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New mechanisms of receptor crosstalk

Shannon Lillian O'Brien

BBiomedSc

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Abstract

Dysregulation of the Renin Angiotensin System (RAS), and its cognate receptor, the angiotensin (AngII) type 1 receptor (AT_1R), has been implicated in the pathogenesis of several cardiovascular disorders including hypertension, fibrosis, congestive heart failure and stroke. Despite the development of pharmacological agents that target the RAS, the persistent burden of cardiovascular disease remains. My PhD focuses on the AT_1R, specifically its ability to 'crosstalk' and transactivate signalling pathways downstream of the Epidermal Growth Factor Receptors (EGFR). This paradigm of EGFR transactivation thereby enables the AT_1R to potentially regulate cell growth, differentiation, apoptosis and promote cancerous cell growth. Part of ongoing work in the Thomas laboratory is to better understand the roll of previously identified novel proteins in AT_1R-EGFR transactivation, including CHKA (Choline Kinase Alpha), BMX (non-receptor tyrosine kinase) and TRIO (Triple Functional Domain PTPRF Interacting). It is therefore the focus of my research to use molecular and cellular approaches to interrogate the mechanistic basis for EGFR transactivation and better define the relative role of these novel proteins in AT_1R-EGFR signalling.

Although it is of great interest to identify and characterise the molecular, temporal and spatial mechanisms of AT_1R-EGFR transactivation, demonstrating EGFR transactivation directly, in live cells, and in real-time has been challenging. Typically, end-point assays (e.g., phospho-ERK1/2) are used as a surrogate readout of EGFR activation, however they are often downstream readouts and are not applicable to live cells. Herein, I report the use of a Bioluminescence Resonance Energy Transfer (BRET) based assay to report the recruitment of the EGFR adaptor protein, growth factor receptor-bound protein 2 (Grb2), association with the EGFR. In live HEK293 cells, both epidermal growth factor (EGF) and AngII stimulation produced sustained ligand-dependent recruitment of Grb2 to the EGFR. The BRET assay was applied to a variety of cell lines and also used to screen a panel of 19 GPCRs, with the AT_1R and vasopressin receptor showing distinct EGFR transactivation. Additionally, HER2, a member of the EGFR family (also known as ErbB2) and preferred dimerisation partner of the EGFR, was also transactivated by the AT_1R. Mechanistically, we observed that the AT_1R-mediated ERK1/2 activation was completely dependent on $G_{q/11}$ as well as EGFR tyrosine kinase activity, whereas EGFR-Grb2 recruitment was independent of $G_{q/11}$ and only

partially dependent upon the EGFR tyrosine kinase activity. This $G_{q/11}$ independence was confirmed using a G protein-uncoupled AT_1R mutant that demonstrated transactivation comparable to that seen in the AT_1R wild type. Finally, we provide evidence that both AngII- and EGF-stimulation promotes a physical association between AT_1R-EGFR, however the molecular requirements vary for each receptor.

In addition, I report in this thesis the concept of 'reverse-transactivation', whereby a significant proportion of the total EGF signalling is dependent upon the co-activation of the AT_1R and its capacity to couple to G proteins. A BRET-based assay was used, both in transfected HEK293 cells and primary isolated vascular smooth muscle cells (VSMC), to demonstrate that EGF-stimulation of the EGFR leads to the activation and arrestin-binding of the AT_1R. This EGF-mediated recruitment of β-arrestin to the AT_1R was completely dependent upon EGFR tyrosine kinase activation and apparently involves the active, G_q coupled state of the AT_1R. Moreover, a truncated AT_1R, lacking all carboxyl-terminal phosphorylation sites, was incapable of recruiting arrestin following EGF-stimulation, indicating reverse transactivation requires phosphorylation and binding of arrestin to the AT_1R. The rapid internalisation of the AT_1R following AngII-stimulation was not observed following EGF activation, instead causing increased recruitment of AT_1R to the plasma membrane.

In summary, I have developed and applied a sensor BRET based-assays to characterise the molecular, temporal and spatial aspects of AT_1R-EGFR transactivation. We provide evidence of bi-directional receptor crosstalk, whereby both the AT_1R and EGFR have the capacity to transactivate each other, however the molecular mechanisms governing this crosstalk vary for each receptor. Collectively, this powerful new platform, which interrogates the most proximal events of AT_1R-EGFR transactivation, can be used to better define AngII influences on cardiovascular physiology and pathology, and therapeutically lead to the development of new drugs against cardiovascular disease.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications included in this thesis

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Submitted manuscripts included in this thesis

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Contributions by others to the thesis

All the work presented in this thesis is my own, with the following exceptions:

Figure 4.03C & Figure 4.04: Effects of $G_{q/11}$ and β-arrestin1/2 deletion on Grb2 interaction with the EGFR. CRISPR/Cas9 engineered HEK293 cells were kindly provided by Assistant Professor Asuka Inoue (Graduate School of Pharmaceutical Sciences & Faculty of Pharmaceutical Sciences, Tohoku University, Japan). The BRET experiment was performed by Professor Terry Hérbert and Dr Dominic Devost (Pharmacology and Therapeutics, McGill University, Canada)

Statement of parts of the thesis submitted to qualify for the award of another degree

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List of Abbreviations used in this thesis

