and examined for doxycycline (DOX)-inducible expression of ARRDC3. An ~12 to 16-fold induction of HA-ARRDC3 expression was detected in cells incubated with 1 or 10 μg/ml doxycycline for 48 h, respectively compared to untreated control cells (Fig. 1.2B, lanes 4-6). The expression of ARRDC3 was determined by immunoblotting using either anti-HA or -ARRDC3 antibodies (Fig. 1.2B, *top* and *middle* panels, respectively). As expected, parental MDA-MB-231 cells incubated with similar concentrations of doxycycline failed to induce ARRDC3 expression (Fig. 1.2B, lanes 1-3). In addition, incubation of MDA-MB-231 HA-ARRDC3 pSLIK cells with doxycycline using the same conditions did not alter the high level of PAR1 cell surface expression basally as detected by ELISA (Fig. 1.2C). These results indicate that doxycycline specifically induces expression of HA-ARRDC3 in highly invasive MDA-MB-231 breast carcinoma.

Using the HA-ARRDC3 pSLIK doxycycline-inducible system in MDA-MB-231 cells, we next examined if ARRDC3 expression is sufficient to restore agonist-stimulated PAR1 lysosomal degradation. MDA-MB-231 HA-ARRDC3 pSLIK cells were preincubated with or without doxycycline for 48 h, then treated with or without agonist, lysed, immunoprecipitated with anti-PAR1 specific antibodies and then immunoblotted to detect PAR1 protein. In cells not exposed to agonist, endogenous PAR1 migrated as a high molecular weight ~75 kDa protein that was not detected in IgG control immunoprecipitates (Fig. 1.3A, lanes 2,7 and 1,6). In HA-ARRDC3

pSLIK expressing MDA-MB-231 cells not incubated with doxycycline and lacking ARRDC3 expression, prolonged treatment with the PAR1-specific agonist peptide failed to promote substantial loss of PAR1 protein as expected (Fig. 1.3A, lanes 2-5). These results are consistent with a defect in sorting of activated PAR1 from endosomes to lysosomes in invasive breast cancer as previously reported [15,16]. However, in MDA-MB-231 cells expressing doxycycline-induced ARRDC3, agonist peptide induced a significant loss of PAR1 protein following 60, 90 or 120 min of stimulation (Fig. 1.3A, lanes 7-10). The kinetics of activated PAR1 degradation is reminiscent of a typical time-course of receptor degradation observed in other cell types [33]. To determine if ARRDC3 affects PAR1 internalization, agonist-induced loss of PAR1 from the cell surface was measured by ELISA. In MDA-MB-231 HA-ARRDC3 pSLIK cells not treated with doxycycline and not expressing ARRDC3, activation of PAR1 with peptide agonist resulted in a robust ~60% loss of receptor from the cell surface (Fig. 1.3B), which was indistinguishable from agonist-induced loss of cell surface PAR1 observed in MDA-MB-231 cells re-expressing ARRDC3. These findings suggest that ARRDC3 is required for agonist-promoted PAR1 degradation but not for receptor internalization.

To further assess ARRDC3 function as a key mediator of PAR1 degradation, we examined whether ARRDC3 regulated PAR1 degradation when proteolytically activated by its natural ligand thrombin. In MDA-MB-231 HA-ARRDC3 pSLIK cells not treated with doxycycline and lacking ARRDC3 expression, thrombin stimulation resulted in a shift in PAR1 mobility indicative of receptor cleavage but failed to cause a substantial loss of PAR1 protein (Fig. 1.4A, lanes 2-3). PAR1 protein was not detected in IgG immunoprecipitates (Fig. 1.4A, lanes 1 and 4). In contrast, thrombin caused a significant ~50% loss of PAR1 protein in MDA-MB-231 cells expressing ARRDC3 (Fig. 1.4A, lanes 5-6), suggesting that ARRDC3 is necessary for receptor lysosomal trafficking. However, thrombin-promoted rapid and robust PAR1 internalization in MDA-MB-231 HA-ARRDC3 pSLIK cells with and without ARRDC3 expression (Fig. 1.4B), indicating that receptor internalization occurs independent of ARRDC3 expression. These

results suggest that ARRDC3 is required for agonist-induced PAR1 trafficking from endosomes to a lysosomal degradative pathway in invasive breast carcinoma.

1.4.3 ALIX is required for ARRDC3-mediated PAR1 degradation in invasive breast carcinoma

We previously showed that ARRDC3 regulates PAR1 degradation in HeLa cells by modulating the function of ALIX, an adaptor protein that binds directly to PAR1 and ESCRT-III to facilitate lysosomal sorting [33,35]. Thus, we next determined if ARRDC3 regulates PAR1 degradation in invasive breast carcinoma through an ALIX-dependent pathway. To assess ALIX function, MDA-MB-231 HA-ARRDC3 pSLIK cells were transfected with ALIX specific siRNAs to deplete cells of endogenous ALIX expression (Fig. 1.5, *middle panel*). In MDA-MB-231 cells transfected with non-specific siRNA and not treated with doxycycline, ALIX expression was easily detectable, whereas ARRDC3 expression was markedly low and thrombin failed to induce PAR1 degradation (Fig. 1.5, lanes 1-2). In contrast, non-specific siRNA transfected MDA-MB-231 cells expressing ALIX, and re-expressing ARRDC3 showed a significant ~40% decrease in PAR1 protein following thrombin stimulation (Fig. 1.5, lanes 3-4). These findings suggest that ARRDC3 expression is sufficient to restore agonist-induced PAR1 degradation in MDA-MB-231 cells with ALIX expression. However, siRNA-mediated knockdown of ALIX blocked thrombin-stimulated PAR1 degradation in cells expressing ARRDC3 (Fig. 1.5, lanes 7-8). These findings indicate that ALIX expression is required for ARRDC3-mediated agonist-stimulated PAR1 degradation in invasive breast carcinoma.

1.4.4 Sorting of activated PAR1 to lysosomes requires ARRDC3

To determine if ARRDC3 directly mediates activated PAR1 sorting to MVBs/lysosomes, we used immunofluorescence confocal microscopy and LysoTracker to label lysosomes. To ensure that the cell permeable LysoTracker probe labelled acidic lysosomal organelles, we

immuno-stained MDA-MB-231 HA-ARRDC3 pSLIK cells with antibodies targeted against the lysosome associated membrane protein-1 (LAMP-1), a resident lysosomal membrane protein. LysoTracker accumulated in the lumen of organelles with distinct LAMP-1 expression at the limiting membrane as detected by confocal microscopy and line-scan analysis (Fig. 1.6A), suggesting that LysoTracker is a valid marker of lysosomes in MDA-MB-231 cells. MDA-MB-231 HA-ARRDC3 pSLIK cells incubated with or without doxycycline and LysoTracker were then labelled with anti-PAR1 antibodies at 4°C. Under these conditions, only the cell surface cohort of PAR1 bind antibody. Cells were washed and then stimulated with the PAR1-specific agonist peptide for 90 min. In untreated 0 min control cells, PAR1 localized mainly to the cell surface and not in LysoTracker labelled organelles, irrespective of doxycycline induction of ARRDC3 (Fig. 1.6B and C, *top panels*). After 90 min of agonist stimulation, PAR1 redistributed from the cell surface to endocytic puncta and failed to co-localize with LysoTracker in cells which displayed minimal ARRDC3 expression (Fig. 1.6B and D). In contrast, however, in cells treated with doxycycline and -expressing HA-ARRDC3 (Fig. 1.6C), activated and internalized PAR1 accumulated within LysoTracker labelled organelles as shown by confocal microscopy and line-scan analysis (Fig. 1.6C and E). Collectively, these data indicate that ARRDC3 expression is both necessary and sufficient to target activated PAR1 to lysosomes for degradation.

1.4.5 ARRDC3 attenuates PAR1-mediated persistent signaling and cellular invasion

Dysregulated PAR1 trafficking in invasive breast carcinoma results in persistent signaling and contributes to cellular invasion [15,16]. Since ARRDC3 expression is sufficient to restore activated PAR1 lysosomal sorting, we examined if ARRDC3 expression was sufficient to attenuate activated PAR1 signaling. In these studies, we focused on activation of JNK, an effector of PAR1-stimulated G_{12} signaling and known mediator of PAR1-stimulated breast carcinoma invasion [26,27]. MDA-MB-231 cells express JNK1 and JNK2 [27], which are phosphorylated on threonine-183 and tyrosine-185 residues following activation of upstream

MAP kinases induced by RhoA signaling [49]. To assess the function of ARRDC3 on PAR1 signaling, MDA-MB-231 HA-ARRDC3 pSLIK cells were treated with or without doxycycline, stimulated with thrombin and phosphorylation of JNK1/2 determined by immunoblotting. In MDA-MB-231 cells without doxycycline treatment and minimal ARRDC3 expression, thrombin induced a prolonged and robust ~7-fold increase in JNK1/2 phosphorylation that peaked at 20 min (Fig. 1.7), consistent with enhanced and persistent PAR1 signaling. However, in MDA-MB-231 HA-ARRDC3 pSLIK cells treated with doxycycline and re-expressing ARRDC3, thrombin induced a transient and ~3-fold increase in JNK1/2 phosphorylation (Fig. 1.7), a response substantially diminished compared to cells lacking ARRDC3 expression. These results suggest that the impact of ARRDC3 on PAR1 trafficking is important for regulating appropriate cellular signaling.

We next examined the function of ARRDC3 and JNK signaling on thrombin-induced breast carcinoma cellular invasion. In MDA-MB-231 HA-ARRDC3 pSLIK cells with no doxycycline treatment and low ARRDC3 expression, incubation with thrombin induced an ~3-fold increase in cellular invasion compared to untreated control cells (Fig. 1.8A, *top panels*). This is consistent with previously reported effects of thrombin on breast carcinoma cellular invasion [15,18]. However, in MDA-MB-231 cells exhibiting doxycycline-induced ARRDC3 expression, thrombin-induced invasion was markedly reduced and equivalent to unstimulated control cells (Fig. 1.8A, *bottom panels*), suggesting that ARRDC3 suppresses thrombin-activated PAR1-driven breast carcinoma invasion. To determine if JNK1/2 is important for cellular invasion, MDA-MB-231 HA-ARRDC3 pSLIK cells were pretreated with the selective JNK inhibitor SP600125 or DMSO vehicle control. Thrombin stimulated a marked increase in JNK1/2 phosphorylation in MDA-MB-231 cells that was virtually abolished in cells treated with the JNK inhibitor SP600125, whereas thrombin-induced p38 phosphorylation was unaffected (Fig. 1.8B, *inset*). Importantly, thrombin-induced breast carcinoma invasion was markedly inhibited in cells pre-incubated with the JNK inhibitor SP600125 compared to DMSO treated control cells (Fig.

1.8B). Together, these findings indicate that ARRDC3 functions as a tumor suppressor by controlling GPCR trafficking and consequently signaling that promotes breast cancer invasion (Fig. 1.9).

1.5 Discussion

While the survival rate for patients diagnosed at early stage breast cancer has improved, this is not the case for patients diagnosed with advanced metastatic breast cancer, which lacks druggable targets. We propose that highly druggable GPCRs are a promising class of targets for metastatic cancer. In fact, several lines of evidence indicate that the GPCR PAR1 is an attractive therapeutic target for advanced breast cancer [11,50]. PAR1 confers tumor cell motility, invasiveness, survival and self-renewal in breast cancer and other cancer types [51]. In addition, expression of PAR1 correlates with high breast tumor grade and poor patient prognosis [14]. Despite compelling evidence supporting the oncogenic function of PAR1 in driving breast cancer progression, the defects that contribute to dysfunction of PAR1 in cancer remain largely unknown. We previously showed that dysregulation of PAR1 trafficking results in persistent signaling and consequently promotes tumor invasion and growth [15,16], but the underlying defects resulting in aberrant PAR1 trafficking are not known. In the present study, we sought to determine the mechanism responsible for aberrant PAR1 trafficking and signaling in invasive breast carcinoma. We found that expression of the α-arrestin ARRDC3, a key regulator of PAR1 trafficking [35], is suppressed in basal-like invasive breast carcinoma, which exhibit high PAR1 expression. We further show that re-expression of ARRDC3 in basal-like invasive breast carcinoma is sufficient to restore normal PAR1 lysosomal trafficking, which occurs through an ALIX-dependent endosomal-lysosomal sorting pathway (Fig. 1.9). Importantly, ARRDC3 re-expression also attenuated thrombin-stimulated JNK signaling and breast carcinoma invasion. These studies are the first to demonstrate a role for the tumor

suppressor ARRDC3 in regulation of GPCR trafficking and signaling in invasive basal-like breast carcinoma.

The α-arrestin ARRDC3 shares structural homology to the β-arrestin family of adaptor proteins [52], which have important regulatory roles in mammalian GPCR signaling and trafficking [37]. ARRDC3 has been shown to regulate endosomal sorting of the β2AR, a classic GPCR [36-38]. However, the precise role of ARRDC3 in this pathway remains controversial given the dominant role of β-arrestins in regulation of β2AR function [36,38]. ARRDC3 possesses arrestin-like N- and C-domains and C-terminal PPxY motifs that bind to WW domains of HECT-domain containing E3 ubiquitin ligases [53,54]. Despite the presence of arrestin-like domains, the α-arrestin ARRDC3 appears to lack a polar core, which is essential for β-arrestin binding to activated and phosphorylated GPCRs [55]. In addition, β-arrestins lack C-terminal PPxY motifs, indicating that α-arrestin and β-arrestin proteins likely have divergent functions. We previously showed that ARRDC3 plays a critical role in regulating PAR1 lysosomal trafficking in HeLa cells. Unlike the β2AR, however, ARRDC3 appears to regulate PAR1 lysosomal sorting by modulating ALIX ubiquitination via recruitment of the E3 ubiquitin ligase WWP2 [35]. Ubiquitination of ALIX enhances its dimerization and binding to activated PAR1 and ESCRT-III to facilitate receptor lysosomal degradation [33,35]. However, there is limited knowledge as to how loss of ARRDC3 disrupts cellular homeostasis to promote breast cancer. In the present study, we found that ARRDC3 expression is suppressed in basal-like invasive breast carcinoma, which exhibit high PAR1 expression and dysregulated receptor trafficking [15,16]. We further report that re-expression of ARRDC3 is sufficient to restore normal agonist-induced PAR1 lysosomal degradation in invasive breast carcinoma. In addition, ARRDC3 controls PAR1 lysosomal sorting through an ALIX-dependent pathway in invasive breast carcinoma, consistent with results from our previous HeLa cell studies [35]. These

findings indicate that ARRDC3 tumor suppressor function is linked to regulation of GPCR trafficking.

ARRDC3 has been identified as a tumor suppressor in breast and prostate cancer [39-41,56]. Using combined high-resolution analysis of genome copy number and gene expression in primary basal and luminal breast cancers, ARRDC3 expression was reported to be low or absent in basal-like breast cancer, the most aggressive and metastatic subtype of breast carcinoma [40]. ARRDC3 expression was also shown to decrease with tumor grade, metastasis, and recurrence and was found in a gene cluster on chromosome 5 deleted in 17% of basal-like breast cancers, compared 0% deletion in luminal breast cancers [39,40]. In addition to gene deletion, ARRDC3 expression is suppressed by epigenetically silencing in basal-like breast carcinoma cells through ARRDC3 promoter deacetylation or hypermethylation and inactivation of the tumor suppressor gene [41,57]. Endogenous small non-coding micro-RNAs that control ARRDC3 gene expression have also been reported to function in regulation of ARRDC3 gene expression in prostate cancer [58]. The strong correlation between ARRDC3 expression and tumor progression indicates that loss of ARRDC3 expression is linked to dysregulation of important cancer drivers. One target of ARRDC3 previously described is the membrane protein integrin β4 [39], which is enriched in triple-negative breast cancer and a marker of poor prognosis [59,60]. Here, we now report that in addition to functioning in regulation of integrin β4, loss of ARRDC3 in invasive breast carcinoma results in aberrant PAR1 lysosomal trafficking, persistent signaling and consequent breast carcinoma cellular invasion. These findings indicate that the tumor suppressor function of ARRDC3 is linked to the regulation of both integrin receptors and GPCRs. While there is currently no known direct link between integrin β4 and PAR1, integrin β1 has been implicated in PAR1-promoted chemotaxis [61], invasion [62], proliferation and ERK1/2 activation [63] in skin, bone and brain tumor cells, respectively. In addition, αvβ5 cooperates with PAR1 in thrombin-mediated lung cancer cell invasion [64]. Thus,

future studies are important to determine if integrin □4 is integrated in PAR1-driven breast cancer progression.

Activated PAR1 stimulates heterotrimeric $G\alpha_{12/13}$ – RhoA signaling [16], which has been implicated in tumorigenesis and cancer progression [65,66]. Several previous studies showed that constitutively active $G\alpha_{12}$ and $G\alpha_{13}$ proteins as well as induction of $G\alpha_{12/13}$ signaling through activation of PAR1 increase invasion of breast carcinoma *in vitro*, whereas inhibition of G_{12} signaling reduced metastasis and improved metastasis-free survival *in vivo* [26]. In the present studies, we report that ARRDC3 tumor suppressor function is linked to the regulation of PAR1-stimulated G_{12}-effector JNK signaling, which mediates breast carcinoma invasion. We discovered that ARRDC3 functions as a negative regulator of PAR1-stimulated breast carcinoma invasion. In addition, ARRDC3 appears to control breast cancer invasion though JNK signaling. In the absence of ARRDC3, PAR1-stimulated a marked and prolonged increase in JNK signaling, however, re-expression of ARRDC3 in breast cancer cells resulted in a significant reduction in both the magnitude and duration of JNK signaling induced by PAR1. JNK has been shown to contribute to malignant transformation, drug resistance and tumor growth in various cancer types [67]. JNK phosphorylates a large number of targets, mainly transcription factors, and regulates cellular proliferation, survival and apoptosis but precisely how JNK contributes to breast carcinoma invasion stimulated by a GPCR is not known and an area of active investigation.

In summary, this study reveals a novel and important role for the tumor suppressor ARRDC3 in the regulation of GPCR trafficking and signaling in invasive basal-like breast cancer. JNK signaling makes important contributions to tumor progression and is an effector of GPCR-$G_{12/13}$ RhoA signaling. The work reported here now suggest that the tumor suppressor ARRDC3 functions not only by regulating PAR1 trafficking but also through modulating appropriate JNK signaling, a critical driver of breast cancer invasion. Thus, in future studies it

will be important to determine how JNK regulates certain substrates to control GPCR-induced breast cancer invasion *in vitro* and the consequences on tumor invasion and growth *in vivo*.

1.6 Acknowledgments

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Chapter 1, in full, is a reprint of the material as it appears in: Arakaki AKS, Pan WA, Lin H, Trejo J. (2018) The a-arrestin ARRDC3 suppresses breast carcinoma invasion by regulating G protein-coupled receptor lysosomal sorting and signaling. *Journal of Biological Chemistry, 293*(9) 3350-3362. The dissertation author was the primary investigator and author of this paper. All co-authors have given written permission for its use and the reproduction of all associated data in this dissertation.