Abbreviated Text	Full name
°C	Degrees celcius
125I	Iodine-125
Å	Angstrom
AAA	Abdominal aortic aneurysm
ACE	Angiotensin converting enzyme
ADAM	A disintegrin and metalloprotease
AGRF	Australian genome research facility
ALP	Alkaline phosphatase
Ang1-7	Angiotensin 1-7
Ang1-9	Angiotensin 1-9
AngI	Angiotensin I
AngII	Angiotensin II
ANOVA	Analysis of variance
ANZSRC	Australia and New Zealand standard research classifications
ARB	Angiotensin receptor blockers
Asn	Asparagine
AT1a	Angiotensin type 1a receptor
AT1b	Angiotensin type 1b receptor
AT_1R	Angiotensin type 1 receptor
AT_2R	Angiotensin type 2 receptor
ATCC	American type tissue culture
AUC	Area under the curve
AUS	Australia
BAL	2,3-dimercaptopropanol or British anti-Lewisite
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BCA	bicinchoninic acid assay
BH	Br homology
BiFC	Bimolecular fluorescence complementation
BMX	Bone marrow kinase X-linked kinase
BrDU	5-bromo-2'-deoxyuridine

BRET	bioluminiscence resonance energy transfer
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
C1/C2	Cysteine rich regulatory domains 1/2
c-Src	cellular Src kinase
C-terminus	carboxyl terminal
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
Cas9	CRISPR associated protein 9
cDNA	Complementary DNA
CHKA	Choline kinase alpha
CHKA1	Choline kinase alpha 1
CHKA2	Choline kinase alpha 2
CHKα	Choline kinase alpha
CHKβ	Choline kinase beta
CHO-K1	Chinese hamster ovary
CK37	Choline Kinase-α Inhibitor
COS-7	Transformed African Green Monkey Kidney Fibroblast Cells
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CVD	Cardiovascular disease
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
ECL	Extracellular loop
EDT	1,2-ethanedithol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB	Epidermal growth factor receptor family of receptors
ERK1/2	Extracellular signal-regulated protein kinase 1 and 2
ET-1	Endothelin-1
ETK	*Escherichia coli* tyrosine kinase

FBS	Fetal bovine serum
FlAsH	Fluorescein arsenical hairpin
FLIPR	Fluorescence Imaging Plate Reader
FoR	Field of research
FRET	Fluorescence resonance energy transfer
G protein	Heterotrimeric guanine nucleotide binding protein
GCaMP	Genetically encoded calcium indicator
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GPCR54	G protein-coupled receptor 54
Grb2	Growth factor receptor-bound protein 2
GRK	G protein-coupled receptor kinase
GTP	Guanine triphosphate
Gα	G protein alpha subunit
Gα12	G protein α12
Gα13	G protein α13
Gαi	G protein αi
Gαq/11	G protein αq/11
Gαs	G protein αs
Gβγ	G protein beta/gamma subunit
HA	Human influenza hemagglutinin
HB-EGF	Heparin-binding epidermal growth factor
HBSS	Hanks' balanced salt solution
HEK-adherent-293	Adherent human embryonic kidney 293
HEK293	Human embryonic kidney 293
HEK293FT	High transfection efficiency expressing SV40 large T antigen human embryonic kidney 293
HeLa	Human cervical cancer cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Human epidermal growth factor receptor
HIT	Heteromer identification technology

HMEC	Human mammary epithelial cell
HT-29	Human colorectal adenocarcinoma
ICL	Intracellular loop
IGF	Insulin growth factor
IP3	Inositol triphosphate
JNK	Jun kinase
JM	Juxtamembrane
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
L	Litre
L1/L2	ligand-binding domain 1/2
LPA	Lysophosphatidic acid
LB	Lysogeny broth
Leu	Leucine
LPA	Lysophosphatidic Acid
LPL	Low-density lipoprotein
LVH	Left ventricular hypertrophy
MAPK	MAP Kinase
MASR	MAS receptor
mCherry	monomeric cherry fluorescent protein
MEK	Mitogen-activated protein kinase kinase
MEM	Minimum essential medium
miRNA	micro RNA
mL	Millimetre
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mm	Millimetre
MMP	Matrix metalloprotease
mol	Molar
MrgD	MAS-related G protein-coupled receptor, D
mRNA	Messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate

NanoLuc	Nano luciferase
NaOH	Sodium hydroxide
NBCS	Neonatal bovine calf serum
NEB	New England biolabs
ng	nanogram
NHA	N-terminus HA tag
NHMRC	National Health and Medical Research Council
NIH-3T3	Mouse embryonic fibroblast
nm	nanometre
ns	Non-significant
P-Choline	phosphocholine
p38 MAPK	P38 mitogen-activated protein kinases
PA	Phosphatidic acid
PAR1	Protease-activated receptor
PBS	Phosphate buffered saline
pcDNA3	Plasmid cytomegalovirus promoter deoxyribonucleic acid
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor receptor
Phe	Phenylalanine
PIP2	Phosphatidycholine-4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PL	Parental
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PP2	4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine
Pro	Proline
PTB	Phosphotyrosine binding
PtdCho	Phosphatidylcholine
PVDF	Polyvinylidene fluoride
Rab	Ras in the brain
Rac1	Ras-related C3 botulinum toxin substrate 1