1.7 Figures

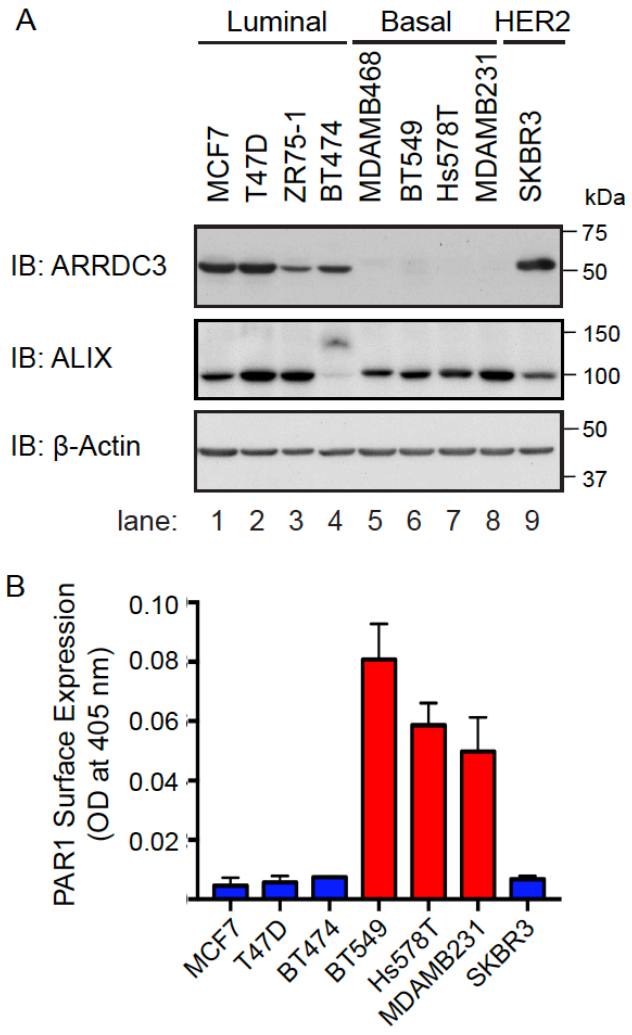

Figure 1.1: PAR1 and ARRDC3 protein expression are inversely correlated in breast carcinoma cell lines. *A,* Equivalent amounts (20 μg) of cell lysates from various breast cancer cell lines were immunoblotted for ALIX and ARRDC3 expression. β-actin expression was determined as a control. *B,* PAR1 cell surface expression was determined by ELISA. Data (mean ± S.D., n=3) shown and is representative of three independent experiments.

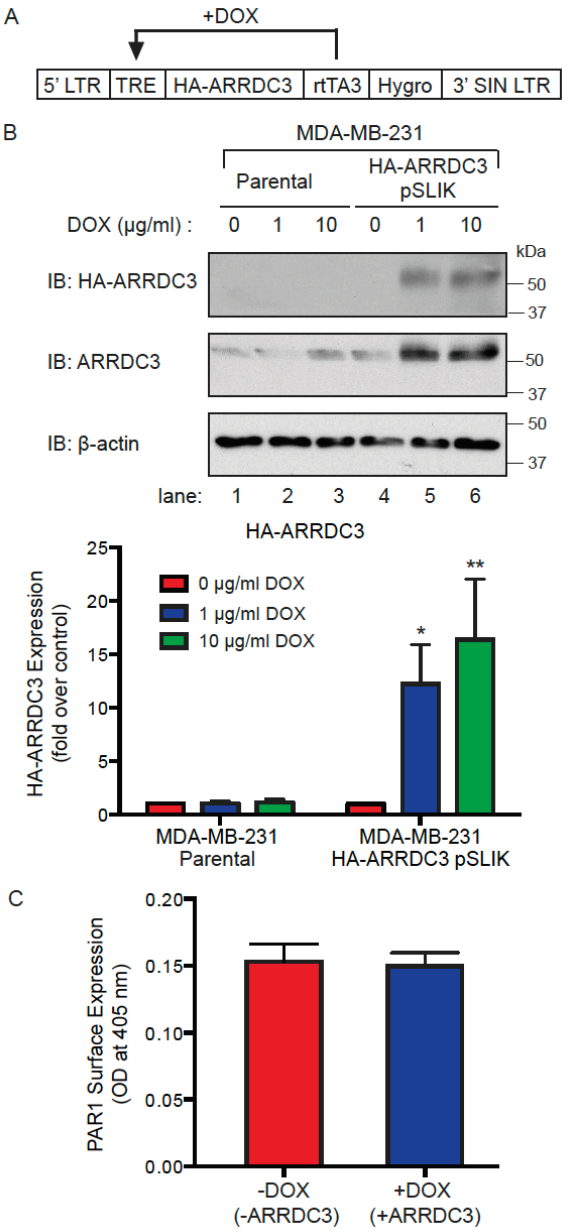

Figure 1.2: Induction of ARRDC3 expression in MDA-MB-231 breast carcinoma cell line using pSLIK vector system. *A,* Schematic of the tetracycline inducible single lentivector for inducible knockdown (pSLIK) system for HA-tagged ARRDC3 expression. *B,* Cell lysates from MDA-MB-231 parental and HA-ARRDC3 pSLIK expressing cells were collected after 48 h of incubation with DOX treatment at 0, 1 μg/ml, or 10 μg/ml. Cell lysates were immunoblotted to detect endogenous ARRDC3 or HA-ARRDC3 expression. β-actin expression was detected as a control. The data shown (mean ± S.D., n=3) were quantified by densitometry and shown as the fold change in ARRDC3 expression relative to 0 min control following doxycycline incubation. Statistical significance was determined by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; n=3). *C,* Expression of PAR1 on the cell surface was determined before and after DOX (1 μg/ml) treatment for 48 h and determined by ELISA. The data (mean ± S.D., n=3) shown as optical density (O.D.) determined at 405 nm are representative of three independent experiments.

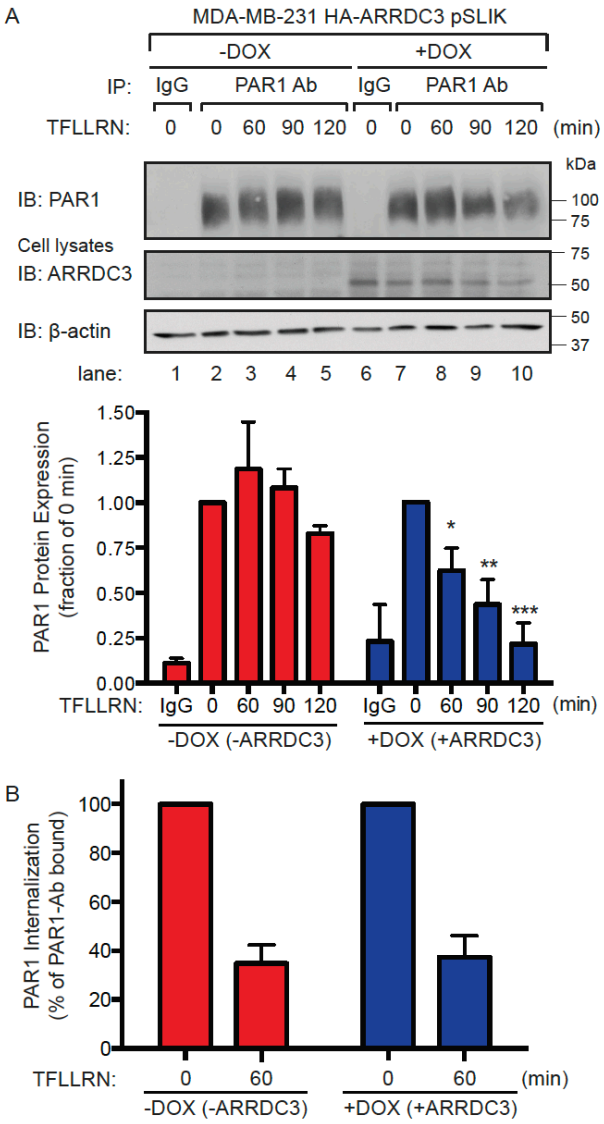

Figure 1.3: Agonist peptide-induced PAR1 lysosomal degradation is restored in cells re-expressing ARRDC3. *A,* MDA-MB-231 HA-ARRDC3 pSLIK cells were treated with or without 1 μg/ml DOX for 48 h. Cells were then stimulated with 100 μM TFLLRN peptide agonist for the indicated times, lysed and IP'ed using the anti-PAR1 WEDE antibody or anti-IgG antibody as a control. IPs were immunoblotted with anti-PAR1 rabbit polyclonal antibody. Cell lysates were immunoblotted with anti-HA-antibody to detect ARRDC3. β-actin expression was determined as a control. The data shown (mean ± S.D., n=3) were quantified by densitometry and represented as PAR1 expression relative to unstimulated control (0 min). Statistical significance determined by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n=3). *B,* MDA-MB-231 HA-ARRDC3 pSLIK cells were treated with or without 1 μg/ml DOX for 48 h, labeled with anti-PAR1 WEDE antibody at 4°C and then stimulated with or without 100 μM TFLLRN for the indicated times. Cells were then fixed and the amount of cell surface PAR1 determined by ELISA. The data (mean ± S.D., n=3) are represented as the percentage of PAR1 remaining on the cell surface relative to untreated control (0 min) and representative of three independent experiments.

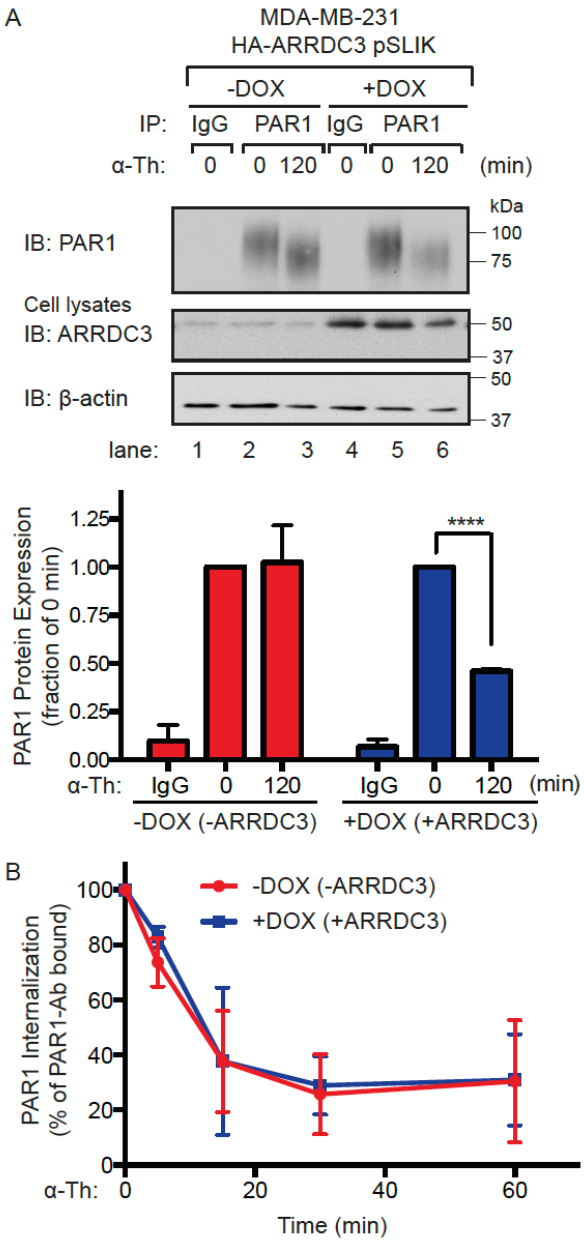

Figure 1.4: Thrombin-induced PAR1 lysosomal degradation is restored in cells re-expressing ARRDC3. *A,* MDA-MB-231 HA-ARRDC3 pSLIK cells incubated with or without 1 μg/ml DOX for 48 h were treated with 10 nM α-thrombin for the indicated times, lysed and IP'ed with anti-PAR1 WEDE antibody or anti-IgG control. Cell lysates were immunoblotted for HA-ARRDC3 and β-actin expression. The data (mean ± S.D., n=3) are represented as the fraction of PAR1 protein remaining relative to 0 min control. Statistical significance determined using an unpaired *t*- test (****, $P < 0.0001$; n=3). *B,* MDA-MB-231 HA-ARRDC3 pSLIK cells were incubated with or without 1 μg/ml DOX for 48 h, pre-labeled with anti-PAR1 WEDE antibody at 4°C and then treated with or without 10 nM α-thrombin. The amount of PAR1 remaining on the cell surface was determined by ELISA. The data (mean ± S.D., n=3) are expressed as the percent of PAR1 remaining relative to 0 min control and representative of three independent experiments.

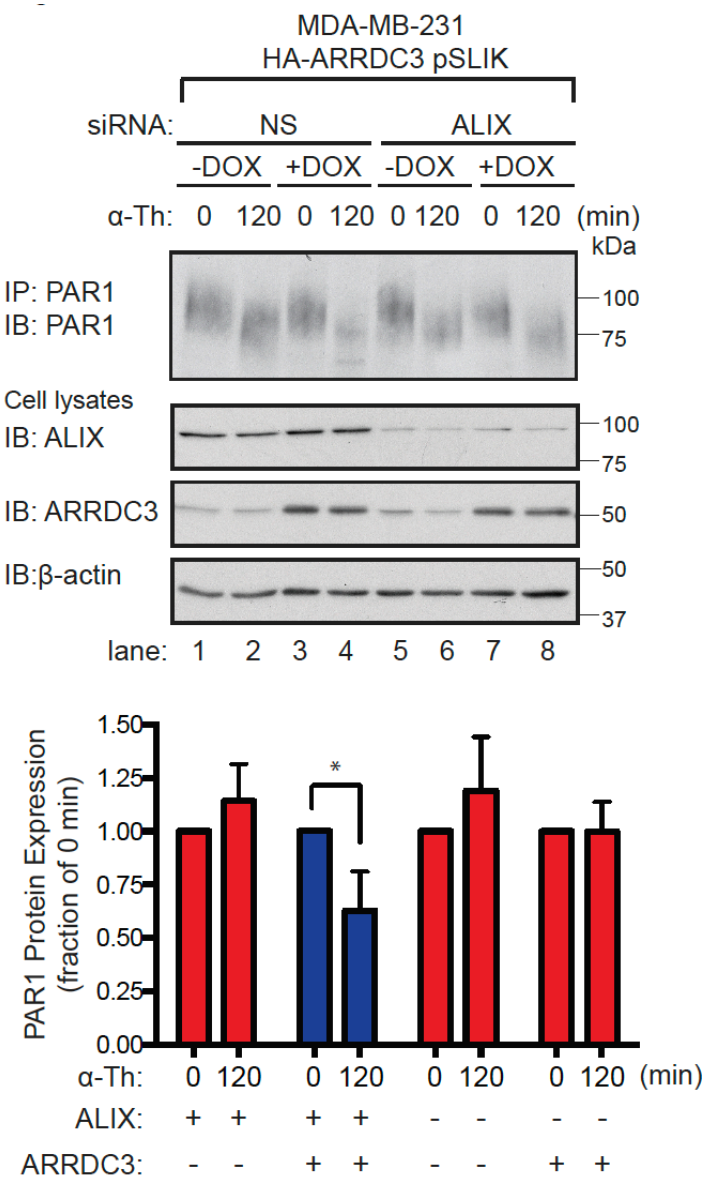

Figure 1.5: ALIX is required for ARRDC3-mediated degradation of activated PAR1. MDA-MB-231 HA-ARRDC3 pSLIK cells were transfected with 25 nM non-specific (NS) or 25 nM of ALIX siRNA using a combination of 12.5 nM ALIX #1 and 12.5 nM ALIX #3 siRNAs and then incubated with or without 1 μg/ml DOX for 48 h. Cells were then stimulated with 10 nM α-thrombin for the indicated times, lysed and IP'ed using the anti-PAR1 WEDE antibody. IPs were immunblotted with anti-PAR1 rabbit antibody to detect PAR1 expression. ALIX, HA-ARRDC3 and β-actin expression were determined by immunoblotting cell lysates. The data (mean ± S.D., n=3) are represented as the fraction of PAR1 remaining relative to 0 min control and representative of three independent experiments. Statistical significant was determined by unpaired *t*-test (*, $P < 0.05$; n=3).

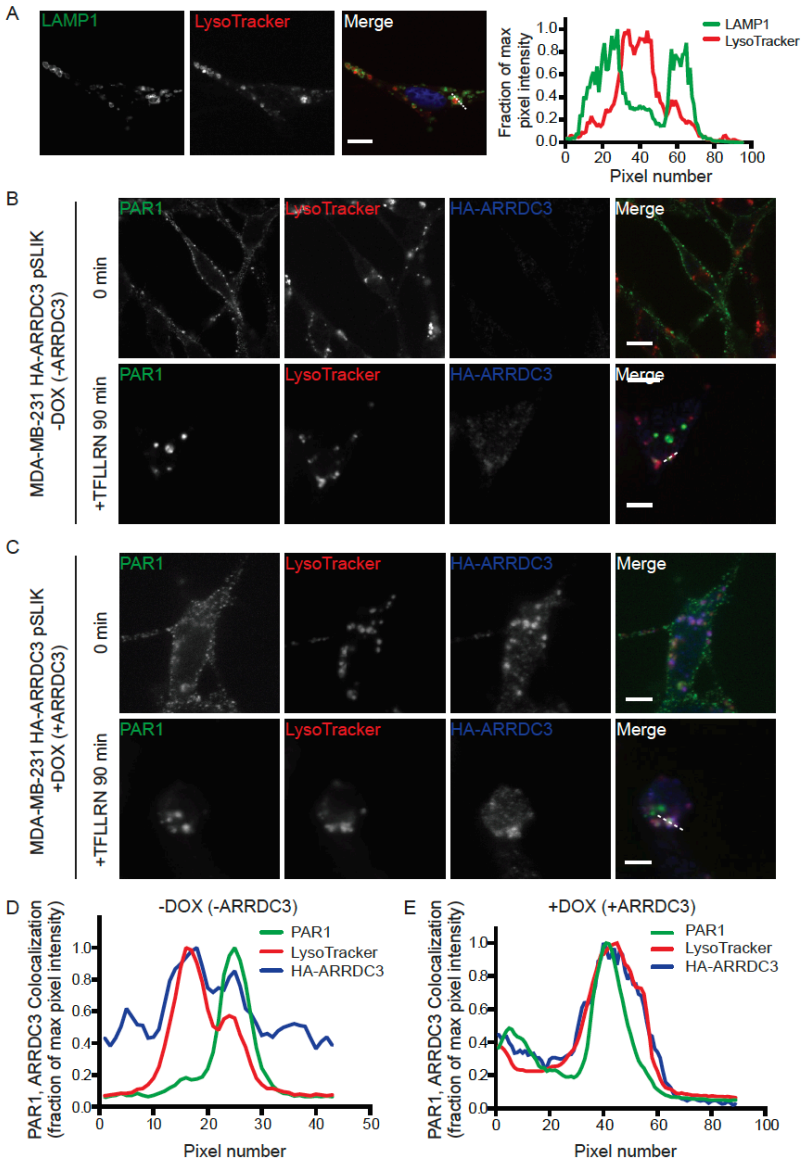

Figure 1.6: ARRDC3 expression is required for activated PAR1 lysosomal trafficking. *A,* MDA-MB-231 HA-ARRDC3 pSLIK cells incubated with 100 nM LysoTracker were fixed, processed, immuno-stained for LAMP1 and imaged by confocal microscopy. Images are represented of many cells examined in three independent experiments, scale bars = 10 um. Line scan analysis of the white dotted line region is plotted as the fraction of maximum pixel intensity versus pixel number (distance) and demonstrates LysoTracker (*red*) accumulates in the lumen of LAMP1-positive lysosomes (*green*). MDA-MB-231 HA-ARRDC3 pSLIK cells treated without (*B*) or with (*C*) 1 µg/ml DOX for 48 h were incubated with 2 mM leupeptin and 100 nM LysoTracker at 37°C. Cells were then incubated with anti-PAR1 antibody to label the surface cohort, stimulated with 100 µM TFLLRN, fixed, processed and imaged by confocal microscopy. Images are representative of many cells examined in three independent experiments. Scale bars, 10 µm. Line-scan analysis of the white dotted line region is plotted as described above and indicate that activated PAR1 (*green*) accumulates in the lumen of LAMP1-positive lysosomes (*red*) in presence of ARRDC3 (*blue*) *E*, but not in lysosomes in the absence of ARRDC3 expression, *D*.

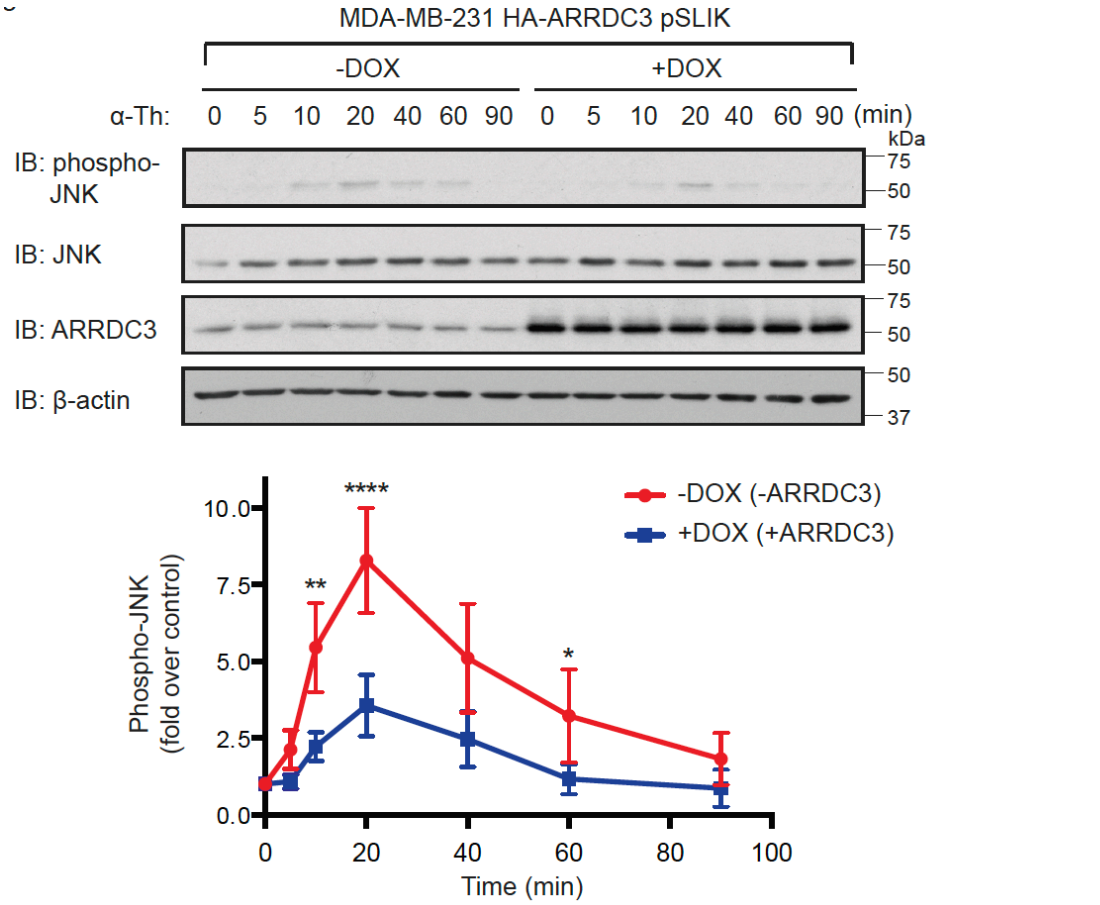

Figure 1.7: ARRDC3 re-expression attenuates PAR1-stimulated JNK signaling. MDA-MB-231 HA-ARRDC3 pSLIK cells treated with or without 1 μg/ml DOX for 48 h were stimulated with 10 nM α-thrombin for the indicated times. Cells were lysed, immunoblotted for phospho-JNK1/2, total JNK, HA-ARRDC3 and β-actin expression. The data shown (mean ± S.D., n=3) were quantified by densitometry and represented as the fold-increase in JNK1/2 phosphorylation relative to 0 min control. Statistical significance was determined by one-way ANOVA (**, $P < 0.01$; ****, $P < 0.0001$; n=3).

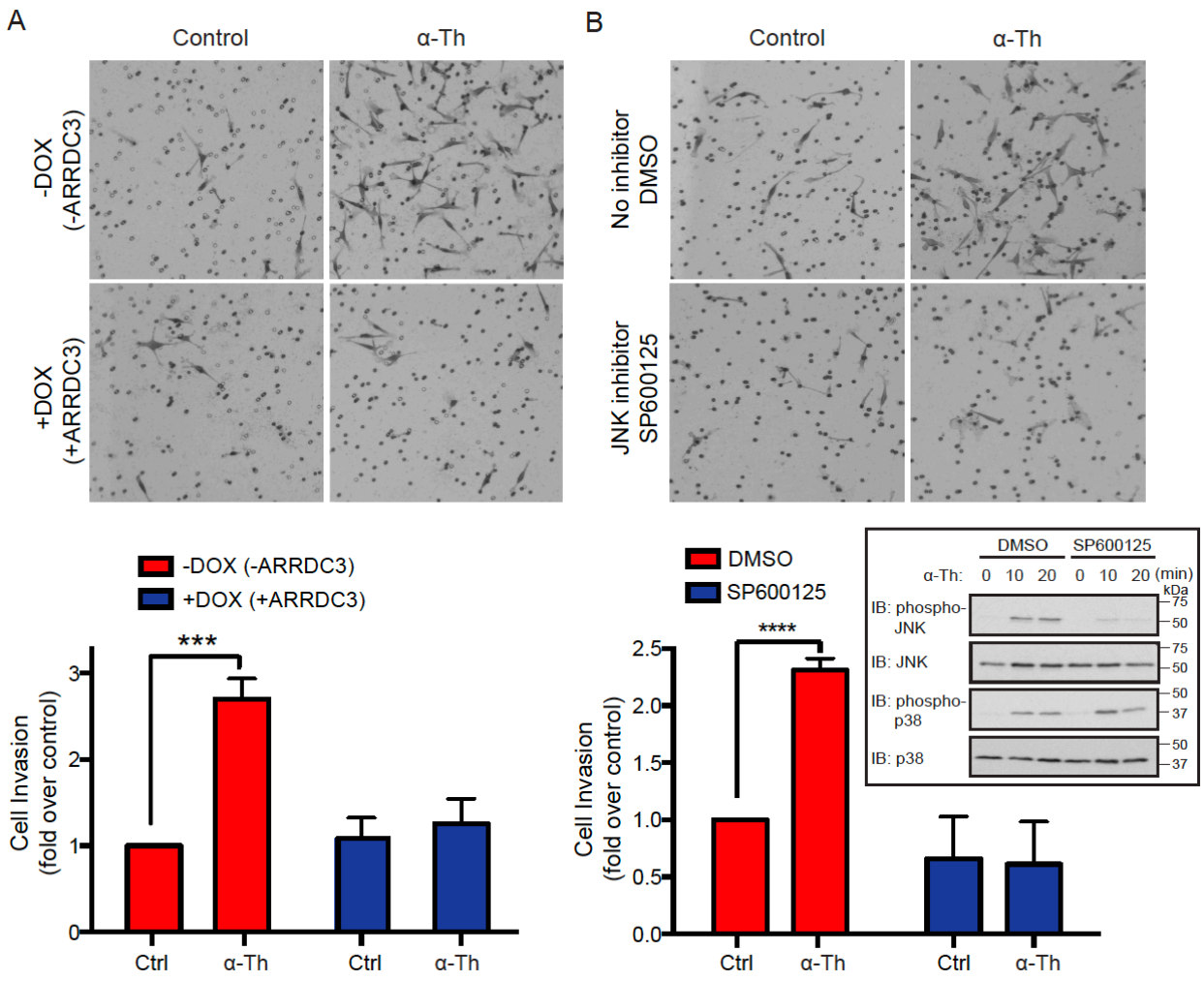

Figure 1.8: PAR1-stimulated breast carcinoma invasion is suppressed by ARRDC3 and JNK inhibition. *A,* MDA-MB-231 HA-ARRDC3 pSLIK cells treated with or without 1 μg/ml DOX for 48 h, were serum-starved cells, seeded onto transwells coated with matrigel and incubated with or without 1 pM α-thrombin (□-Th) for 5 h at 37°C. Cells were fixed, stained and imaged. Images shown are representative of three independent experiments. The data (mean ± S.D., n=3) were quantified from nine different fields of view at 10x magnification for each condition and represented as the fold change over untreated control cells. Statistical significance was determined by unpaired *t*-test (***, $P < 0.001$; n=3). *B,* MDA-MB-231 HA-ARRDC3 pSLIK cells were pre-incubated with DMSO or 20 μM SP600125 JNK inhibitor for 2 h at 37°C, seeded onto transwells coated with matrigel and then treated with 1 pM α-Th. Cells were processed and statistical significance determined by unpaired *t*-test (****, $P < 0.0001$; n = 3). The *inset* shows effects of DMSO and SP600125 on α-Th-stimulated phosphorylation of JNK and p38, cell lysates were immunoblotted for total JNK and p38 as a control.

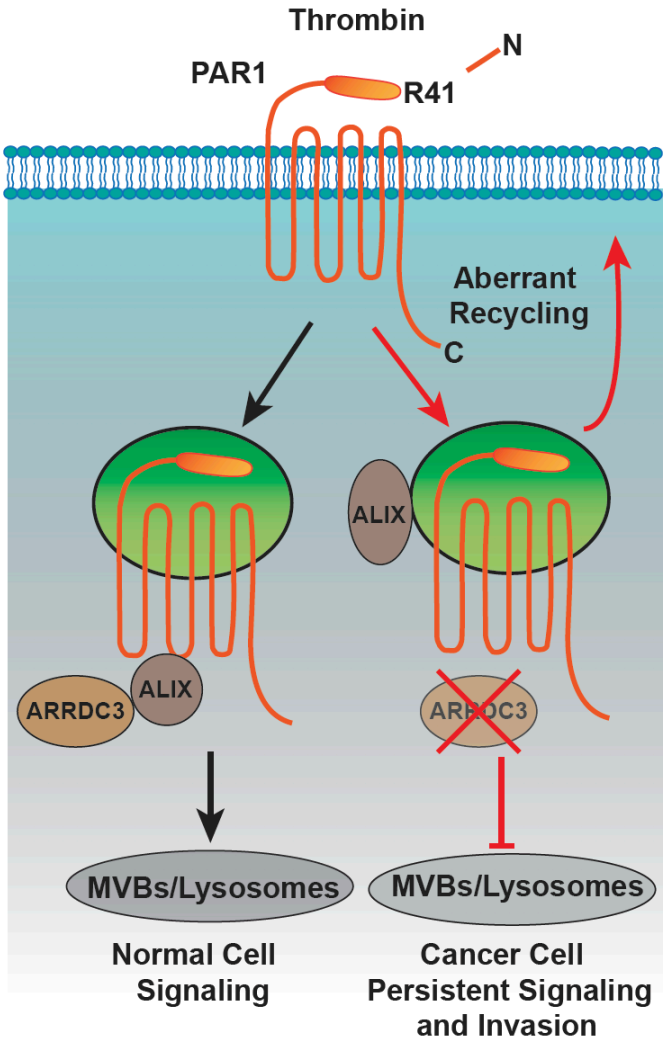

Figure 1.9: Model of ARRDC3 and PAR1 trafficking. Thrombin binds to and cleaves the PAR1 N-terminus at arginine (R)-41, exposing a new N-terminal domain that acts like a tethered ligand. Due to the irreversible proteolytic mechanism of PAR1 activation, internalization and lysosomal sorting is critical for termination of G protein signaling. Unlike most classic GPCRs, activated PAR1 is rapidly sorted from endosomes to lysosomes through a non-canonical pathway mediated by ARRDC3 and ALIX. In invasive breast cancer, activated PAR1 is internalized and recycled and fails to sort to lysosomes for degradation and consequently signals persistently which promotes tumor cell invasion and growth. We discovered that loss of ARRDC3 of expression in invasive breast cancer is responsible for defective PAR1 trafficking that results in persistent signaling and cellular invasion.

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CHAPTER 2: ARRDC3 functions as a metastasis suppressor by regulating GPCR activation of the Hippo pathway in breast cancer

2.1 Abstract

The α-arrestin domain containing protein-3 (ARRDC3) is a tumor suppressor, with reduced or lost expression in triple-negative breast carcinoma (TNBC). TNBC is highly aggressive, lacks druggable targets and results in higher rates of metastasis, recurrence and mortality. Thus, understanding the mechanisms and targets of ARRDC3 tumor suppressor function in TNBC is important. ARRDC3 regulates trafficking of protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR) implicated in breast cancer progression. Loss of ARRDC3 in TNBC causes overexpression of PAR1 and results in aberrant signaling. Moreover, dysregulation of GPCR-induced Hippo pathway signaling is associated with breast cancer progression. However, the mechanisms responsible for dysregulation of Hippo signaling induced by GPCRs are not known. Here, we report that the Hippo pathway transcriptional co-activator TAZ, and not YAP, is the major effector of PAR1 and other GPCR signaling and is required for PAR1-stimulated cell migration and invasion of TNBC. In addition, ARRDC3 suppresses PAR1-induced Hippo signaling via sequestration of TAZ, which occurs independent of ARRDC3-regulated PAR1 trafficking. The ARRDC3 C-terminal PPXY motifs and the TAZ WW domain are critical for mediating this interaction, and are required for TAZ cytoplasmic retention and suppression of TNBC migration and lung metastasis *in vivo*. These studies are the first to demonstrate a role for ARRDC3 in regulating GPCR-induced TAZ activity in TNBC, and further reveal multi-faceted tumor suppressor functions of ARRDC3.

2.2 Introduction

Basal-like triple-negative breast cancer (TNBC) is highly aggressive, lacks targeted treatment options and exhibits therapeutic resistance [1,2]. Arrestin-domain containing protein 3 (ARRDC3) is an emerging tumor suppressor for highly metastatic breast cancer, with lost or decreased expression due to gene deletion or epigenetic silencing in basal-like TNBC [3-5]. The loss of ARRDC3 expression correlates with increased breast cancer metastasis, tumor recurrence and poor prognosis [3]. In contrast, ARRDC3 overexpression in TNBC reverses epithelial-to-mesenchymal transition and reduces chemo-resistance [6]. However, the mechanism by which ARRDC3 exerts its tumor suppressor function in basal-like metastatic breast cancer is poorly understood.

The a-arrestin ARRDC3 is structurally similar to the multi-functional b-arrestin scaffold protein and possesses an arrestin-like N- and C-domain but differs by the presence of C-terminal PPXY motifs. The ARRDC3 PPXY motifs are known to mediate interaction with WW domains [7]. In basal-like breast carcinoma, ARRDC3 was previously reported to modulate trafficking of integrin β4 [8], a protein marker for poor prognosis enriched in TNBC [9]. Our group showed that ARRDC3 regulates trafficking of protease-activated receptor-1 (PAR1) [10,11], a G protein-coupled receptor (GPCR) implicated in breast cancer progression. PAR1 expression is markedly increased in breast cancer biopsies and correlates with metastasis and poor prognosis [12,13]. Overexpression of PAR1 also occurs in TNBC [10], due in part to defective lysosomal trafficking, resulting in persistent signaling, cellular invasion and tumor growth [10,14,15]. Intriguingly, PAR1 expression is high and ARRDC3 expression is low or absent in TNBC [8,10,13]. Moreover, re-expression of ARRDC3 is sufficient to rescue PAR1 defective lysosomal trafficking in TNBC and thus attenuates PAR1-mediated persistent signaling and invasion [10]. These studies indicate that ARRDC3 tumor suppressor function is linked to regulation of receptor trafficking, but whether ARRDC3 displays other tumor suppressor functions to control GPCR signaling in TNBC is not known.

The Hippo pathway is dysregulated in many cancers and triggers tumorigenesis, metastasis and drug resistance [16,17]. Core components of the Hippo pathway include the MST1/2 kinase, which phosphorylates LATS1/2 kinases that directly phosphorylates the transcriptional co-activators yes-associated protein (YAP) and WW-domain-containing transcription regulator protein-1 (TAZ), key effectors of the Hippo pathway. Phosphorylation of YAP and TAZ promotes cytoplasmic retention, whereas dephosphorylation triggers nuclear translocation and gene expression [16]. Hippo signaling is dynamically regulated by soluble factors that act mainly through GPCRs including PAR1, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) receptors [18-20]. Activation of PAR1 with the peptide agonist TRAP6 causes YAP and TAZ dephosphorylation, nuclear translocation and both were required for induction of gene expression [18]. While YAP and TAZ have previously been shown to function largely redundantly [21], recent studies suggest an emerging role for TAZ in invasive breast cancer. Increased TAZ expression and activity is associated with high-grade human breast cancers, metastasis and correlates with poor prognosis [22-24]. Although GPCRs activate both YAP and TAZ, it is not known if YAP and TAZ have distinct or overlapping functions in Hippo pathway signaling induced by GPCRs in TNBC.

Here we examined whether YAP and TAZ activation exert redundant or distinct functions in GPCR-stimulated Hippo signaling and whether ARRDC3 regulates GPCR-induced Hippo signaling in TNBC. Our studies demonstrate that TAZ, and not YAP, is the major effector of GPCR-stimulated Hippo signaling in TNBC. Remarkably, we further show that ARRDC3 binds to and suppresses TAZ, and not YAP, activation, resulting in reduction of gene expression, breast carcinoma migration and metastasis *in vivo*. Thus, ARRDC3 inhibits GPCR-induced TAZ activity independent of receptor trafficking, indicating that ARRDC3 is a multifunctional tumor suppressor protein in TNBC.

2.3 Materials & Methods

Reagents and Antibodies - Human α-thrombin was obtained from Enzyme Research Technologies (South Bend, IN). Vorapaxar (SCH530348, #1755) was purchased from Axon Medchem (Reston, VA) and tetracycline-free FBS (#631101) from Takara Bio USA. Dabigatran (α-thrombin inhibitor) was from Boehringer Ingelheim Pharma (Biberach, Germany). Rabbit anti-phospho-YAP S127 (#4911), rabbit anti-YAP (#14074), rabbit anti-phospho-TAZ S89 (#59971), rabbit anti-TAZ (#4883), rabbit anti-MST1 (#3682), rabbit anti-MST2 (#3952), rabbit anti-SAV1 (#13301), rabbit anti-MOB1 (#13730), rabbit anti-β-TRCP (#4394), rabbit anti-phospho-JNK1/2 (#9251), mouse anti-JNK1 (#3708), rabbit anti-phospho-LATS1 S909 (#9157), rabbit anti-LATS1 (#3477), mouse HA-tag Alexa Fluor 488 Conjugate (#2350), rabbit anti-pan-TEAD (#13295), rabbit anti-HA (#3724) and normal Rabbit IgG (#2729) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (#A-21244), goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (#A-11008), DAPI (#D-106), and ProLong Gold Antifade Mountant (#P36930) and doxycycline chow (200 mg/kg doxycycline pellets, #14727450) were purchased from ThermoFisher Scientific (Waltham, MA). Mouse monoclonal anti-CTGF E-5 (#sc-365970) and monoclonal anti-ALIX antibody (#sc-53538) were from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit polyclonal anti-ANKRD1 antibody (#11427-1-AP) was purchased from Proteintech (Rosemont, IL). Monoclonal anti-β-actin antibody (#A5316), SLIGKV peptide (#S-9063), rat tail collagen (#C3897) and anti HA-peroxidase (3F10) were purchased from Sigma-Aldrich (Carlsbad, CA). 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). Doxycycline hydrochloride (#J67043) was purchased from Alfa Aesar (Haverhill, MA). Mouse monoclonal anti-HA antibody (HA.11) (#MMS-101R) was purchased from Covance (Princeton, New Jersey) and lysophosphatidic acid (#3854) and shingosine-1-phosphate (#1370) were purchased from Tocris Bioscience (Minneapolis, MN). Anti-GAPDH

antibody (#GTX627408) was purchased from GeneTex (Irvine, CA). Protein A-Sepharose CL-4B beads were from GE Healthcare. Mouse IgG (#010-0102) were purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse anti-rabbit IgG, light chain specific (#211-032-171) and goat anti-mouse IgG, light chain specific (#115-035-174) was purchased from Jackson Immuno Research Laboratories (West Grove, PA).