RAS	Renin Angiotensin System
RET	Resonance energy transfer
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay
Rluc	Renilla Luciferase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen specie
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src homology 2
SHP-2	Src homology- 2 domain containing phosphatase
shRNA	Short hairpin RNA
SII	Sar1-Ile4-Ile8
SIP1	Sphingosine-1-phosphate
siRNA	short interfering RNA
SMC	Smooth muscle cell
Sos	Sons of sevenless
TAE	Tris-acetate-EDTA
TLC	Thin layer chromatography
TM	Transmembrane
TMPS	Triple membrane passing signal
TRIO	Triple functional domain PTPRF interacting protein
TSHR	Thyroid stimulating hormone receptor
Tyr	Tyrosine
UV	Ultraviolet
UVP	UV Transilluminator
V	Voltage
Vn	N-terminal part of a split Venus

$V_{1b}R$	Vasopressin V1b receptor
Vc	C-terminal part of a split Venus
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cells
WR	Working reagent
YFP	Yellow fluorescent protein
α	Alpha
β	Beta
β-arr	β-arrestin
β_1AR	β1-adrenergic receptor
β_2AR	β2-adrenergic receptor
γ	Gamma
μ	Micro

Chapter One

General Introduction.

1.0 General Introduction

1.1 Cardiovascular Disease – Cellular Remodelling

Cardiovascular disease (CVD) refers to diseases of the heart and blood vessels, including stroke, heart attack and failure, as well as vascular dysfunction. The prevalence of heart disease has increased in Australia over the last decade, with 30% of all deaths contributed to CVD (Heart Foundation Australia, 2014). Despite the development of improved pharmaceuticals, the disease burden on the community and economy is significant, and alternative approaches are required to improve prevention and treatment methodologies. Cellular remodelling, through the actions of cellular growth (hypertrophy), fibrosis, cellular migration and proliferation, has been shown to contribute to the pathogenesis of CVD, making it a potential therapeutic target for intervention (Ferrario, 2016).

The Renin Angiotensin System (RAS) plays a central role in the development and regulation of cellular remodelling, mediating a broad range of homeostatic and modulatory processes. RAS dysregulation also promotes the development of pathological states through actions in cellular growth and remodelling, endothelial dysfunction, angiogenesis, inflammation and cancer (Lijnen, Petrov & Fagard, 2000; Mehta & Griendling, 2007; Nakashima, Suzuki, Ohtsu et al., 2006; Ruiz-Ortega, Rupérez, Esteban et al., 2005; Wegman-Ostrosky, Soto-Reyes, Vidal-Millán et al., 2015). Accordingly, therapeutic agents that target the RAS such as Angiotensin (AngII) converting enzyme (ACE) inhibitors and AngII receptor blockers (ARBs) modify the process of cardiac remodelling and remain frontline therapeutics (Cuspidi, Negri & Zanchetti, 2008). Thus, the blockade of the RAS regresses cellular remodelling and in turn inhibits CVD progression, most likely by attenuating the direct effects of AngII on the heart.

1.2 Angiotensin II and the AT_1 Receptor

1.2.1 The Renin Angiotensin System

The peptide hormone AngII (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) plays an integral role in cardiovascular and renal pathophysiology and physiology, having been shown to influence a large range of cardiovascular homeostatic and modulatory processes. AngII is produced as part of the RAS cascade as outlined in Figure 1.01. The synthesis of renin is the initial rate-limiting step and the initiator of RAS signalling (Skeggs, Kahn, Lentz et al., 1957). Renin is initially produced and stored in the granular cells within the juxtaglomerular apparatus of the kidney as its inactive form, prorenin. Once cleaved and secreted into the bloodstream, prorenin undergoes processing to form the biologically active form, renin (Bajwa & Kwatra, 2012). The classical endocrine view of the RAS involves renin cleaving liver-borne angiotensinogen to generate the decapeptide, Angiotensin I (AngI). Circulating AngI is cleaved by the angiotensin converting enzyme (ACE) and is converted to the octapeptide, AngII (Gul, Ramdas, Mandavia et al., 2012). AngII then exerts its effects through integrated actions on the cardiovascular system, central nervous system and the kidney. AngII stimulates vasopressin release (increasing water retention by the kidney), vasoconstriction (thereby increasing total peripheral resistance), thirst (via the central nervous system) and aldosterone release from the adrenal cortex (promoting water retention and the reabsorption of sodium chloride), ultimately leading to an increase in blood pressure (Atlas, 2007).

Undoubtedly, the actions of AngII on its cognate G protein-coupled receptor (GPCR), the Angiotensin type 1 receptor (AT_1R), accounts for the majority of the biological functions attributed to AngII (Gul, Ramdas, Mandavia et al., 2012). AngII can also bind to the angiotensin type 2 receptor (AT_2R), which is thought to have opposing actions in response to AngII. AT_2R activation contributes to the cardioprotective effects of ARBs (Brede, Hadamek, Meinel et al., 2001; Ferrario, 2010), although this cardioprotective role is lost in hearts that excessively overexpress the AT_2R (Xu, Sun, Carretero et al., 2014). More recently, the simplistic view that the RAS system is a linear biochemical cascade comprised of two proteolytic steps has been replaced by a much more complex system involving various mediators, receptors and multifunctional enzymes as shown in Figure 1.01 (Dzau, 1988).