Cell Culture and Transfections - MDA-MB-231 cells were maintained in Leibowitz-15 media supplemented with 10% fetal bovine serum (v/v). MDA-MB-231 HA-ARRDC3 WT and AAXA pSLIK cells were generated as previously described [10] and cultured in Leibowitz-15 media supplemented with 10% tetracycline-free FBS. All cell lines were purchased from ATCC and grown according to ATCC instructions. siRNA transfections were performed using Oligofectamine (Life Technologies) per the manufacturer's instructions. Signaling, migration and invasion assays described were performed 48 h after transfection.

siRNA Sequences - All single siRNAs were purchased from Qiagen: non-specific (ns) siRNA sequence with concentration following, 5'-CUACGUCCAGGAGCGCACC-3' (25 nM); YAP #1 target sequence, 5'-AAGACATCTTCTGGTCAGAGA-3' (25 nM); YAP #5 target sequence, 5'-CAGGTGATACTATCAACCAAA-3' (25 nM); TAZ #1 target sequence, 5'-CTGCGTTCTTGTGACAGATTA-3' (25 nM); TAZ #4 target sequence, 5'-ACAGTAGTACCAAATGCTTTA-3' (25 nM); ALIX #1 target sequence, 5′-AAGTACCTCAGTCTATATTGA-3′ (12.5 nM); ALIX #3 target sequence, 5′-AATCGAGACGCTCCTGAGATA-3′ (12.5 nM). For all conditions, cells were transfected with one siRNA with the exception of ALIX which was treated with ALIX #1 and ALIX #3 siRNAs.

Immunofluorescence Confocal Microscopy - MDA-MB-231 cells were serum-starved overnight, treated with 2 mM leupeptin for 1 h at 37°C, stimulated with α-thrombin and processed and imaged as previously described [10]. Slides were immunostained with anti-HA Alexa Fluor 488 conjugate antibody, DAPI to stain nuclei and either anti-YAP or anti-TAZ antibody. The ratio of nuclear to cytoplasmic YAP and TAZ localization was quantified using the

ImageJ intensity ratio nuclei cytoplasm tool on six to ten fields of view for each condition, from three biological independent replicates. When quantifying the percentage of cells displaying equal nuclear and cytoplasmic staining (N=C), cells showing 45-55% ratio nuclear-cytoplasmic were considered equal.

Signaling Assays and Immunoblotting - Signaling assays were performed essentially as described previously; briefly, cells were serum-starved overnight then treated with agonist for the indicated times [10]. To assess persistent signaling, media was changed with added dabigatran (α-thrombin inhibitor) after 30 min of α-thrombin treatment. Cell lysates were collected and immunoblotted as previously described [10]. Immunoblots were quantified by densitometry using ImageJ software (NIH, Bethesda, MD).

Quantitative RT-PCR - Cells were collected in trizol when cells were about 80% confluent. RNA was extracted using Direct-zol RNA Miniprep Plus kit (#R2072, Zymo Research, Irvine, CA) and the SuperScript IV VILO Master Mix with ezDNase enzyme kit (#11766050, ThermoFisher Scientific) was used for reverse transcription PCR, according to the manufacturer's instructions. Note that 750 ng of total RNA was used for RT-PCR. Quantitative PCR was performed using the TaqMan Fast Advanced Master Mix (#4444964, ThermoFisher Scientific) in technical triplicate. Three biological independent replicates were performed and YAP and TAZ expression were normalized to S18 expression and compared to YAP expression in MDA-MB-231 cells. TaqMan Gene Expression Assay probes (Thermo Fisher Scientfic): YAP1, Hs00902712_g1; TAZ, Hs00210007_m1; 18S, Hs03003631_g1.

Immunoprecipitation - HEK293T cells were transiently transfected as described in the text. 48 h after transfection, cells were lysed with NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 10 μg/ml leupeptin, aprotinin, trypsin protease inhibitor, pepstatin, 100 μg/ml benzamide, 1 mM PMSF, 50 mM β-glycerophosphate, 20 mM NaF, 1 mM Na3VO4 and 20 mM *N*-ethylmaleimide). Cell lysates were homogenized by needle and syringe, and cleared by centrifugation. Supernatants were pre-cleared with Protein A-Sepharose beads and

protein concentrations determined by BCA assay (Thermo Fisher Scientific). Equal amounts of normalized lysates were immunoprecipitated with appropriate antibodies overnight at 4°C followed by incubation of BSA-blocked Protein-A beads for 2 h. Immunoprecipitates were washed and eluted in 2X Laemmli sample buffer containing 200 mM DTT. Cell lysates and immunoprecipitates were analyzed by immunoblotting. To assess TEAD interaction with YAP and TAZ, MDA-MB-231 pSLIK cells were treated with doxycycline and thrombin as described in the text, then collected and processed, as above.

Migration and Invasion Assays - Migration and invasion assays were performed essentially as previously described [10]. Migration assays were performed on membranes coated with rat tail collagen while invasion assays were performed with BioCoat Matrigel invasion chambers with or without α-thrombin or 0.5% FBS added. Cell migration and invasion was quantified by cell count in nine fields of view at 10X magnification for each condition, from three biological independent replicates.

Tail vein injection mouse model of metastasis - NSG mice were obtained from the University of California, San Diego in-house breeding colony. GFP-transduced MDA-MB-231 HA-ARRDC3 WT ($4x10^5$ cells in 50 μl PBS) or HA-ARRDC3 AAXA ($3x10^5$ cells in 50 μl PBS) pSLIK cells were pre-treated with or without doxycycline *in vitro* for 48 h then injected into the lateral tail vein of 5-8 week old NSG mice. Mice injected with cells that were pretreated with doxycycline were given or doxycycline chow (200 mg/kg doxycycline pellets) while mice not treated were given normal chow. Mice were sacrificed two weeks post-injection; lungs were imaged and metastatic nodules counted with the Zeiss Axio Zoom.V16 microscope. All procedures were performed according to an animal protocol approved by the UC San Diego Institutional Animal Care and Use Committee.

Data Analysis - Statistical significance was determined by unpaired *t*-test or one-way ANOVA using Prism 8.0 software (GraphPad). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

2.4 Results

2.4.1 Hippo pathway activation induced by thrombin requires PAR1

To define the role of PAR1 in Hippo signaling induced by its natural ligand thrombin, nuclear translocation and dephosphorylation of YAP and TAZ transcriptional co-activators were examined in TNBC MDA-MB-231 cells. In unstimulated serum-starved MDA-MB-231 cells, YAP and TAZ were localized mainly in the cytoplasm (**Fig. 1A-F**). Incubation with thrombin for 30 min caused significant translocation and nuclear accumulation of YAP (**Fig. 1A-C)** and TAZ (**Fig. 1D-F**) assessed by immunofluorescence confocal microscopy. The major initiator of YAP and TAZ nuclear translocation is dephosphorylation and was next assessed. Thrombin promoted a rapid and significant decrease in phosphorylation of YAP S127 and TAZ S89 at 30 min that was sustained for 2 h (**Fig. 1G-I**). However, in cells preincubated with vorapaxar, a PAR1 selective antagonist, thrombin-induced YAP S127 and TAZ S89 dephosphorylation was significantly inhibited (**Fig. 1G-I**). Thrombin also promoted a significant increase in connective tissue growth factor (CTGF) and ankyrin repeat domain 1 (ANKRD1) expression, major effectors of Hippo signaling in MDA-MB-231 cells (**Fig. 1G and J-K**), which was significantly reduced by vorapaxar (**Fig. 1G and J-K**). These results indicate that PAR1 is required for thrombin-induced Hippo signaling in TNBC.

2.4.2 TAZ and not YAP is the major effector of GPCR-stimulated Hippo signaling

High TAZ expression and activity has been implicated in breast cancer progression [22,23,25]. To understand whether TAZ alone, or along with other Hippo pathway components, correlates with invasiveness, we profiled a panel of invasive TNBC that exhibit high PAR1 expression and luminal non-invasive breast carcinoma that display low PAR1 expression [10,14]. In contrast to variable expression of Hippo pathway components including YAP observed in both invasive and non-invasive breast carcinoma, high expression of TAZ was detected primarily in TNBC (**Fig. 2A, lanes 6-9**) and not in luminal non-invasive or HER2-

positive breast carcinoma (**Fig. 2A, lanes 1-5 and 10**). The relative expression of YAP and TAZ mRNA transcript abundance was also determined by qPCR. There was no significant difference in TAZ and YAP mRNA transcript abundance in BT549 and Hs578T TNBC cells (**Fig. 2B**). However, a significant but modest increase in TAZ mRNA transcripts compared to YAP was detected in parental MDA-MB-231 cells (**Fig. 2B**). A similar difference in TAZ versus YAP mRNA abundance was observed in MDA-MB-231 cells stably expressing doxycycline-regulated HA-ARRDC3 single lentivector for inducible knockdown (pSLIK) (**Fig. 2B**), a cell system optimized to interrogate ARRDC3 function [10]. Thus, transcriptional and post-transcriptional processes likely regulate YAP and TAZ protein expression resulting in higher TAZ expression in TNBC compared to luminal breast carcinoma. PAR1 expression is similarly high in TNBC, whereas ARRDC3 expression is low or absent [10,14].

YAP and TAZ are thought to function redundantly in most cell types and are both expressed in TNBC (**Fig. 2A**) [21]. To determine if YAP and TAZ function redundantly in GPCR-stimulated Hippo signaling, induction of CTGF and ANKRD1 expression by thrombin-activated PAR1 was assessed in YAP and TAZ siRNA depleted MDA-MB-231 HA-ARRDC3 pSLIK cells. Knockdown of YAP expression with two different siRNAs failed to significantly affect thrombin-induced CTGF and ANKRD1 expression compared to non-specific siRNA control cells (**Fig. 2C-E**). Conversely, depletion of TAZ by siRNA caused significant inhibition of thrombin-stimulated expression of CTGF and ANKRD1 compared to non-specific siRNA control cells (**Fig. 2C-E**), suggesting that YAP and TAZ have distinct functions. TAZ and not YAP also emerged as the major effector of thrombin-induced Hippo pathway activation in parental MDA-MB-231 cells (**Supplementary Fig. S1A, B**). Next, we used Hs578T cells to determine if YAP and TAZ differentially regulate thrombin-promoted Hippo pathway activation in other TNBC. Similar to MDA-MB-231 cells, thrombin-stimulated CTGF expression was blocked by deletion of TAZ but not YAP in Hs578T cells (**Fig. 2F and G**). Thrombin failed to induce ANKRD1 expression in

Hs578T cells and was not examined (**Supplementary Fig. S1C, D**). These data indicate TAZ, and not YAP, is the major effector of thrombin-induced Hippo signaling in TNBC.

To determine if YAP and TAZ differentially function in Hippo signaling stimulated by other GPCRs in TNBC, we examined signaling by the LPA receptors (LPARs), PAR2, and S1P receptors (S1PRs) in MDA-MB-231 HA-ARRDC3 pSLIK cells. Similar to thrombin, activation of the LPA receptor with its cognate ligand induced a significant increase in CTGF expression in non-specific siRNA cells that was significantly inhibited in TAZ but not YAP knockdown cells (**Fig. 3A**). Depletion of TAZ was also more effective than knockdown of YAP expression at reducing LPA-stimulated induction of ANKRD1 expression (**Fig. 3A**). Similarly, cells treated with either SLIGKV, a PAR2 selective peptide agonist, or S1P also caused a marked increase in CTGF and ANKRD1 expression in the non-specific siRNA transfected cells and was significantly inhibited in TAZ but not YAP deficient cells (**Fig. 3B, C**). These findings indicate that TAZ, and not YAP, functions as the main effector of GPCR-stimulated Hippo signaling in TNBC.

The specific roles of YAP versus TAZ in thrombin-stimulated cell migration and invasion are not known and were examined in TNBC. Thrombin stimulated a significant increase in migration of control non-specific siRNA transfected cells, comparable to FBS-induced migration observed in MDA-MB-231 HA-ARRDC3 pSLIK cells (**Fig. 3D**). In contrast, thrombin-induced cell migration was significantly inhibited in TAZ depleted cells (**Fig. 3D**), whereas FBS-induced cell migration remained intact (**Fig. 3D**). Cell migration induced by thrombin or FBS was not impaired in YAP deficient cells (**Fig. 3D**), consistent with a role for TAZ and not YAP. Moreover, thrombin caused a significant increase in invasion of control non-specific siRNA cells (**Fig. 3E**), that was similar in YAP depleted cells (**Fig. 3E**). However, loss of TAZ expression significantly inhibited thrombin-stimulated breast carcinoma cell invasion (**Fig. 3E**). Thus, YAP and TAZ differ in their capacity to regulate PAR-induced breast carcinoma cell migration and invasion, with TAZ emerging as the key effector of GPCR-stimulated Hippo signaling in TNBC.

2.4.3 ARRDC3 suppresses GPCR-stimulated Hippo signaling independent of receptor trafficking

The loss of ARRDC3 tumor suppressor function is essential for PAR1 promoted breast cancer progression [10], raising the possibility that ARRDC3 may regulate Hippo pathway signaling induced by PAR1. Since ARRDC3 expression is lost or suppressed in TNBC [3,8,10], MDA-MB-231 cells stably expressing HA-ARRDC3 pSLIK were used to allow doxycycline-inducible expression of HA-ARRDC3. In control MDA-MB-231 HA-ARRDC3 pSLIK cells not treated with doxycycline and deficient in ARRDC3, incubation with thrombin caused a marked increase in CTGF and ANKRD1 expression (**Fig. 4A**). However, thrombin-stimulated CTGF and ANKRD1 expression was significantly inhibited in doxycycline-induced ARRDC3-expressing cells (**Fig. 4A**), suggesting that ARRDC3 suppresses Hippo signaling. Similar to thrombin-activated PAR1, ARRDC3 inhibited induction of CTGF and ANKRD1 expression by ligands acting at the LPARs, PAR2 and S1PRs (**Fig. 4B-D**). Thus, ARRDC3 regulates Hippo signaling induced by multiple GPCR ligands in TNBC.

ARRDC3 functions as a tumor suppressor by facilitating PAR1 lysosomal trafficking through an ALIX dependent pathway in invasive breast carcinoma (**Fig. 5A**). Both ARRDC3 and ALIX prevent activated PAR1 recycling to the plasma membrane and thus inhibit $G\alpha_{12/13}$-mediated JNK signaling [10], since loss of either one blocks lysosomal degradation. ARRDC3 and ALIX are both required for activated PAR1 lysosomal trafficking (**Fig. 5A**) [10,11]. To determine if ARRDC3 suppresses activated PAR1-stimulated Hippo signaling independent of receptor trafficking, we examined whether blockade of activated PAR1 lysosomal trafficking by siRNA knockdown of ALIX results in decreased Hippo signaling in the presence of ARRDC3 (Fig. 5A). MDA-MB-231 HA-ARRDC3 pSLIK cells transfected with non-specific siRNA or ALIX-specific siRNAs were treated with or without doxycycline to induce ARRDC3 expression followed by thrombin stimulation. MDA-MB-231 cells transfected with non-specific siRNA expressing ALIX and not ARRDC3 were stimulated with thrombin and showed a significant

increase in JNK phosphorylation as well as induction of CTGF and ANKRD1 expression (**Fig. 5B, lanes 1-3, and C-E**). As expected, re-expression of ARRDC3 in cells expressing ALIX significantly reduced thrombin-stimulated JNK phosphorylation (**Fig. 5B, lanes 4-6 and C-E**), which has been attributed to restoration of PAR1 lysosomal trafficking [10], and further attenuated induction of CTGF and ANKRD1 expression (**Fig. 5B, lanes 4-6 and C-E**). In cells lacking both ALIX and ARRDC3 expression, thrombin caused a significant increase in JNK phosphorylation and induction of CTGF and ANKRD1 expression (**Fig. 5B, lanes 7-9 and C-E**). However, in cells lacking ALIX, re-expression of ARRDC3 retained the capacity to suppress thrombin-induced CTGF and ANKRD1 expression, despite blockade of PAR1 lysosomal trafficking resulting in enhanced JNK phosphorylation (**Fig. 5B, lanes 10-12 and C-E**). These findings indicate that ARRDC3 regulates PAR1-stimulated Hippo signaling via a mechanism that is independent of ALIX and receptor trafficking in TNBC.

2.4.4 ARRDC3 suppresses thrombin-induced TAZ and not YAP dephosphorylation, nuclear translocation

Hippo pathway activation occurs via a core kinase cascade that phosphorylates and activates LATS1/2, which phosphorylates and inactivates YAP and TAZ, but how ARRDC3 integrates into the Hippo pathway is not known and was examined in MDA-MB-231 HA-ARRDC3 pSLIK cells. In cells with or without ARRDC3 expression, basal phosphorylation of LATS1, YAP and TAZ was observed in unstimulated cells (**Fig. 6A, lanes 1 and 5)**, indicating that Hippo signaling is turned on. After thrombin incubation, a significant decrease in phosphorylation of LATS1 as well as YAP and TAZ was detected in cells lacking ARRDC3 expression (**Fig. 6A, lanes 1-5, and B-D**). However, in ARRDC3-expressing cells, thrombin stimulated TAZ dephosphorylation was significantly blocked with no difference in LATS and YAP dephosphorylation kinetics compared to control cells (**Fig. 6D, lanes 6-10 and B-D**),

suggesting that ARRDC3 regulates thrombin-induced Hippo signaling by controlling TAZ activity in invasive breast carcinoma.

TAZ contains a single WW domain that mediates protein-protein interaction with PPXY motifs, whereas ARRDC3 contains two C-terminal PPXY motifs that bind to WW domains (**Fig. 6E**). However, it is not known if ARRDC3 and TAZ co-associate and was examined in HEK293T cells. Wild type TAZ and HA-ARRDC3 showed robust co-association in anti-HA co-immunoprecipitates (co-IPs) but not in IgG control (**Fig. 6F, lanes 1, 2**). Neither ARRDC3 nor TAZ were detected in co-IPs in cells expressing only TAZ (**Fig. 6F, lane 5**), indicating specific interaction. In contrast, wild type TAZ failed to interact with the HA-ARRDC3 AAXA double mutant, where the critical prolines (P) and tyrosine (Y) of the C-terminal PPXY motifs were converted to alanine (A) compared to wild type ARRDC3 (**Fig. 6E, F, lanes 2, 3 and G**), suggesting that the PPXY motifs are critical for ARRDC3-TAZ interaction. Moreover, deletion of the single WW domain of TAZ also resulted in a marked loss of interaction with ARRDC3 wild type (**Fig. 6E, F, lanes 2, 4 and G**). Thus, ARRDC3 and TAZ interaction occurs via WW domain interaction with PPXY motifs.

To determine if ARRDC3 regulates TAZ function via the PPXY motifs, thrombin-induced TAZ dephosphorylation and nuclear translocation was examined in MDA-MB-231 expressing HA-ARRDC3 wildtype or AAXA mutant. In contrast to wild type ARRDC3, which suppresses TAZ dephosphorylation (**Fig. 6A, C**), thrombin-induced TAZ dephosphorylation was not affected in ARRDC3 AAXA mutant expressing cells (**Fig. 6H, lanes 5-8, and I**). As expected, ARRDC3 AAXA expression failed to affect thrombin-stimulated YAP de-phosphorylation (**Supplemental Fig. S2A, B**). These results suggest that ARRDC3 interaction with TAZ suppresses dephosphorylation induced by thrombin. Nuclear localization of TAZ stimulated by thrombin was also significantly inhibited by wild type ARRDC3 (**Fig. 6J, K and Supplementary Fig. S2C-D**). In contrast to wildtype ARRDC3, the ARRDC3 AAXA mutant failed to block thrombin-stimulated TAZ nuclear translocation (**Fig. 6L, M)**). Neither ARRDC3 wildtype nor AAXA mutant expression

had any effect on thrombin-stimulated nuclear translocation of YAP (**Supplementary Fig. S2E-H**).

2.4.5 ARRDC3-TAZ interaction inhibits TAZ-TEAD binding and is required for suppression of thrombin-induced CTGF and ANKRD1 expression and cell migration

After desphosphorylation and translocation to the nucleus, YAP and TAZ, which lack DNA binding motifs, interact with TEA domain family members (TEAD) 1-4 DNA binding transcription factors to regulate gene transcription (Zhao Genes Dev 2007, Lamar PNAS 2012). To determine if ARRDC3 regulates YAP or TAZ activity, TEAD binding to YAP and TAZ was examined in MDA-MB-231 HA-ARRDC3 pSLIK cells by co-IP. In cells lacking ARRDC3 expression, thrombin-induced a significant increase in YAP-TEAD and TAZ-TEAD interaction (**Fig. 7A, lanes 1-4 and B,C**). While thrombin-induced YAP-TEAD interaction was retained in wild type ARRDC3-expressing cells (**Fig. 7A, lanes 5-8 and B**), TAZ-TEAD interaction was significantly inhibited by wild type ARRDC3 (**Fig. 7A, lanes 5-8 and C**). Contrary to the effect of WT ARRDC3 re-expression on TAZ-TEAD binding, expression of ARRDC3 AAXA mutant showed significant thrombin-induced increase in both YAP-TEAD and TAZ-TEAD interaction (**Fig. 7D-F**), suggesting that the PPXY motifs facilitating ARRDC3-TAZ binding are necessary to block subsequent thrombin-mediated TAZ-TEAD binding.

Next, the functional consequences of ARRDC3 regulation of TAZ was examined in MDA-MB-231 HA-ARRDC3 pSLIK cells. As shown previously, thrombin-induced significant expression of the YAP/TAZ targeted genes CTGF and ANKRD1 in cells lacking ARRDC3 expression (**Fig. 7A, lanes 1-5 and B,C**), whereas expression of ARRDC3 resulted in significant inhibition of thrombin-induced CTGF and ANKRD1 expression (**Fig. 7A, lanes 6-10, and B,C**). In contrast, expression of ARRDC3 AAXA mutant failed to block thrombin-induced CTGF and ANKRD1 expression (**Fig. 7D, lanes 5-8 and E, F**), compared to the response observed in cells lacking ARRDC3 (**Fig. 7D, lanes 1-4 and E, F**). Thus, the suppression of TAZ

activation by ARRDC3 is sufficient to block CTGF and ANKRD1 gene expression, indicating that YAP function is not necessary for thrombin-induced Hippo signaling in invasive breast carcinoma.

The effect of ARRDC3 interaction with TAZ on breast carcinoma cell migration was also examined using MDA-MB-231 HA-ARRDC3 AAXA mutant pSLIK cells. In cells lacking ARRDC3 induction, thrombin stimulated a significant increase in cellular migration (**Fig. 7G**), which was similarly observed in cells incubated with FBS (**Fig. 7G**). However, in wild type ARRDC3-expressing cells thrombin-induced cell migration was markedly reduced compared to FBS-promoted cellular migration, which remained intact in cells expressing ARRDC3 (**Fig. 7G**). In contrast, expression of the ARRDC3 AAXA mutant defective in TAZ binding failed to block thrombin-stimulated cell migration (**Fig. 7H**), whereas FBS induced cell migration remained intact and was comparable to that observed in ARRDC3 deficient cells (**Fig. 7H**). These results indicate that ARRDC3 suppresses thrombin-induced TAZ-dependent breast carcinoma cell migration.

2.4.6 ARRDC3-TAZ interaction is required for suppression of breast carcinoma metastasis

The role of ARRDC3 in breast tumor metastasis is not known and was examined using a tail-vein injection model. MDA-MB-231 HA-ARRDC3 wild type and AAXA mutant pSLIK cells transduced with GFP and pre-treated with or without doxycycline for 48 h *in vitro* were injected into the tail-vein of immunocompromised NSG mice. Mice injected with pre-treated doxycycline cells were fed doxycycline chow to induce ARRDC3 expression while mice injected with non-treated cells were fed normal chow, and lung metastasis was quantified by immunofluorescence microscopy. After 2 weeks, a high metastatic tumor burden was observed with a large number of GFP-positive nodules detected in the lung tissue of mice injected with control HA-ARRDC3 wild type pSLIK cells and not treated with doxycycline (**Fig. 8A, B**). In contrast, the number of

detected metastatic nodules were significantly reduced in doxycycline-treated mice injected with wild type ARRDC3 pSLIK cells (**Fig. 8A, B**), suggesting that ARRDC3 suppresses metastasis. Control mice injected with HA-ARRDC3 AAXA mutant pSLIK cells and not treated with doxycycline also exhibited high tumor burden (**Fig. 8A, B**). However, unlike mice injected with wild type HA-ARRDC3 pSLIK cells, mice injected into the tail-vein with ARRDC3 AAXA mutant pSLIK cells and treated with doxycycline formed abundant metastatic foci (**Fig. 8A, B**), suggesting that the ARRDC3 AAXA mutant fails to suppress breast carcinoma metastasis. Induction of HA-ARRDC3 wildtype and AAXA mutant expression in MDA-MB-231 pSLIK cells was confirmed from in cells taken from the pool of cells prior to injection treated with doxycycline (**Fig. 8C**). Collectively, these *in vivo* metastasis results combined with the cellular and biochemical data above indicate that ARRDC3 functions specifically to inhibit TAZ and not YAP activity induced by GPCRs resulting in suppression of Hippo-mediated induction of CTGF and ANKRD1 expression, cell migration and breast carcinoma metastasis *in vivo* (**Fig. 8D**).

2.5 Discussion

Basal-like TNBC remains a critical subtype contributing to breast cancer mortality due to its high metastatic potential and lack of molecular targets [1,2]. GPCRs, including PAR1, play significant roles in breast cancer progression yet are currently underutilized as therapeutic targets [20,26,27]. Hippo signaling, which normally prevents YAP and TAZ activation, is turned-off predominantly by GPCRs including PAR1, LPARs, PAR2 and S1PRs to promote proliferation and invasion [18,19]. However, the mechanisms responsible for dysregulation of the Hippo signaling induced by GPCRs in TNBC is not known. Here, we show that the transcriptional co-activator TAZ, and not YAP, is the major effector of GPCR-induced Hippo signaling in TNBC and promotes cell migration and invasion. We further demonstrate that ARRDC3 suppresses GPCR-induced Hippo signaling through TAZ, which occurs independent of ARRDC3 regulation on receptor trafficking. The ARRDC3 C-terminal PPXY motifs mediate interaction with the WW

domain of TAZ, resulting in TAZ cytoplasmic retention and inhibition of Hippo signaling. Our study also indicates that the capacity of ARRDC3 to suppress breast carcinoma migration and metastasis *in vivo* is dependent on ARRDC3 engagement with TAZ. Thus, ARRDC3 exhibits multiple tumor suppressor functions including regulation of receptor trafficking and control of GPCR-induced activity of TAZ in TNBC.

GPCRs preferentially signal via TAZ, and not YAP, in TNBC. Although YAP and TAZ are largely functionally redundant, TAZ has emerged as an important driver of breast cancer progression. Both overexpression of TAZ and nuclear localization, indicative of high TAZ activity, are correlated with high-grade, metastatic breast cancer and poor prognosis [22,23]. In addition, TAZ overexpression promotes breast carcinoma proliferation, migration, invasion and epithelial to mesenchymal transition [22], whereas loss of TAZ expression impairs migration and invasion, metastatic colonization and chemoresistance [22,24]. A previous study showed that combined depletion of both YAP and TAZ inhibited breast carcinoma invasion induced by TRAP6, a synthetic peptide agonist that activates both PAR1 and PAR2 [18,28]. Here, we show that activation of PAR1 with thrombin, its natural agonist, stimulates both YAP and TAZ dephosphorylation and is blocked by the PAR1 specific-antagonist vorapaxar, indicating that PAR1 triggers Hippo signaling in TNBC. Moreover, loss of TAZ but not YAP expression in TNBC is sufficient to block PAR1-stimulated CTGF and ANKRD1 gene expression, migration and invasion. TAZ, and not YAP, was also shown to be the major effector for gene induction stimulated by other GPCR agonists including LPA, SLIGKV and S1PR1 in TNBC (Fig. 3). A dominant role for TAZ was similarly demonstrated for the G protein-coupled estrogen receptor (GPER) in invasive ductal carcinoma [29]. In this study, activation of GPER was shown to induce gene expression, migration, invasion and tumor growth through a TAZ-dependent pathway in ER+ breast carcinoma [29]. These findings suggest that TAZ plays a pivotal role in Hippo signaling induced by GPCRs in both TNBC and ER+ breast carcinoma.

ARRDC3 is a multi-functional tumor suppressor in invasive breast carcinoma. ARRDC3 expression is low or absent in basal-like breast carcinoma including TNBC, resulting from gene deletion or epigenetic silencing [3,4]. However, the mechanisms by which ARRDC3 exerts its tumor suppressor functions are poorly understood. Given that the a-arrestin ARRDC3 shares structural homology with the multi-faceted b-arrestin scaffolds [30], ARRDC3 likely also exhibits multiple functions. ARRDC3 was shown to regulate trafficking of the integrin β4 and suppressed migration, invasion and tumor growth of TNBC [8]. We demonstrated that ARRDC3 is both necessary and sufficient for regulating lysosomal trafficking of PAR1 and suppressed persistent signaling and invasion of TNBC [10]. Here, we report that ARRDC3 displays an additional tumor suppressor function through direct regulation of GPCR-stimulated Hippo signaling in TNBC. We found that ARRDC3 suppressed Hippo-mediated CTGF and ANKRD1 expression induced by several GPCR agonists acting through PAR1, LPARs, PAR2 and S1PRs (Fig. 4), indicating that ARRDC3 functions broadly to control Hippo signaling. Moreover, ARRDC3 suppression of Hippo signaling occurs independent of receptor trafficking, since blockade of PAR1 trafficking by depletion of ALIX was not sufficient to enhance Hippo signaling in the presence of ARRDC3 (Fig. 4). PAR1 and a subset of other GPCRs utilize a non-canonical ALIX and ARRDC3 pathway for lysosomal sorting [11,31,32], which is distinct from the canonical ubiquitin-dependent lysosomal sorting pathway used by most classic GPCRs. In addition, ARRDC3 regulates Hippo signaling induced by activated PAR2 (Fig. 4) while PAR2 traffics to lysosomes via the canonical ubiquitin-mediated pathway independent of ALIX and ARRDC3 [31,33]. Finally, neither trafficking of LPARs nor S1PR1 are likely to be regulated by ALIX or ARRDC3, since they both utilize the canonical ubiquitin-driven lysosomal sorting pathway for degradation [34,35]. Yet, LPA- and S1P-induced Hippo signaling is suppressed by ARRDC3. Together, these studies support our findings that ARRDC3 functions on the Hippo pathway independently of its role on GPCR trafficking.

ARRDC3 suppresses GPCR-stimulated Hippo signaling by sequestering TAZ in the cytoplasm, thereby preventing gene induction, migration and metastasis of TNBC. A previous study in *Drosophila* reported that Leash, a homolog of ARRDC3, interacts with Yorkie, the homolog of YAP/TAZ, and inhibits Hippo signaling by facilitating Yorkie degradation [36,37]. This discovery gave credence to the idea that mammalian ARRDC3 might regulate Hippo signaling through interaction with YAP and TAZ. In addition, ARRDC3 contains C-terminal PPXY motifs that directly interact with WW domains, conserved regions that fold into a triple stranded b-sheet present in both YAP and TAZ. In colorectal cancer cells, ARRDC3 co-associates with YAP and enhances YAP degradation, and thereby suppresses tumorigenesis and chemotherapy sensitization [38]. Similarly, in renal cell carcinoma, ARRDC3 and YAP were shown to interact via the ARRDC3 PPXY motifs and WW domains of YAP, resulting in YAP degradation and suppression of tumor progression [39]. Here, we report that ARRDC3 interacts with TAZ via the PPXY motifs of ARRDC3 and the single WW domain of TAZ. Unlike studies of YAP in colorectal and renal carcinoma, ARRDC3 does not regulate the stability of TAZ in TNBC. Instead, ARRDC3 suppresses PAR1-induced TAZ dephosphorylation and nuclear translocation in TNBC (Fig. 6), but fails to modulate YAP activity. Moreover, ARRDC3 interaction with TAZ is required for suppression of PAR1-stimulated CTGF and ANKRD1 gene expression and cell migration as well as *in vivo* metastasis of TNBC (Fig. 6-8). The precise mechanism by which ARRDC3 prevents PAR1-induced dephosphorylation of TAZ is not known, but may involve the specificity and stability of the ARRDC3-TAZ versus YAP interaction, since YAP contains several structural features not present in TAZ including an additional WW domain, SH3 binding motif, and an N-terminal proline-rich region [21]. In addition, post-translational modifications such as phosphorylation and ubiquitination may influence ARRDC3-TAZ interaction and function [40-43], but this has not been investigated.

GPCRs are highly druggable and currently represent the target of 34% of all FDA approved therapeutics that represent 475 drugs; however only 8 drugs targeting GPCRs are in

use for oncology [44]. Despite the fact that GPCRs are widely dysregulated in cancer and contribute to tumorigenesis by promoting proliferation, invasion and evasion of the immune system, this receptor class remains under-utilized as drug targets in oncology [44]. Thus, ongoing investigations aimed at unraveling GPCR function at the molecular and cellular level in invasive breast cancer may reveal new targets or combination of targets for the development of new therapeutic strategies for the treatment of triple-negative breast cancer. In summary, our study reveals an unanticipated predominant role of TAZ in GPCR-mediated gene induction, migration and invasion of TNBC, and a multi-function role for ARRDC3 as a tumor suppressor in regulation of GPCR-stimulated TAZ activity. These studies further indicate that TAZ could be utilized as a drug target due to its critical role in TNBC migration, invasion and metastasis driven by dysregulated GPCRs.

2.6 Acknowledgement

Chapter 2, in full, has been submitted for publication of the material as it currently appears in: Arakaki AKS, Pan WA, Wedegaertner H, Roca-Mercado I, Chinn L, Trejo J. a-arrestin ARRDC3 tumor suppressor function is linked to GPCR-induced TAZ activation and breast cancer metastasis. *J Cell Science.* The dissertation author was the primary investigator and author of this paper.

2.7 Figures

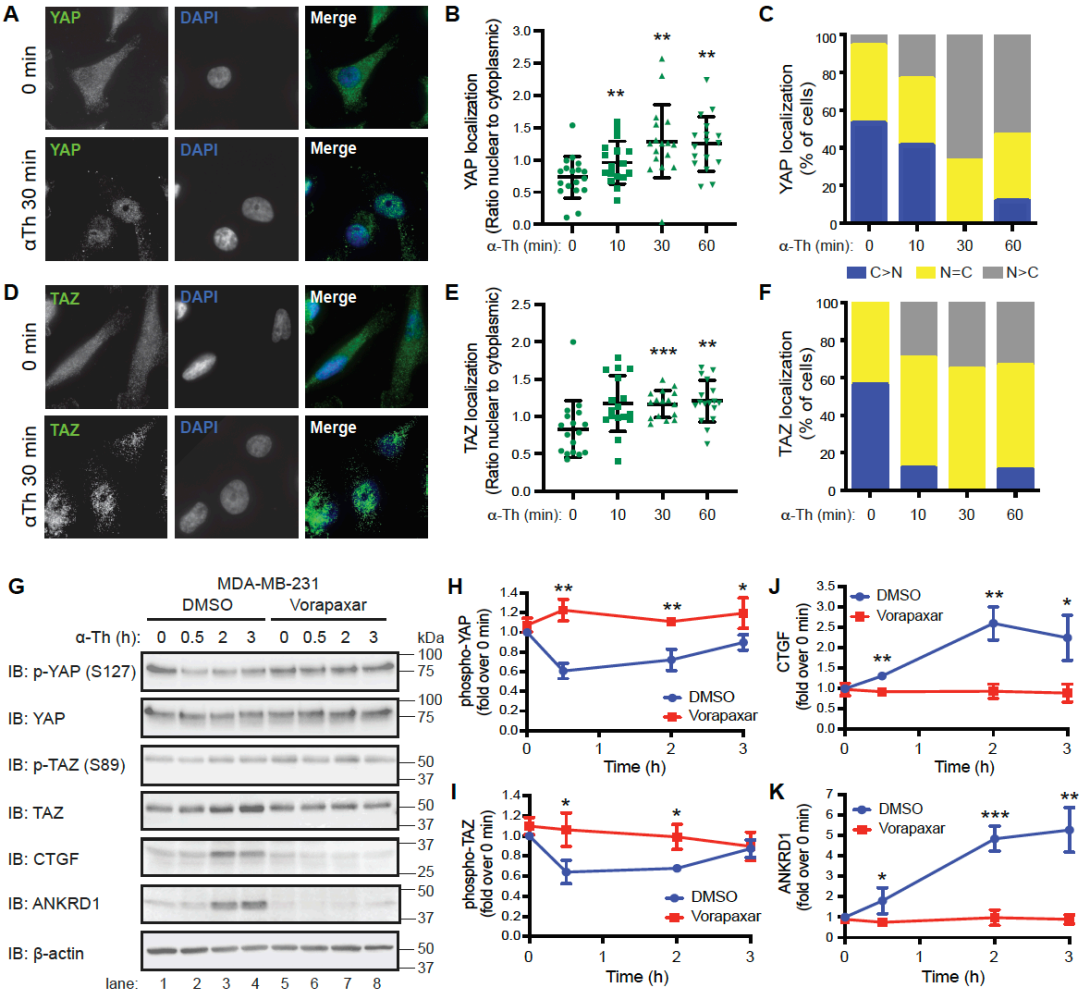

Figure 2.1: Thrombin activates the Hippo pathway in metastatic breast cancer cells through PAR1. A-F, YAP and TAZ subcellular localization was determined by immunofluorescence staining of endogenous YAP (**A**) and TAZ (**D**) (*green*) and DAPI (*blue*) used to stain nuclei after α-thrombin treatment. **A** and **D,** Images are representative of many cells examined in three independent experiments, showing 0 and 30 min time points. Scale bars, 10 μm. **B** and **E**, Ratio nuclear to cytoplasmic YAP (**B**) or TAZ (**E**) localization at the indicated times. Statistical significance was determined by one-way ANOVA of each time point compared to 0 min (n=18). **C** and **F**, Percentage of cells displaying greater cytoplasmic (*blue*), nuclear (*gray*) or equal (*yellow*) staining of YAP (**C**) or TAZ (**F**) at the indicated time points. **G-K**, MDA-MB-231 cells were pretreated with DMSO or the PAR1-specific antagonist Vorapaxar (10 μM) for 1 h then treated with 10 nM α-thrombin for the indicated times. The data shown (mean ± S.D., n=3) are represented as the fold-change in YAP phosphorylation (**H**), TAZ phosphorylation (**I**), CTGF expression (**J**) and ANKRD1 expression (**K**) relative to 0 min DMSO control. Statistical significance was determined by unpaired *t*-test at each time point.