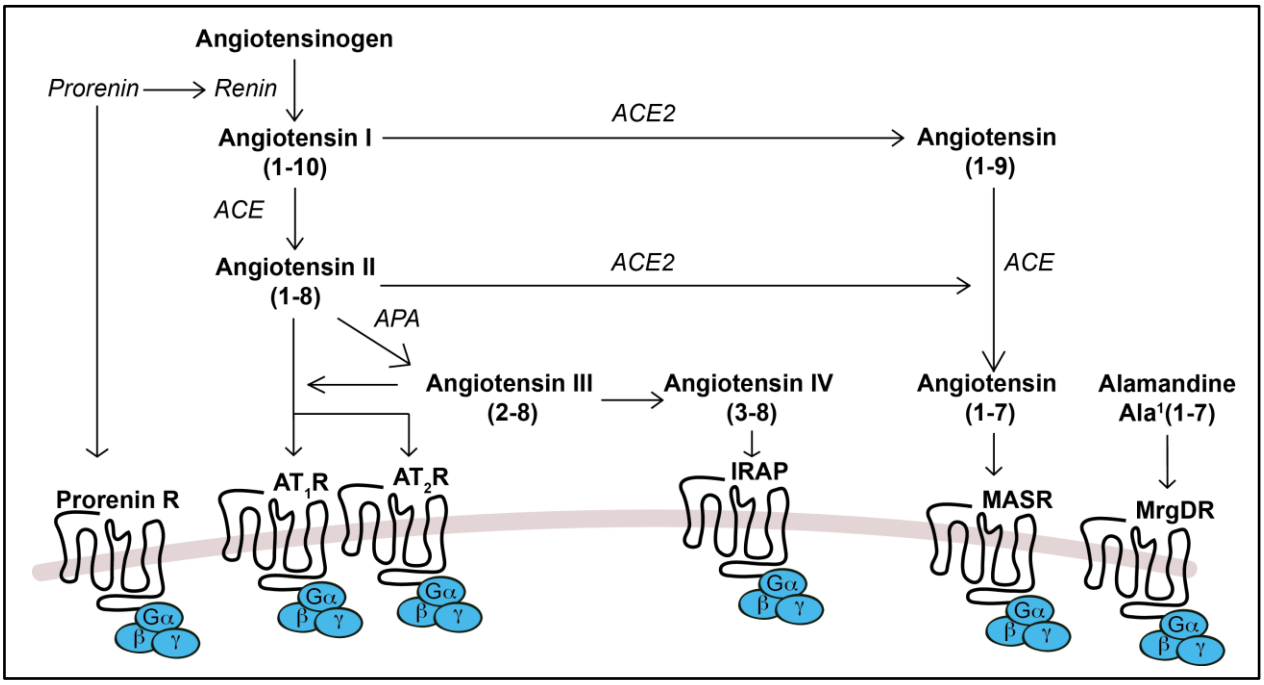


Figure 1.01: The Renin Angiotensin System

A simplified schematic of the major components of the RAS. The classical RAS pathway was initially considered to involve sequential cleavage of angiotensinogen by Renin and ACE to yield AngII. AngII exerts the majority of its physiological actions through binding and activating the AT_1R, in addition to the AT_2R. However, during the past two decades, there have been additional advancements in the RAS biochemical axis, including the discovery of an ACE homologue, ACE2, which produces the Ang1-7 fragment that may act via the MAS receptor. Angiotensin converting enzyme (ACE), aminopeptidase A (APA), insulin-regulated aminopeptidase (IRAP), MAS receptor (MASR), Mas-related GPCR type D (MrgDR)

Additional advancements in the RAS regulation include the recently identified ACE homologue, ACE2, considered the cardio-protective axis of the RAS (Shi, Mao, Xu et al., 2010) (Figure 1.01). ACE2, predominantly expressed in the heart, blood vessels and kidney (Oudit, Crackower, Backx et al., 2003), cleaves the carboxyl-terminal amino acid from AngII to form Angiotensin1-7 (Ang1-7), also formed by the action of ACE on Angiotensin1-9. Ang1-7 reportedly binds to the MAS receptor (MASR, a GPCR), and is believed to counter the actions of AngII leading to vasodilation and a decrease in cell proliferation, fibrosis and oxidative stress (Zhang, Chen, Zhong et al., 2014), however more recent studies have shown that Ang1-7 cardioprotective effects are thought to involve both the MASR and AT_1R as well as other unknown targets (Teixeira, Parreiras-e-Silva, Bruder-Nascimento et al., 2017). Furthermore, almandine, similar structurally to Ang1-7 (a substitution at the first amino acid

position) does not bind to the MAS receptor, instead acting through the Mas-related GPCR type D (MrgD) receptor to promote vasodilation and antihypertensive effects (Etelvino, Peluso & Santos, 2014; Lautner, Villela, Fraga-Silva et al., 2013). Other advancements include the identification of the prorenin receptor, which binds renin and prorenin and enhances the catalytic conversion of angiotensinogen to AngI, increasing AngII generation (Nguyen, Delarue, Burcklé et al., 2002). Activation of the prorenin receptor can mediate intracellular pathways independent of the generation of AngII, having been shown to be an accessory protein of vacuolar H^+-ATPase and implemented in Wnt signalling, low-density lipoprotein (LPL) clearance, glucose metabolism and profibrotic signalling (Cruciat, Ohkawara, Acebron et al., 2010).

It is now well established that local RAS systems exist in many tissues and that these function as an independent entity from the blood-borne RAS (Coble, Grobe, Johnson et al., 2015; Ferrario, 2010; Leung & Carlsson, 2001; Satou & Gonzalez-Villalobos, 2012; Satou, Shao & Navar, 2015; Sparks, Crowley, Gurley et al., 2014; Yang, Smolders & Dupont, 2011). For over 30 years, researchers have known of the existence of renin and renin activity, as well as the presence of the mRNA for angiotensinogen, renin and ACE in a variety of tissues (e.g., brain, heart, kidney, vasculature etc.). Of particular note is the cardiac RAS, with the myocardium capable of synthesising and secreting major components of the RAS (Crackower, Sarao, Oudit et al., 2002; Dzau & Re, 1994).

1.2.2 The AT_1 Receptor

The AT_1R is expressed in a range of organs including the heart, liver, kidney, brain, adrenals, vasculature and lung (De Gasparo, Catt, Inagami et al., 2000). The AT_1R is an approximately 40-41kDa seven transmembrane (TM) domain-containing protein composed of 359 amino acids. All GPCRs, including the AT_1R, share a common molecular topology with a hydrophobic core of seven transmembrane spanning α-helices that form an extracellular/intra-membrane ligand binding pocket, an extracellular N-terminus and an intracellular carboxyl terminal (C-terminus) tail. The AT_1R and AT_2R were cloned simultaneously over two decades ago, and led to the discovery of two distinct AT_1R subtypes in the rat and mice, the AT1a and AT1b receptor located on chromosome 17 and 2, respectively (Koike, Horiuchi, Yamada et al., 1994; Murphy, Alexander, Griendling et al.,

1991; Sandberg, Ji, Clark et al., 1992). This contrasts humans, which have a single AT_1R encoded by the AGTR1 gene on chromosome 3q. Although the AT1a and AT1b receptor are located on two different chromosomes, the two subtypes display over 90% homology in the coding region of the cDNA sequence and 95% homology at the amino acid level (Martin, White, Li et al., 1995). In contrast, the coding region of the AT_1R and AT_2R cDNA exhibit only 30% homology.

Both the AT1a and AT1b receptor subtypes share nearly identical ligand binding affinities and signalling properties, while expression appears to be tissue dependent, with the AT1b receptor abundant in the pituitary gland and adrenal cortex, whereas the AT1a receptor is more abundant in the heart, kidney, liver, smooth muscle and lungs (Gasc, Shanmugam, Sibony et al., 1994; Guo, Uno, Ishihata et al., 1995). Genetic ablation of either or both subtypes show that the AT1a receptor plays an important role in the regulation of blood pressure and is the dominant subtype in cardiovascular physiology (Ito, Oliverio, Mannon et al., 1995; Timmermans, Wong, Chiu et al., 1993). The AT1a receptor is the predominant receptor used to characterise AT_1R physiology and pharmacology, and for this reason, all work in this thesis was performed using the AT1a receptor subtype, heretofore referred to as the AT_1R for simplicity.

1.3 AT_1 Receptor Signalling & Regulation

1.3.1 G protein-coupled receptors

Seven transmembrane spanning GPCRs represent the largest superfamily of cell surface receptor proteins and are arguably the most physiologically important class of receptor signal transducers (Katritch, Cherezov & Stevens, 2013). The total number of confirmed human GPCRs is in excess of 800 unique full-length members (Fredriksson, Lagerstrom, Lundin et al., 2003). GPCRs are considered the gatekeepers of intracellular communication and can subsequently bind a myriad of signalling molecules, including photons, ions, odorants, peptides, lipids, proteins and bioamines (Vischer, Watts, Nijmeijer et al., 2011). Generally, GPCRs transduce information provided by the extracellular stimuli into appropriate downstream effectors via their coupling to heterotrimeric guanine nucleotide binding proteins (G proteins) (Johnston & Siderovski, 2007). The binding of agonist to GPCR selects for a

receptor conformation state that allows for the disassociation of the G protein, and the activation of a variety of intracellular second messengers. However, dysregulation and mutations of GPCRs, and the resulting signalling pathways, has been implicated in the pathogenesis of many diseases, and it is of no surprise that GPCRs represent the largest family of drug targets (Insel, Tang, Hahntow et al., 2007).

1.3.2 AT_1 Receptor binding and activation

Despite the critical role of AT_1R in cardiovascular pathophysiology and the widespread clinical use of AT_1R blockers to treat CVD, its structure at the atomic level has remained elusive until the recent revelation of the crystal structure for the human AT_1R in complex with a variety of inverse agonists and antagonists (Zhang, Han, Batyuk et al., 2017; Zhang, Unal, Desnoyer et al., 2015; Zhang, Unal, Gati et al., 2015). As already mentioned, the AT_1R exhibits the canonical seven transmembrane α-helical architecture, comprising of an extracellular N-terminus, three extracellular (ECL-3) and intracellular (ICL-3) loops, an amphipathic helix 8 and lastly an intracellular carboxyl terminus (Zhang, Unal, Gati et al., 2015). The four putative extracellular domains each contain a cysteine residue that is capable of forming two disulphide bonds, (De Gasparo, Catt, Inagami et al., 2000; Ohyama, Yamano, Sano et al., 1995). One of which is unique to the AT_1R and connects the N-terminus with ECL3, helping to shape the extracellular side of the AT_1R. The other, which is conserved among GPCRs, connects helix III with ECL3 and is important for receptor folding expression and conformational stability (Zhang, Unal, Gati et al., 2015).