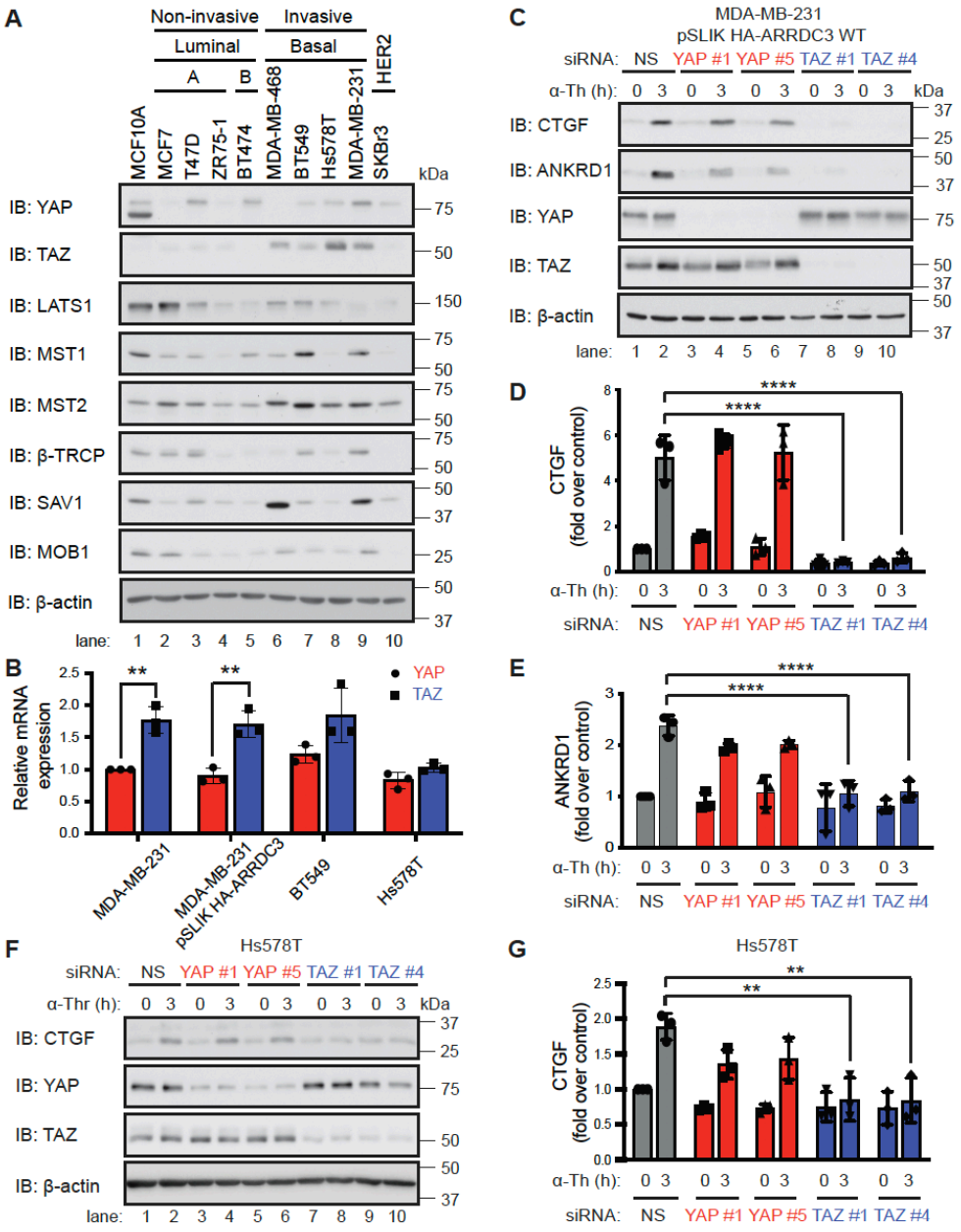

Figure 2.2: TAZ, but not YAP, is required for thrombin-mediated activation of the Hippo pathway in invasive breast cancer cell lines. A, Equivalent amounts (20 μg) of cell lysates from various breast cancer cell lines were immunoblotted for Hippo pathway components: YAP, TAZ, LATS1, MST1, MST2, β-TRCP, SAV1, and MOB1 expression. β-actin expression was determined as a control. **B**, YAP (*red*) and TAZ (*blue*) mRNA expression in invasive breast cancer cell lines was quantified by qPCR. The data (mean ± S.D., n=3) are normalized to S18 mRNA expression and are represented as the fold-change relative to YAP expression in parental MDA-MB-231. Statistical significance was determined by unpaired t-test. MDA-MB-231 HA-ARRDC3 pSLIK (**C-E**) and Hs578T (**F-G**) cells were transfected with the indicated siRNAs, serum-starved overnight then stimulated with 10 nM α-thrombin. The data shown (mean ± S.D., n=3) are represented as the fold-increase in CTGF expression (**D, G**) and ANKRD1 expression (**E**) relative to 0 min NS transfected control. Statistical significance was determined by one-way ANOVA.

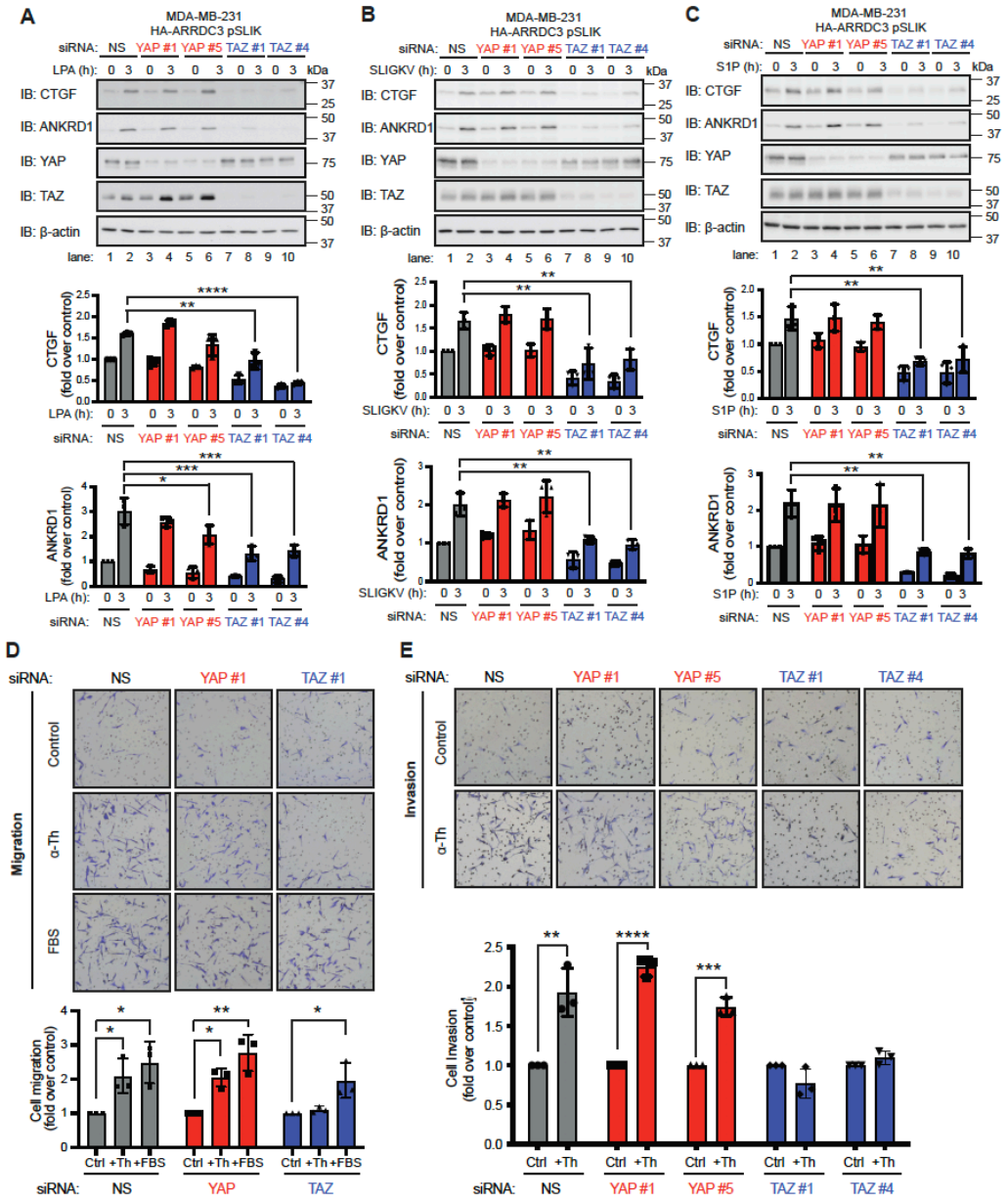

Figure 2.3: TAZ, but not YAP is required for GPCR activation of the Hippo pathway and for thrombin-mediated migration and invasion. A-C, MDA-MB-231 HA-ARRDC3 pSLIK cells were transfected with the indicated siRNAs, serum-starved overnight then stimulated with 100 nM lysophosphatidic acid (LPA, **A**), 1 μM SLIGKV peptide agonist for PAR2 (**B**), or 100 nM shingosine-1-phosphate (S1P, **C**) for 3 h. The data shown (mean ± S.D., n=3) are represented as the fold-increase in CTGF and ANKRD1 expression relative to 0 min NS transfected control. Statistical significance was determined by one-way ANOVA. **D** and **E**, MDA-MB-231 HA-ARRDC3 pSLIK cells were transfected with the indicated siRNAs, serum-starved overnight seeded onto transwells for migration assay (**D**) or invasion assay (**E**) and incubated with or without 100 pM (**D**) or 1 pM (**E**) α-thrombin, or 0.5% FBS (**D**). Images shown are representative of three independent experiments. The data (mean ± S.D., n=3) were quantified and represented as the fold change over untreated control cells. Statistical significance was determined by unpaired *t*-test.

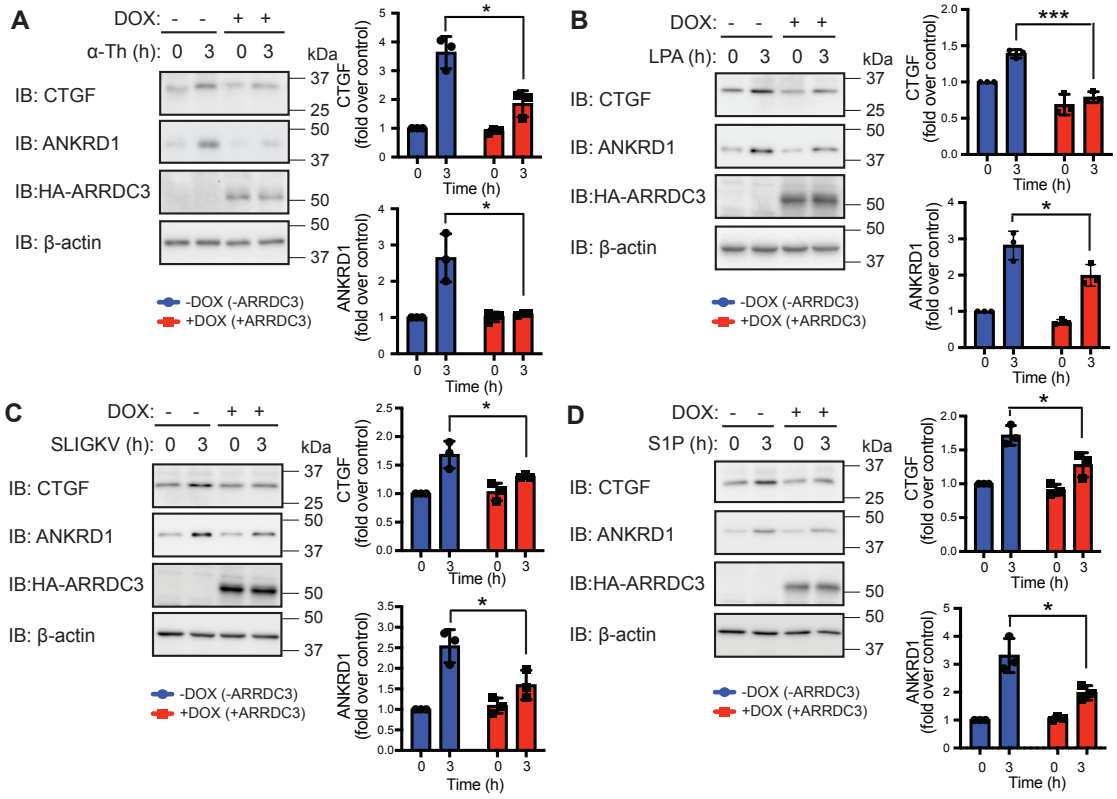

Figure 2.4: ARRDC3 re-expression suppresses GPCR activation of the Hippo pathway. A-D, MDA-MB-231 HA-ARRDC3 pSLIK cells were treated with or without 10 μg/ml doxycycline (DOX) for 48 h, starved overnight then stimulated with 10 nM α-thrombin (**A**), 100 nM LPA (**B**), 1 μM SLIGKV (**C**), or 100 nM S1P (**D**) for 3 h. The data shown (mean ± S.D., n=3) are represented as the fold-increase in CTGF and ANKRD1 expression relative to 0 min -DOX control. Statistical significance was determined by unpaired *t*-test.

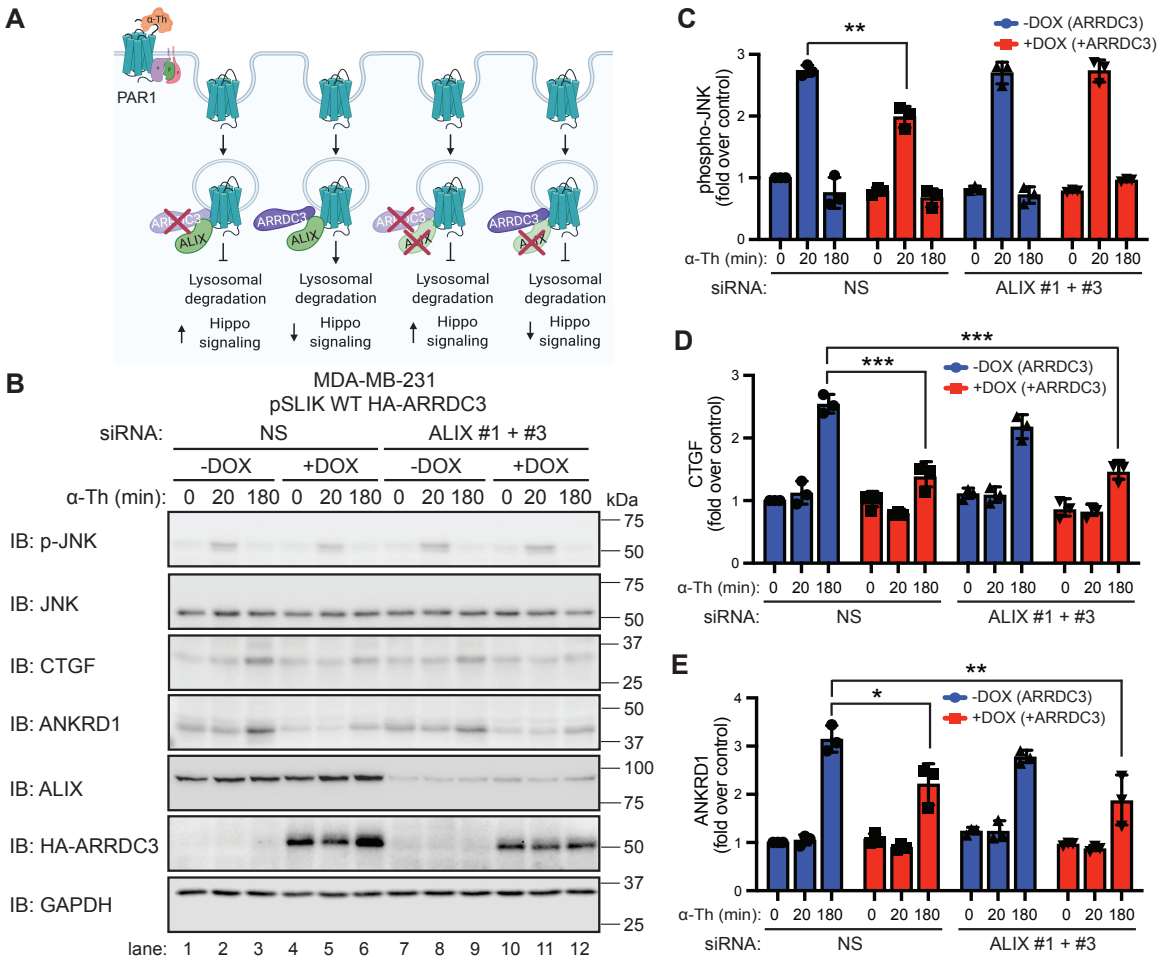

Figure 2.5: ARRDC3 regulates Hippo signaling independent of its function on PAR1 trafficking and degradation. A, Illustration of ARRDC3 and ALIX requirement for facilitating lysosomal degradation and hypothesis of ARRDC3 effect on Hippo signaling. **B-E**, MDA-MB-231 HA-ARRDC3 pSLIK cells were transfected with non-specific (NS) or ALIX #1 and #3 siRNAs and then incubated with or without DOX for 48 h. Cells were serum-starved overnight then stimulated with 10 nM α-thrombin for the indicated times. The data shown (mean ± S.D., n=3) are represented as the fold-increase in JNK phosphorylation (**C**), CTGF expression (**D**), and ANKRD1 expression (**E**) relative to 0 min -DOX, NS transfected control. Statistical significance was determined by one-way ANOVA.

Figure 6

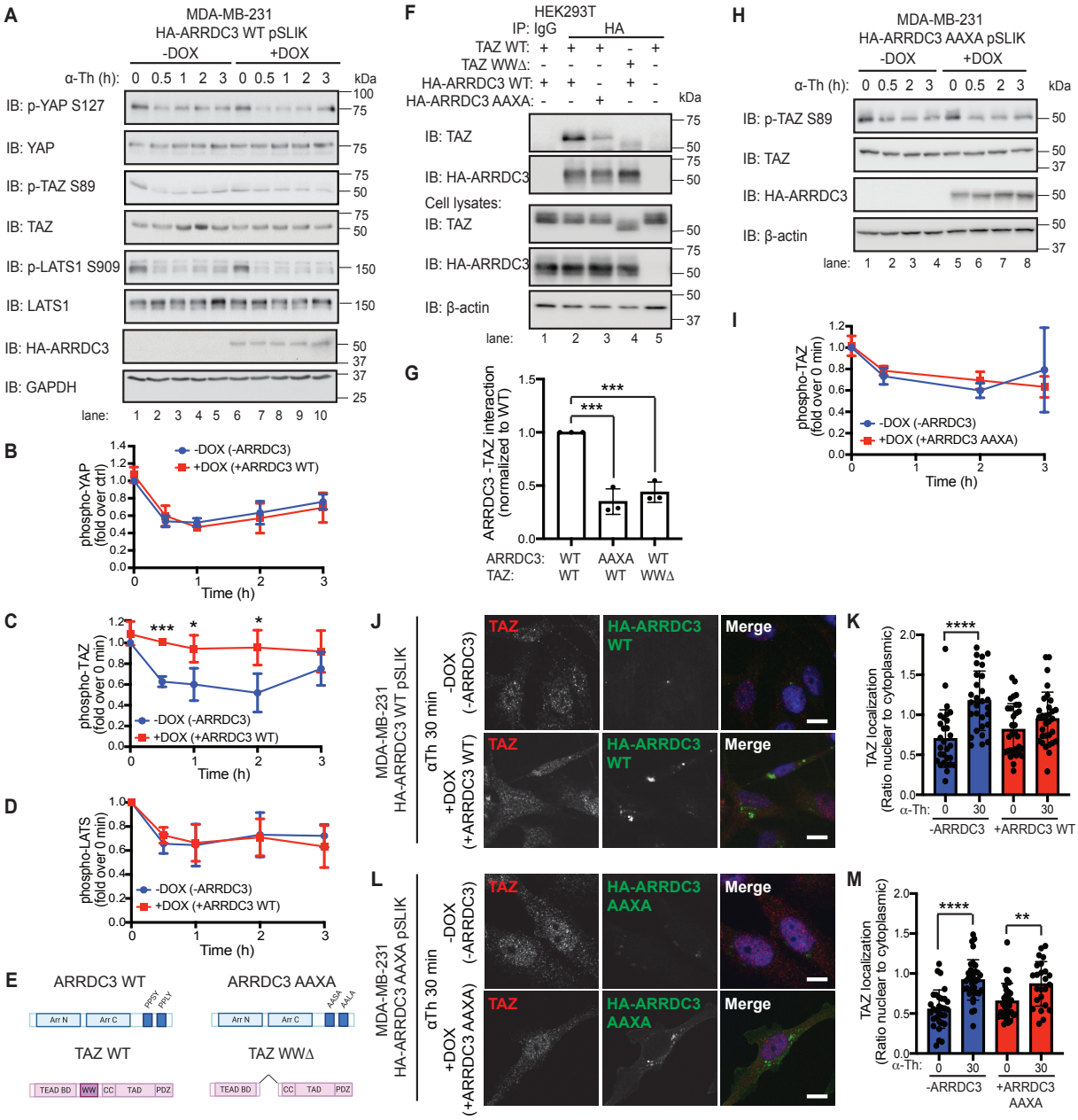
A
MDA-MB-231
HA-ARRDC3 WT pSLIK
-DOX
+DOX
α-Th (h): 0 0.5 1 2 3 0 0.5 1 2 3
kDa
IB: p-YAP S127
IB: YAP
IB: p-TAZ S89
IB: TAZ
IB: p-LATS1 S909
IB: LATS1
IB: HA-ARRDC3
IB: GAPDH
lane: 1 2 3 4 5 6 7 8 9 10
B
phospho-YAP (fold over ctrl)
-DOX (-ARRDC3)
+DOX (+ARRDC3 WT)
Time (h)
C
phospho-TAZ (fold over 0 min)
-DOX (-ARRDC3)
+DOX (+ARRDC3 WT)
Time (h)
D
phospho-LATS (fold over 0 min)
-DOX (-ARRDC3)
+DOX (+ARRDC3 WT)
Time (h)
E
ARRDC3 WT
ARRDC3 AAXA
Arr N
Arr C
PPSY
PPLY
AASA
AALA
TAZ WT
TAZ WWΔ
TEAD BD
WW
CC
TAD
PDZ
F
HEK293T
IP: IgG HA
TAZ WT: + + + - +
TAZ WWΔ: - - - + -
HA-ARRDC3 WT: + + - + -
HA-ARRDC3 AAXA: - - + - -
kDa
IB: TAZ
IB: HA-ARRDC3
Cell lysates:
IB: TAZ
IB: HA-ARRDC3
IB: β-actin
lane: 1 2 3 4 5
G
ARRDC3 -TAZ interaction (normalized to WT)
***
***
ARRDC3: WT AAXA WT
TAZ: WT WT WWΔ
H
MDA-MB-231
HA-ARRDC3 AAXA pSLIK
-DOX
+DOX
α-Th (h): 0 0.5 2 3 0 0.5 2 3
kDa
IB: p-TAZ S89
IB: TAZ
IB: HA-ARRDC3
IB: β-actin
lane: 1 2 3 4 5 6 7 8
I
phospho-TAZ (fold over 0 min)
-DOX (-ARRDC3)
+DOX (+ARRDC3 AAXA)
Time (h)
J
MDA-MB-231
HA-ARRDC3 WT pSLIK
αTh 30 min
-DOX (-ARRDC3)
+DOX (+ARRDC3 WT)
TAZ
HA-ARRDC3 WT
Merge
K
TAZ localization (Ratio nuclear to cytoplasmic)
****
α-Th: 0 30 0 30
-ARRDC3
+ARRDC3 WT
L
MDA-MB-231
HA-ARRDC3 AAXA pSLIK
αTh 30 min
-DOX (-ARRDC3)
+DOX (+ARRDC3 AAXA)
TAZ
HA-ARRDC3 AAXA
Merge
M
TAZ localization (Ratio nuclear to cytoplasmic)
****
**
α-Th: 0 30 0 30
-ARRDC3
+ARRDC3 AAXA

Figure 2.6: ARRDC3 re-expression blocks thrombin-mediated TAZ dephosphorylation and nuclear localization through co-association between ARRDC3 and TAZ. MDA-MB-231 WT (**A-D**) and AAXA mutant (**H-I**) HA-ARRDC3 pSLIK cells were treated with or without 10 μg/ml DOX for 48 h, serum-starved overnight, then stimulated with 10 nM α-thrombin by persistent signaling assay for various times. The data shown (mean ± S.D., n=3) are represented as the fold-change in YAP phosphorylation (**B**), TAZ phosphorylation (**C** and **I**), and LATS phosphorylation (**D**) relative to 0 min -DOX control. Statistical significance was determined by unpaired *t*-test comparing -DOX and +DOX at each time point. **E**, Illustration of constructs of ARRDC3 WT and AAXA mutant as well as of TAZ WT and WW domain deletion (WWΔ) mutant. **F** and **G**, HEK293T cells transiently expressing HA-ARRDC3 WT, TAZ WT, HA-ARRDC3 AAXA, or TAZ WW domain deletion were immunoprecipitated with HA antibody to pulldown ARRDC3. Proteins in immunoprecipitation and cell lysate input were analyzed by immunoblotting. The data (mean ± S.D., n=3) are quantified by densitometry and co-association of ARRDC3-TAZ (**G**) is represented as fold over WT control. Statistical significance determined using one-way ANOVA. **J-M**, TAZ subcellular localization following thrombin treatment was determined by immunofluorescence staining of endogenous TAZ (*red*) in MDA-MB-231 WT (**J, K**) and AAXA mutant (**L, M**) HA-ARRDC3 pSLIK cells; HA (*green*) stained for ARRDC3 and DAPI (*blue*) for cell nuclei. Scale bars, 10 μm. **K** and **M**, Quantification of the ratio nuclear to cytoplasmic TAZ localization. Statistical significance was determined by one-way ANOVA of each time point compared to 0 min.

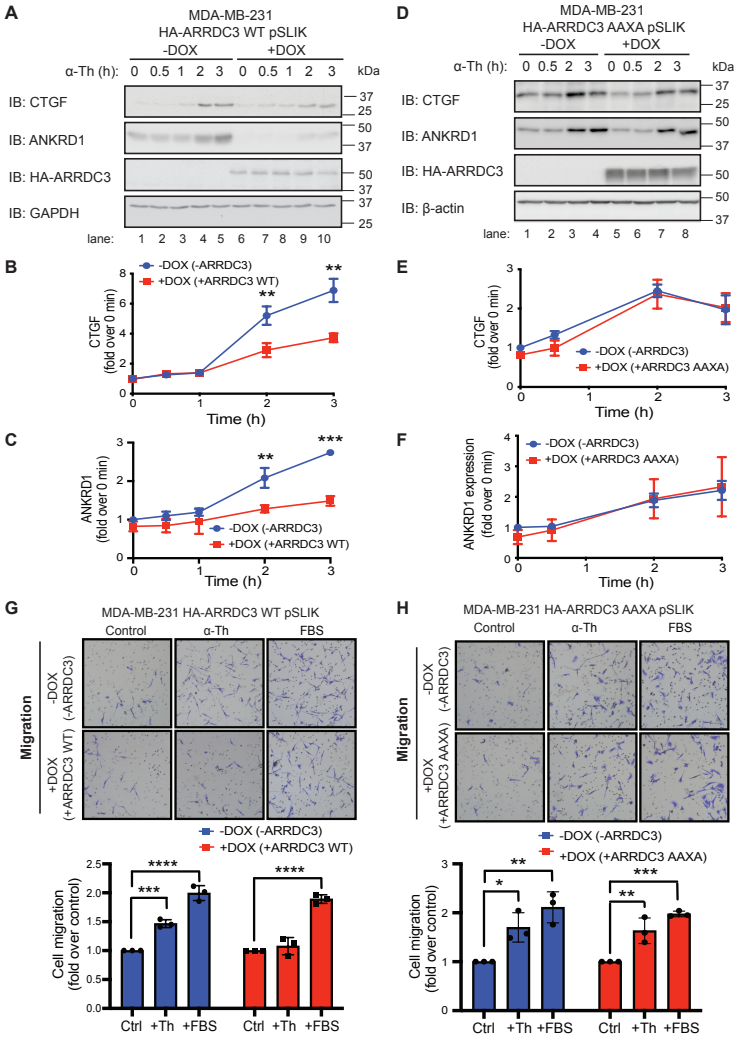

Figure 2.7: ARRDC3 re-expression inhibits TAZ-TEAD binding and attenuates downstream Hippo signaling and thrombin-induced migration, dependent on the PPXY motifs of ARRDC3. MDA-MB-231 WT (**A-C** and **G**) and AAXA mutant (**D-F** and **H**) HA-ARRDC3 pSLIK cells were treated with or without 10 μg/ml DOX for 48 h, serum-starved overnight, then stimulated with 10 nM α-thrombin by persistent signaling assay. **A-C** and **D-F**, Cells were lysed and IP'ed with anti-TEAD antibody or anti-IgG control. IP samples and cell lysates were immunoblotted. The data (mean ± S.D., n=3) are quantified and co-association of YAP-TEAD (**B** and **E**) and TAZ:TEAD (**C** and **F**) is represented as fold over -DOX 0 min control. Statistical significance determined using an unpaired *t*- test (n=3). **G** and **H**, The data shown (mean ± S.D., n=3) are represented as the fold-change in CTGF and ANKRD1 expression relative to 0 min -DOX control. Statistical significance was determined by unpaired *t*-test comparing -DOX and +DOX at each time point. **I** and **J,** MDA-MB-231 WT (**I**) and AAXA mutant (**J**) HA-ARRDC3 pSLIK cells were treated with or without 10 μg/ml DOX for 48 h, serum-starved overnight, seeded onto transwells for migration and incubated with or without 100 pM α-thrombin or 0.5% FBS for 5 h at 37°C. Images shown are representative of three independent experiments. The data (mean ± S.D., n=3) were quantified and represented as the fold change over untreated control cells. Statistical significance was determined by unpaired *t*-test.

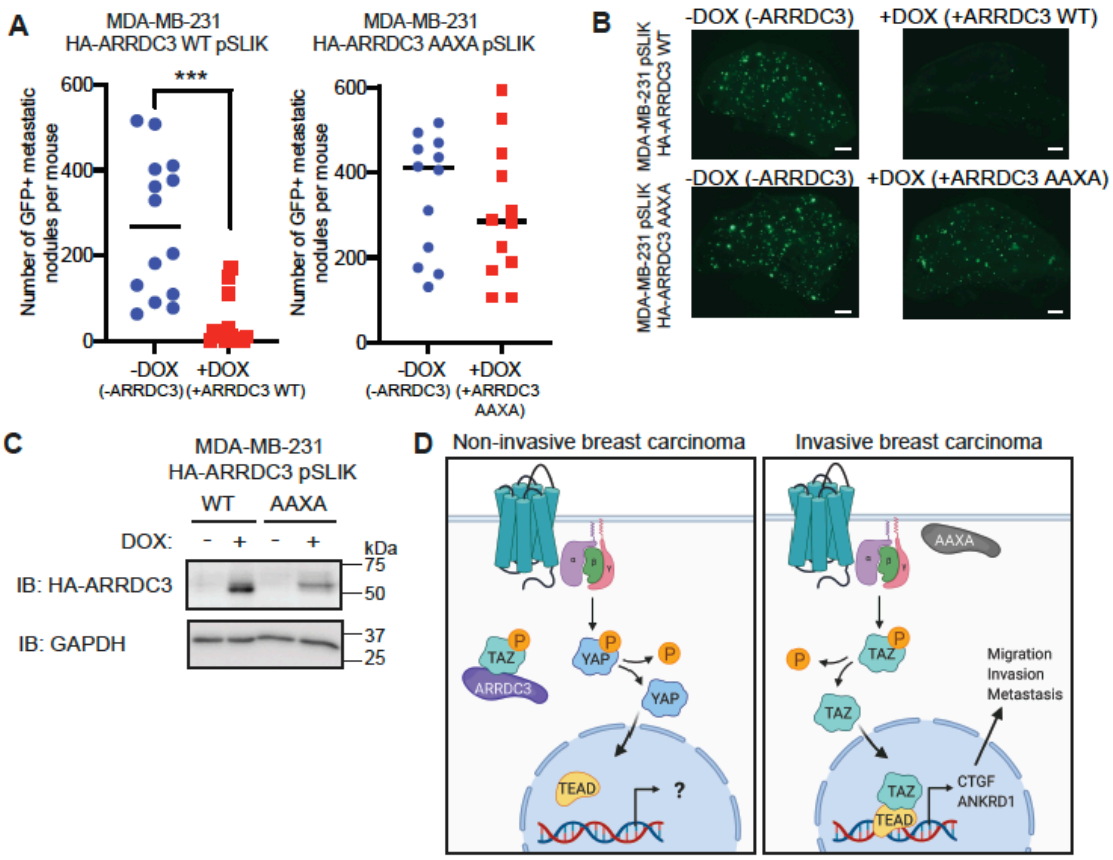

Figure 2.8: ARRDC3 re-expression blocks *in vivo* breast cancer metastasis, dependent on the PPXY motifs of ARRDC3. GFP-labeled MDA-MB-231 WT or AAXA mutant HA-ARRDC3 pSLIK cells were injected into the tail vein of NSG mice. **A**, Quantification of GFP-positive metastatic nodules in the lungs of the mice collected 2 weeks after injection. Statistical significance determined by unpaired t-test with Welch's correction (WT, n=14 mice per group; AAXA, n=12 mice per group). **B**, Representative fluorescent images of GFP-positive metastatic lesions in the lungs of mice. GFP signal indicates tumor cell extravasation, seeding, growth and colonization in the lung. Scale bars, 1 mM. **C**, Verification of HA-ARRDC3 WT or HA-ARRDC3 AAXA re-expression in MDA-MB-231 pSLIK cells collected prior to injection. Lysates immunoblotted for HA-ARRDC3 and GAPDH expression. **D**, When ARRDC3 is present such as in normal mammary epithelial cells or in luminal non-invasive breast carcinoma cells, ARRDC3 co-associates with TAZ, leading to its cytoplasmic retention and attenuated GPCR-mediated Hippo pathway signaling. However, when ARRDC3 is absent such as in invasive basal-like breast carcinoma or is lacking PPXY motifs and thus functionally inactive, TAZ moves into the nucleus upon activation of GPCRs, binds to TEAD family of transcriptional co-activators, induces CTGF and ANKRD1 expression and promotes cell migration, invasion and metastasis.

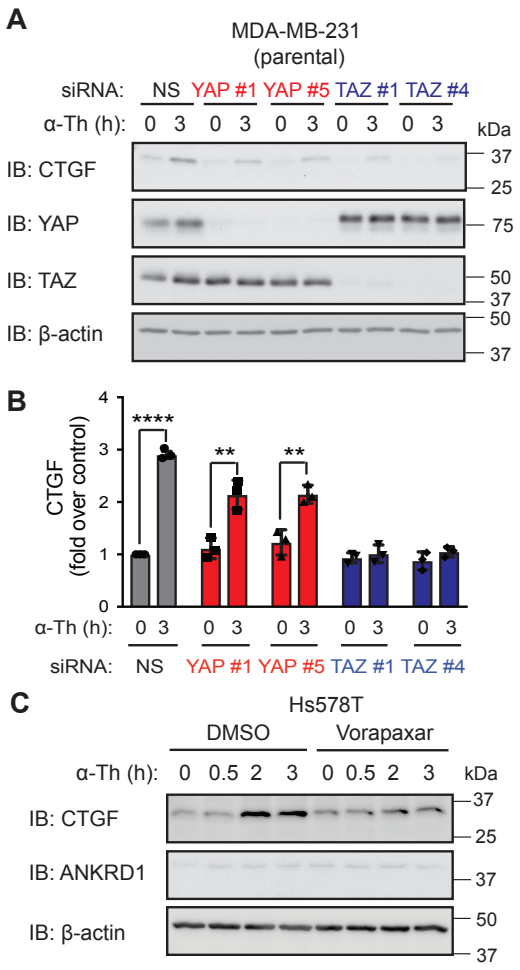

Figure S2.1. TAZ but not YAP is required for GPCR activation of the Hippo pathway in parental MDA-MB-231. A and **B,** Parental MDA-MB-231 cells were transfected with respective siRNAs, serum-starved overnight then stimulated with 10 nM α-thrombin for 3 h. The data shown (mean ± S.D., n=3) is represented as the fold-increase in CTGF expression (**B**) relative to 0 min NS transfected control. Statistical significance was determined by one-way ANOVA. **C,** Hs578T cells were serum-starved overnight, pretreated with DMSO or the PAR1-specific antagonist Vorapaxar for 1 h then treated with 10 nM α-thrombin for the indicated times. Cells were lysed and immunoblotted for CTGF, ANKRD1 and β-actin expression.