A full discussion of structural and functional characteristics of AngII-AT_1R interactions is beyond the scope of this thesis, however specific AngII residues and AT_1R amino acids are critical for ligand binding. Upon AngII stimulation, the AT_1R undergoes conformational changes in TM3 and TM6 helices, allowing for TM2 and TM7 interaction (Miura, Zhang, Boros et al., 2003). Amino acid residues of TM2, 3, 4, 5, 6 and 7 form the ligand binding pocket and force AngII to adopt a vertical binding configuration, with its amino terminus interacting with the ECL2 and the carboxyl terminus interacting more deeply within the TM core (Fillion, Cabana, Guillemette et al., 2013). The major contacts of AngII that activate the AT_1R are the amino acids Phe^8 (Holloway, Qian, Pipolo et al., 2002; Miura, Feng, Husain et al., 1999; Nikiforovich, Zhang, Yang et al., 2006; Noda, Feng, Liu et al., 1996; Yamano,

Ohyama, Kikyo et al., 1995) and Tyr^4 (Miura, Feng, Husain et al., 1999; Nikiforovich, Mihalik, Catt et al., 2005). The phenylalanine at position 8 is critical for agonist activity with mutation of Phe^8 shown to weaken agonist activity and produce an agonist-to-antagonist transition (Wilkes, Masaro, Schiller et al., 2002). In the absence of receptor activation, the receptor is held in an inactive conformation via two intramolecular hydrogen bonds that exist between Asn111 and Asn295 (Balmforth, Lee, Warburton et al., 1997), with the recent elucidation of AT_1R crystal structure confirming this (Zhang, Unal, Gati et al., 2015). The α-carboxyl group of Phe^8 docks with Lys199, enabling Phe^8 side chain to interact with His256 and Phe259 and stabilise an active receptor conformation (Noda, Saad & Karnik, 1995; Oliveira, Costa-Neto, Nakaie et al., 2007). Phe^8 interaction with His256 facilitates Tyr^4 coordination with Asn111 and destabilises this hydrogen bond network and allows Tyr292 to interact with the conserved residue Asp74 (Groblewski, Maigret, Larguier et al., 1997; Inoue, Nakamura & Inagami, 1997). It is thought that this set of interactions play a vital role in relaying the conformational changes in the ligand binding pocket to the cytoplasmic domain and coupling to downstream signalling.

1.3.3 Classical $G\alpha_{q/11}$ AT_1 Receptor Signalling

As per most GPCRs, the classical mechanism of AT_1R signal transduction involves receptor coupling to heterotrimeric G proteins. The G protein complex is composed of three polypeptides: Gα, which binds and hydrolyses guanine triphosphate (GTP), and a Gβγ dimer (Milligan & Kostenis, 2006). Prior to ligand binding, the inactive Gα subunit is bound to guanine diphosphate (GDP) and associates with the Gβγ dimer (Gilchrist, 1996). Following ligand binding, a conformational change follows, decreasing Gα affinity for GDP (Oldham & Hamm, 2008). GDP disassociates from the complex, and GTP binds, rendering the Gα subunit active. Gα-GTP disassociates from both the receptor and the Gβγ dimer, allowing both the Gα-GTP and Gβγ to interact with downstream effector enzymes and regulate cell signalling. The cellular response is terminated following the hydrolysis of Gα-GTP to Gα-GDP, thus allowing the reformation of the inactive form of the heterotrimeric G protein complex.

GPCRs can interact with a variety of Gα proteins, including $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12,}$ and $G\alpha_{13,}$ subsequently activating a multitude of second messengers and intracellular protein kinases (Higuchi, Ohtsu, Suzuki et al., 2007). The 'classical' AT_1R signalling responsible for vasoconstriction, primarily couples to the heterotrimeric G protein $G\alpha_{q/11}$ (Belmonte & Blaxall, 2012). $G\alpha_{q/11}$ activation results in PLC-β activation and subsequent hydrolysis of phosphatidycholine-4,5-biphosphate (PIP2) to form two key second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG) (Balakumar & Jagadeesh, 2010). IP3 binds to its receptor located at the sarcoplasmic reticulum, which initiates the release of calcium (Ca^{2+}). Ca^{2+} release is further enhanced with free Ca^{2+} additionally binding to Ca^{2+}-release channels and/or AngII directly stimulating cell membrane voltage-gated Ca^{2+} channels. Depending on the tissue, Ca^{2+} then binds to calmodulin or troponin (myocardium), activating myosin light chain kinase (MLCK). This triggers phosphorylation of myosin light chain and initiates vascular smooth muscle contraction. The additional second messenger derived from PIP2 hydrolysis, DAG, is a lipid second messenger and a potent activator of Protein Kinase C (PKC) through binding to a conserved C1 domain, leading to PKC plasma translocation (Wang, 2006). PKC is also a well-known mediator of the mitogen-activated protein (MAPK) pathway, a critical mediator of signal transduction in mammalian cells that contributes to the vasoconstrictive properties of receptor activation (Lu & Xu, 2006).

A number of receptor mutations within conserved regions have been show to perturb classical, G protein-mediated signalling from the AT_1R. Of the residues introduced previously, substitution of Asp74 to asparagine or glycine and Tyr292 to phenylalanine uncouples the receptor from G protein activation (Bihoreau, Monnot, Davies et al., 1993). Mutation of Asn111 to glycine (N111G), biases the receptor towards the $G_{q/11}$ pathway, resulting in an approximate ~50% increase in IP3 production compared to wild type AT_1R (Groblewski, Maigret, Larguier et al., 1997; Noda, Feng, Liu et al., 1996). This constitutively active mutant [N111G]AT_1R is not phosphorylated (Cabana, Holleran, Leduc et al., 2015; Thomas, Qian, Chang et al., 2000). The highly conserved D(E)RY motif in helix III has also been shown to mediate receptor activation/inactivation as well as G protein coupling. Mutation of DRY to AAY impairs receptor internalisation and attenuates classical $G\alpha_{q/11}$-mediated signalling (Feng, Ding, Ren et al., 2005; Gáborik, Jagadeesh, Zhang et al., 2003). Another highly conserved amino acid is Try215 at the base of TM5. Hunyady et al., 1995 demonstrated that following agonist stimulation, mutant receptor Y215F has an impaired

ability to couple to G proteins as well as a significant (but still demonstrable) reduction in receptor internalisation (Hunyady, Bor, Balla et al., 1995). The same group also revealed that the conserved sequence, NPxxY (Asn^{298}, Pro^{299}, Leu^{300}, Phe^{301}, Tyr^{302}), located in the TM7 helix also participates in receptor coupling (Hunyady, Bor, Baukal et al., 1995). Alanine replacement of Asn298, Pro299 and Tyr302 impaired G protein coupling, however showed no substantial changes in receptor internalisation kinetics. Helix VIII has also been shown to bind Ca^{2+}-regulated effector protein, calmodulin (Thomas, Pipolo & Qian, 1999), and is important for receptor coupling to G protein signalling (Sano, Ohyama, Yamano et al., 1997; Thomas, Baker, Motel et al., 1995). When comparing AT_1R structure to that of the AT_2R, a GPCR that poorly couples to G proteins and arrestins, AT_2R helix VIII no longer runs parallel to the plasma membrane and forms an interface with the intracellular cavity of the 7TM bundle, repressing canonical AT_2R activity and sterically blocking various receptor partners (Zhang, Han, Batyuk et al., 2017). Most recently solved GPCR structures suggest that helix VIII stabilises the active-like receptor state but also works as a gate keeper, sterically blocking or recruiting G proteins (Zhang, Han, Batyuk et al., 2017; Zhang, Unal, Gati et al., 2015). These AT_1R mutants provide evidence that a number of topologically distinct contacts are required for agonist-induced coupling of the AT_1R to $G\alpha_{q/11}$.

1.3.4 AT_1 Receptor Regulation

The co-ordinated actions of two families of proteins, the GPCR serine/threonine kinases (GRKs) and arrestins, are critical for receptor down regulation and desensitisation (Ahn, Shenoy, Wei et al., 2004; Balakumar & Jagadeesh, 2014; Patel, Noor & Rockman, 2010). Following ligand binding, GRKs phosphorylate serine/threonine sites located on the AT_1R C-tail, resulting in the attenuation of G protein-dependent signalling and targets the receptor for internalisation (Magalhaes, Dunn & Ferguson, 2012). GRK-mediated phosphorylation promotes the binding of the cytoplasmic adaptor protein β-arrestin, which facilitate the aggregation of deactivated receptors into clathrin-coated vesicles and enable the internalisation and sorting of receptor into endosomes.

AT_1R phosphorylation can result in homologous or heterologous desensitisation. Homologous desensitisation accounts for specific phosphorylation of an activated GPCR and the attenuation of further signalling. GRK2, 3, 5 and 6 are ubiquitously expressed and

interact with most GPCRs, however the relative contribution of GRKs to AT_1R desensitisation is not well resolved. Heterologous desensitisation is mediated by second messenger-activated kinases such as protein kinase A (PKA) and PKC, which phosphorylate and desensitise the receptor (Cho, Cho, Park et al., 2007; Kelly, Bailey & Henderson, 2008; Xiang, Yu, Guo et al., 2001). AT_1R heterologous desensitisation was originally thought to be independent of β-arrestins, however it has been recently demonstrated that PKC activation (whether it be by Adrenergic-α_{1A} receptor, phorbol myristate ester (PMA) or EGFR activation) mediates AT_1R phosphorylation and β-arrestin2 binding (Tóth, Prokop, Gyombolai et al., 2018).

Arrestins terminate AT_1R signalling and mediate receptor internalisation, serving as key regulators of AT_1R function. The β-arrestin family comprises four cytoplasmic adaptor proteins: β-arrestin1 and β-arrestin2 (also denoted as β-arrestin2 and β-arrestin3, respectively) as well as two closely related visual β-arrestins (Smith & Rajagopal, 2016). β-arrestins share high sequence homology (89%) and exhibit the same structural features. GPCRs tend to either interact with β-arrestins transiently, termed "Class A" GPCRs, or form tight stable complexes with arrestins, known as "Class B" receptors (Oakley, Laporte, Holt et al., 2000). Class B receptors, which include the AT_1R, accumulate in endocytic vesicles and form a stable receptor-arrestin complex. The serine-threonine-rich segment of the carboxyl terminus is essential for AT_1R phosphorylation and endocytosis (Kule, Karoor, Day et al., 2004). Almost complete inhibition of AT_1R phosphorylation is observed in truncated AT_1R in which 34 amino acids are removed from the carboxyl terminus (Thomas, Motel, Kule et al., 1998), with studies revealing that the truncated AT_1R, [TK325]AT_1R mutant, is unable to recruit β-arrestins.