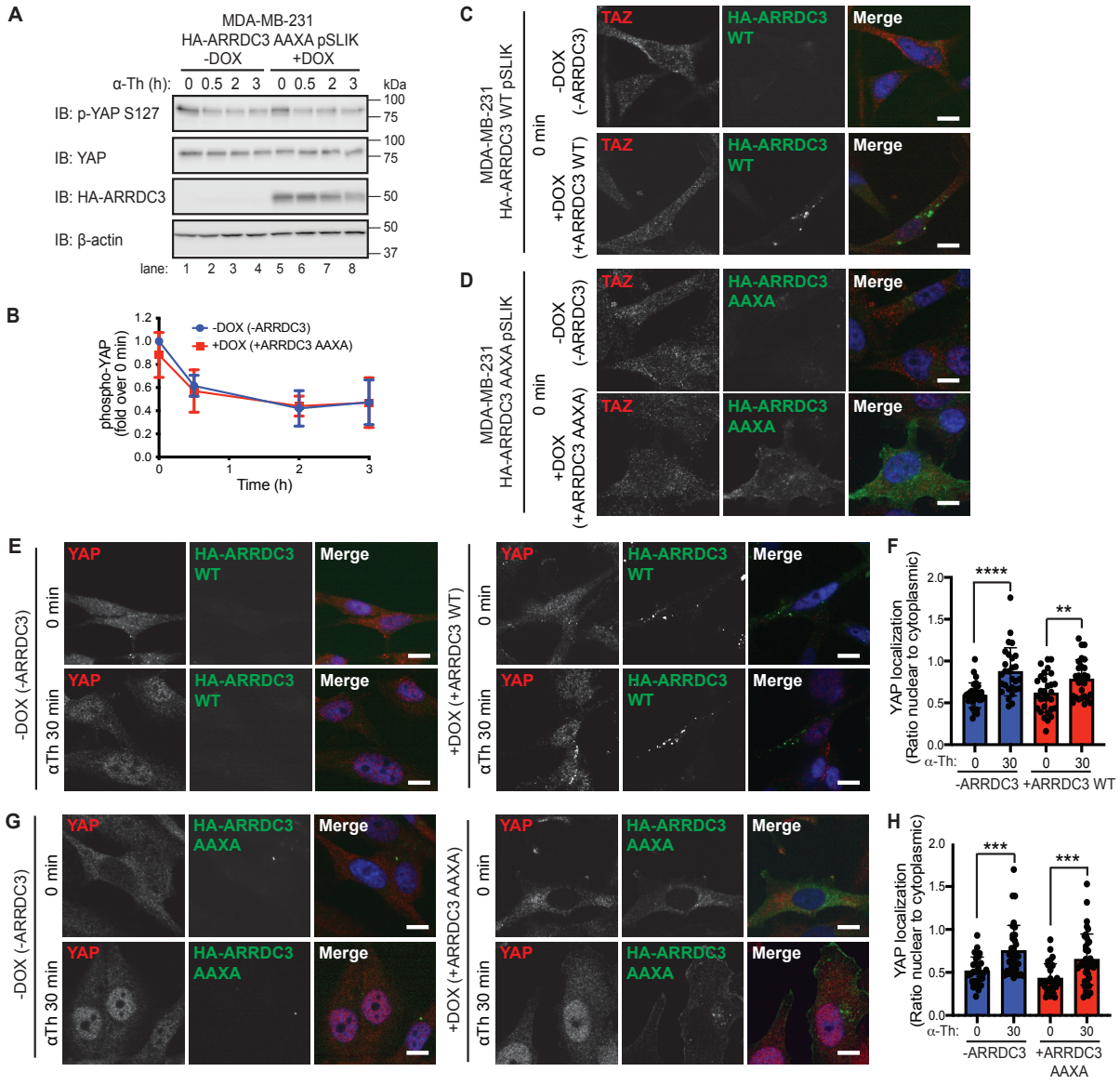

Figure S2.2. Re-expression of ARRDC3 WT and AAXA double mutant has no effect on thrombin-mediated YAP activation. A and **B,** MDA-MB-231 AAXA mutant HA-ARRDC3 pSLIK cells were treated with or without 10 μg/ml DOX for 48 h, serum-starved overnight, then stimulated with 10 nM α-thrombin for various times. The data shown (mean ± S.D., n=3) are represented as the fold-change in YAP phosphorylation (**B**) relative to 0 min -DOX control. Statistical significance was determined by unpaired *t*-test comparing -DOX and +DOX at each time point. **C-H,** TAZ and YAP subcellular localization was determined by immunofluorescence staining of endogenous TAZ (**C** and **D**) and YAP (**E** and **G**) in MDA-MB-231 WT HA-ARRDC3 pSLIK (**C, E**) and MDA-MB-231 AAXA mutant HA-ARRDC3 pSLIK (**D, G**) cells. After α-thrombin treatment, cells were fixed, processed, stained for TAZ or YAP (*red*), HA-ARRDC3 (*green*) and DAPI for nuclei (*blue*) and imaged by confocal microscopy. **C,D,E,G**, Images are representative of many cells examined in three independent experiments. Scale bars, 10 μm. **F,H**, Quantification of the ratio nuclear to cytoplasmic YAP localization from at least 9 fields of view from each biological replicate. Statistical significance was determined by one-way ANOVA of each time point compared to 0 min.

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CONCLUSION

Despite G protein-coupled receptors (GPCRs) displaying important roles in tumorigenesis, invasion, angiogenesis and evasion of the immune system, they remain underutilized as targets in the treatment of cancer [1]. In order to reveal novel therapeutic targets, we need to further understand the mechanisms and biological processes in place that cause GPCRs to be dysregulated in cancer and promote tumor growth and metastasis.

In the work described in this thesis, I primarily focused my studies on the GPCR protease-activated receptor-1 (PAR1) due to the well-established role PAR1 has in promoting breast cancer invasion and metastasis [2-4]. PAR1 protein expression is high in invasive breast carcinoma cell lines due to dysregulated trafficking and degradation of the receptor [4]. Activation of PAR1 as well as other GPCRs including lysophosphatidic acid receptors (LPARs), protease-activated receptor-2 (PAR2) and shingosine-1-phosphate receptors (S1PRs) mediates Hippo pathway signaling, also contributing to proliferation and invasion [5]. LPARs, PAR2 and S1PRs have also been implicated in breast cancer [6] and thus, were additionally studied here. Our lab became interested in understanding the role of the adaptor protein arrestin domain-containing protein 3 (ARRDC3), an α-arrestin family member and recently identified breast cancer tumor suppressor [7], in regulating GPCR trafficking and signaling. β-arrestins are well-characterized in their role in GPCR trafficking and signaling although there are key differences in the structures of α-arrestins and β-arrestins that may contribute to differing functions. Additionally, work in *Drosophila* showed that *Leash,* the homolog of ARRDC3, could interact and promote degradation of *Yki*, the homolog of YAP/TAZ thus suggesting ARRDC3 could potentially regulate Hippo pathway signaling [8]. My thesis work aimed to investigate the role of ARRDC3 in both GPCR trafficking and signaling, in the context of invasive breast carcinoma.

In this work, I utilized a lentiviral vector pSLIK system to re-express ARRDC3 in the model invasive breast carcinoma cell line MDA-MB-231 and assessed the effects of ARRDC3 on GPCR trafficking, degradation and signaling using biochemical assays and imaging techniques, on invasive phenotype using transwell assays, and on *in vivo* metastasis using a tail vein injection model in immunocompromised mice. I further interrogated mechanisms of regulation using RNA interference, molecular biology and biochemical techniques. The studies described in this dissertation provide novel insights into potential therapeutic targets of GPCR-mediated metastasis, particularly identifying TAZ as the major effector of GPCR-mediated Hippo signaling and invasion in invasive breast cancer.

3.1 ARRDC3 controls proper PAR1 trafficking and degradation, thus inhibiting persistent G protein signaling and invasion

Previous to my thesis studies, our lab identified ARRDC3 as a necessary regulator for proper PAR1 trafficking in HeLa cells [9]. ARRDC3 recruits the E3 ubiquitin ligase WW domain-containing protein 2 (WWP2), which then ubiquitinates ALG-interacting protein X (ALIX), a critical step in trafficking of PAR1 to the lysosome for degradation [9]. Due to the identification of ARRDC3 as a tumor suppressor in breast cancer [7] and the well-established role of PAR1 in breast cancer growth and invasion [3,10], we hypothesized that loss of ARRDC3 in aggressive breast cancer patients, due to genetic deletion or epigenetic silencing [7,11], was responsible for dysregulated PAR1 degradation and subsequent increased expression and signaling. In chapter 1, I used the lentiviral vector pSLIK system in MDA-MB-231 cells which lack ARRDC3 and have increased levels of PAR1, to re-express ARRDC3 in these cells and assess the effect on PAR1 trafficking and signaling. I demonstrated that re-expression of ARRDC3 in highly invasive basal-like breast carcinoma is sufficient to restore normal PAR1 lysosomal trafficking, which occurs through an ALIX-dependent endosomal-lysosomal sorting pathway. As a result of proper trafficking and degradation, ARRDC3 re-expression also attenuated thrombin-stimulated

JNK signaling and thrombin-mediated cellular invasion. These studies are the first to demonstrate a role for the tumor suppressor ARRDC3 in regulation of GPCR trafficking and signaling in invasive basal-like breast carcinoma.

Although we have a good understanding of how ARRDC3 regulates lysosomal sorting by modulating ALIX, we currently still do not understand the exact mechanisms as to how ARRDC3 is recruited to the endocytosed PAR1 complex. ARRDC3 is likely to be post-translationally modified by phosphorylation or ubiquitination to alter its activity or localization [12]. Current members of our lab are actively studying how ARRDC3 is regulated and recruited to PAR1 to perform its function in mediating PAR1 degradation. Additionally, we identified JNK signaling, a critical driver of breast cancer progression [13], as a target of ARRDC3 regulation. In the absence of ARRDC3, PAR1-stimulated a marked and prolonged increase in JNK signaling; however, re-expression of ARRDC3 in breast cancer cells resulted in a significant reduction in both the magnitude and duration of JNK signaling induced by PAR1. JNK phosphorylates a large number of targets, mainly transcription factors, and regulates cellular proliferation, survival and apoptosis but precisely how JNK contributes to breast carcinoma invasion stimulated by a GPCR is not known and an area of future investigation.

3.2 ARRDC3 regulates the Hippo pathway independently of its role on PAR1 trafficking, by interacting and sequestering TAZ in the nucleus

Recent studies demonstrated that ARRDC3 interacts with YAP to facilitate degradation and thus inhibit Hippo signaling [14,15]. To make a distinction on the ability of ARRDC3 to inhibit Hippo signaling by either indirectly modulating PAR1 degradation or directly interacting with YAP or TAZ, we utilized two different approaches. We first assessed the role of ARRDC3 re-expression on activation of the Hippo pathway by other GPCRs, including the LPARs, S1PRs and PAR2. These GPCRs are well-established in their ability to mediate Hippo pathway signaling [5]; additionally, we do not expect ARRDC3 to play a role on trafficking and

degradation of these receptors since they lack the YPX_3L motif critical for the lysosomal degradation pathway employed by PAR1 [16]. The LPARs and S1PRs also act in a classical agonist-binding mode of activation as compared to the irreversible proteolytic activation of PAR1. PAR2 lysosomal trafficking is also characterized and does not rely on ARRDC3 [17]. When ARRDC3 is re-expressed in MDA-MB-231 cells and treated with the LPA, S1P, or SLIGKV (PAR2 peptide agonist), we observed a decrease in the agonist-induced downstream Hippo pathway effectors CTGF and ANKRD1 expression, compared to cells lacking ARRDC3. In chapter 1, I showed that both ARRDC3 and ALIX are required for proper PAR1 degradation in breast carcinoma cells, with cells lacking ALIX only or ARRDC3 only failing to rescue PAR1 degradation. I utilized this system to assess if ARRDC3 has a separate role on Hippo signaling. As a control, we assessed JNK activation by measuring p-JNK. In cells expressing both ARRDC3 and ALIX, which we previously demonstrated have rescued PAR1 degradation, thrombin-induced JNK phosphorylation is decreased, whereas all other conditions had sustained signaling. In contrast, when we looked at thrombin-induced CTGF and ANKRD1, we see a decrease in expression of these proteins in conditions that re-express ARRDC3, even when ALIX protein level is knocked down and thus PAR1 degradation is blocked. These studies together suggest this separate role for ARRDC3 in regulating GPCR-mediated activation of the Hippo pathway.

I went on to show that ARRDC3 exhibits this distinct adaptor function through interaction with TAZ. ARRDC3 interaction with TAZ was examined in HEK293T cells expressing HA-ARRDC3 wildtype and TAZ wildtype. We observed co-immunoprecipitation of TAZ WT with HA-ARRDC3 but not with the IgG control, suggesting specific interaction of ARRDC3 and TAZ. In MDA-MB-231 HA-ARRDC3 pSLIK cells, re-expression of ARRDC3 block thrombin-induced TAZ de-phosphorylation and stabilization and further blocks TAZ nuclear translocation and interaction with TEAD1-4. These data together suggest that ARRDC3 associates with TAZ, thereby keeping TAZ in the cytoplasm and preventing it from binding to the TEAD family to

induce downstream gene transcription. TAZ activity and cellular localization can be regulated in many ways. Dephosphorylation of TAZ is a key step in GPCR-mediated activation of the Hippo pathway pathway. Inactivation of LATS1/2 kinase by F-actin formation downstream of GPCR-RhoA activation blocks additional phosphorylation [18,19]. In addition, dephosphorylation of TAZ is mediated by the phosphatase PP1 while YAP is dephosphorylated by PP2 [20,21]. Thus, ARRDC3 interaction with TAZ may block the recruitment of PP1 but not PP2 and should be further studied. There are many reports of TAZ interacting with other PPxY-containing proteins, such as RUNX2 [22,23], Smad2/3-4 [24], Pax3 [25], and parafibromin [26] to promote TAZ activity [27]. Similarly, ARRDC3 may hinder these interactions due to TAZ containing only one WW domain, compared with YAP having two WW domains. Post-translational modifications of both ARRDC3 and TAZ have been reported to affect their activity. Serine/threonine phosphorylation is well-established in regulating TAZ cytoplasmic retention and degradation [28]. In addition, tyrosine phosphorylation of either residues present in WW domains or PPxY motifs can increase the strength of this interaction [29]. TAZ is ubiquitinated by β-TRCP, facilitating proteasomal degradation [30]. ARRDC3 may also recruit E3 ligases to ubiquitinate TAZ; however, it is of note that we did not observe TAZ degradation when re-expressing ARRDC3 in our studies. Instead, ARRDC3 functioned to block TAZ dephosphorylation and sequester TAZ in the cytoplasm upon GPCR stimulation.

3.3 The PPxY motifs of ARRDC3 are critical for its metastasis suppressor function

ARRDC3 contains arrestin-like N- and C-domains and C-terminal PPxY motifs that bind to WW domain containing proteins such as E3 ligases, transcription coactivators, isomersases, scaffold and signaling proteins [29,31]. Indeed, several studies demonstrate that ARRDC3 acts as an adaptor to recruit HECT-domain containing E3 ubiquitin ligases through this interaction [9,32]. ARRDC3 interacts with YAP in colorectal cancer cell lines and in HEK293T cells, dependent on the PPxY motifs of ARRDC3 and WW domains of YAP [14,15]. In the present

study, we found that overexpression of ARRDC3 and TAZ in HEK293T cells results in co-association, and that this is dependent on the PPxY motifs of ARRDC3 and WW domain of TAZ. To further assess the importance of the PPxY motifs, we used MDA-MB-231 cells transfected with a pSLIK lentiviral vector encoding an AAXA double mutant, where the critical prolines (P) and tyrosines (Y) were converted to alanines (A). In cells re-expressing the AAXA mutant of ARRDC3, we observed no change in TAZ dephosphorylation kinetics or CTGF and ANKRD1 induction compared to the non-doxycycline treated control, suggesting the PPxY motifs of ARRDC3 are critical for ARRDC3 function in regulating PAR1-mediated activation of the Hippo pathway. In line with these results, expression of the ARRDC3 AAXA mutant resulted in TAZ nuclear translocation, while re-expression of the WT ARRDC3 protein blocked TAZ nuclear localization.

We also assessed the role of ARRDC3 and its PPxY motifs on migration and metastasis *in vivo.* MDA-MB-231 HA-ARRDC3 pSLIK cells re-expressing WT ARRDC3 showed inhibited thrombin-mediated migration compared to cells lacking ARRDC3. Contrary to ARRDC3 WT re-expression, cells that re-express ARRDC3 AAXA double mutant failed to inhibit thrombin-induced cell migration, suggesting the PPxY motifs are an important determinant in ARRDC3 for suppressing migration. Most strikingly, when we inject MDA-MB-231 cells into the tail vein of immunocompromised mice to assess lung metastasis, we observe a robust block in metastatic nodules in MDA-MB-231 pSLIK cells re-expressing WT ARRDC3 but not the AAXA mutant ARRDC3. These *in vivo* results strongly support our cellular and biochemical data showing that ARRDC3, dependent on PPxY motifs to interact with TAZ, functions in suppressing cell migration and invasion and breast cancer metastasis *in vivo.*

3.4 YAP and TAZ display differential roles in GPCR activation of the Hippo pathway and invasion in breast cancer

The distinct role of TAZ in breast cancer metastasis has been illuminated in recent studies. In breast cancer patient samples, both TAZ overexpression and nuclear staining, indicative of active TAZ, are correlated with high-grade, metastatic disease as well as poor prognosis. Overexpression of TAZ in normal mammary epithelial MCF10A cells promotes cell proliferation, migration, invasion and EMT while TAZ knockdown in invasive breast carcinoma cells impairs cell migration and invasion, metastatic colonization, chemoresistance. TAZ functions in breast cancer metastasis as a central mediator of the self-renewal, tumor initiation capacity and metastatic seeding potential of breast cancer stem cells. Many oncogenic regulators of TAZ activity in breast cancer have been identified. Despite the well-established role of GPCRs in regulating the Hippo pathway, few studies have assessed this differential role for TAZ in GPCR signaling in the context of breast carcinoma. GPCRs coupled with $G\alpha_{12/13}$, $G\alpha_{i}$, and $G\alpha_{q/11}$, such as protease-activated, LPA, and S1P receptors, will activate both YAP and TAZ. These studies showed that knockdown of both YAP and TAZ resulted in inhibition of GPCR-mediated physiological breast cancer cell proliferation and invasion. Additionally, in ER+ breast carcinoma cell lines, activation of the G protein-coupled estrogen receptor (GPER) is dependent on TAZ in mediating Hippo pathway gene transcription, cell proliferation, migration, and *in vivo* tumor growth. Our studies further examined the differential roles of YAP and TAZ in GPCR signaling, migration and invasion in triple negative breast carcinoma cells. We found that TAZ is required for thrombin, LPA, SLIGKV, and S1P-mediated induction of CTGF and ANKRD1, established downstream effectors of the Hippo pathway, as well as agonist-mediated migration and invasion. In contrast, YAP knockdown had little to no effect on these processes. Indeed, this is in line with recent studies in which YAP KO, TAZ KO and double YAP/TAZ KO HEK293A cells were generated using the CRIPSR/Cas9 system; by RNA-seq approaches, their results suggest distinct transcriptional activity of YAP and TAZ. These studies together suggest

that YAP and TAZ have differential roles depending on the cell context or tissue type, and we have shown TAZ plays a major role in GPCR signaling in breast carcinoma. Understanding the differential roles for YAP and TAZ in various types of cancer is crucial in developing targeted treatments against this oncogenic pathway. Our studies support the growing body of literature indicating TAZ as the major target in triple negative, metastatic breast cancer.

3.5 Concluding remarks

The work presented in this dissertation reveals a novel and important role for the tumor suppressor ARRDC3 in regulation of GPCR trafficking and signaling in invasive basal-like breast cancer. The data reported here suggests that ARRDC3 functions not only by regulating proper PAR1 trafficking to control signaling dynamics but also by directly interacting with the Hippo pathway component TAZ to inhibit signaling.

In addition to teasing out the molecular mechanisms involved in this regulatory process, I further showed that ARRDC3 plays a physiological role in inhibiting agonist-induced migration and invasion as well as tumor metastasis *in vivo.* I also determined that the ARRDC3 PPxY motifs are crucial determinants for the tumor and metastasis suppressor activity of ARRDC3. We speculate that the PPxY motifs are important for both interacting with E3 ubiquitin ligases as well as with TAZ to modulate function. ARRDC3 represents a potential biomarker for aggressive disease as well as a potential therapeutic target as some studies have shown that nuclear export inhibitors and therapeutics that target epigenetic regulators can induce the expression of ARRDC3 in basal-like breast carcinoma cells [33].

My work has also added to the growing body of literature supporting a major role for TAZ in breast cancer [34]. This study is the first to investigate the differential role of YAP and TAZ in GPCR-mediated signaling in the context of invasive, basal-like breast carcinoma. We found that TAZ is the critical effector, while YAP was expendable, for mediating GPCR-Hippo signaling as well as migration and invasion in invasive breast cancer cells. Interestingly, YAP is still activated

in these cells by GPCR signaling and it is important to, in the future, determine the function of YAP in invasive breast cancer. Revealing this role for TAZ is particularly exciting as the Hippo pathway has been of great interest to pharmaceutical companies, with the hopes of developing drugs that target and inhibit this pathway. Our studies suggest that TAZ may be a better therapeutic target than YAP in the treatment of breast cancer patients.

3.6 References

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