Structural studies of arrestin have revealed three conserved motifs that maintain the inactive state of arrestin consisting of the C-terminal tail, the polar core and a number of hydrophobic inter-domain interactions (Gurevich & Gurevich, 2004). Arrestin is thought to first engage with the phosphorylated C-terminus of the receptor, disrupting the three-element interaction, releasing the C-terminus of arrestin and destabilising the polar core (Shukla, Manglik, Kruse et al., 2013; Shukla, Westfield, Xiao et al., 2014). This leads to a major conformational change of arrestin; the most prominent conformational change is the movement of the finger loop, which inserts into the crevice of the activated receptor, allowing for a fully engaged

GPCR-arrestin complex (Kang, Zhou, Gao et al., 2015). Notably, a transition from inactive state, to the C-tail engaged arrestin-GPCR complex, to the fully engaged GPCR-arrestin complex has been observed (Sente, Peer, Srivastava et al., 2018). It has been suggested that distinct GRKs imprint specific phosphorylation patterns, so called phosphorylation barcodes onto the receptor, which are then differentially 'read out' by arrestin, resulting in tail- or fully engaged GPCR-arrestin complexes which result in distinct functional outcomes. More recently, an additional mechanism of arrestin activation has been described that does not require the GPCR tail (Latorraca, Wang, Bauer et al., 2018). The receptor transmembrane core can bind distinct surfaces of arrestin and can stimulate arrestin activity independent from the receptor cytoplasmic tail. The intracellular loops of the receptor interact with the arrestin body rather than the marked finger loops, destabilising charged residues that maintain arrestin in its inactive form (Eichel, Jullié, Barsi-Rhyne et al., 2018). A general consensus is that ligand-mediated core conformation can act independently or in concert with phospho-barcoding of the receptor C-tail to achieve specific arrestin conformations and signalling functions (Hilger, Masureel & Kobilka, 2018). This variability in GPCR-arrestin binding is thought to give rise to either the stable GPCR-arrestin complexes commonly seen with "Class B" receptors, or the transiently formed "Class A" GPCR-arrestin complexes.

The prevailing view that arrestins only uncouple and desensitise G protein-mediated responses has also been challenged in recent years. GPCRs have been described that rely on β-arrestins as genuine signal initiators that facilitate the activation and subcellular localisation of signalling cascades (Lefkowitz, Rajagopal & Whalen, 2006; Luttrell, Ferguson, Daaka et al., 1999; Rajagopal, Kim, Ahn et al., 2010; Ren, Reiter, Ahn et al., 2005; Shenoy, Drake, Nelson et al., 2005; Wei, Ahn, Barnes et al., 2004; Wei, Ahn, Shenoy et al., 2003). It is now clear that a number of catalytically active proteins bind arrestins upon ligand-binding, among them is the Src family tyrosine kinases (DeFea, Vaughn, O'bryan et al., 2000; Luttrell, Ferguson, Daaka et al., 1999), components of the MAPK cascade (DeFea, Zalevsky, Thoma et al., 2000; Luttrell, Roudabush, Choy et al., 2001; McDonald, Chow, Miller et al., 2000), cyclic adenosine monophosphate (cAMP) phosphodiesterase (Perry, Baillie, Kohout et al., 2002), diacelglycerol kinase (Nelson, Perry, Regier et al., 2007) and many more. Of particular relevance to this thesis is the discovery that arrestins act as a 'signalling scaffold' that can recruit extracellular signal-regulated kinase (ERK1/2), a MAPK that appears to be a critical component in eliciting mitogenic responses (Whitmarsh, 2007). This arrestin-

mediated ERK1/2 activation influences the spatial and temporal nature of ERK1/2 signal transduction.

1.3.5 Atypical signalling from the AT_1 Receptor

The full diversity of GPCR-mediated signals cannot be entirely explained by the classical heterotrimeric G protein signalling pathway (Maudsley, Martin & Luttrell, 2007). A number of papers have demonstrated that in the absence of G protein signalling, the AT_1R can still signal to downstream ERK1/2 pathways (Kim, Ahn, Rajagopal et al., 2009; Wei, Ahn, Shenoy et al., 2003) Studies have revealed the existence of two independent pathways – a G protein- (occurs ≤2minute) and a β-arrestin-mediated pathway (>5minutes) (Kim, Ahn, Rajagopal et al., 2009; Luttrell, Roudabush, Choy et al., 2001; Wei, Ahn, Shenoy et al., 2003) that facilitates ligand-activated scaffolding of multiple proteins that are linked to cardio-protective downstream signalling molecules. Within endosomal vesicles, β-arrestin is thought to act as a scaffold and subsequently lead to the assembly of kinases involved in the ERK1/2 cascade - Raf-1, MEK and ERK1/2, thus leading to functionally distinct pools of nuclear (G protein-mediated) and cytoplasmic (arrestin-mediated) pools of ERK1/2 (Ahn, Shenoy, Wei et al., 2004).

The identification of G protein-dependent and -independent signalling has lead to the development of biased ligands that can selectively block or engage a subset of the AT_1R signalling repertoire. Several studies have demonstrated that by selectively engaging arrestin-mediated pathways (in the absence of any detectable G protein signalling), biased ligands can promote cardio-protective and antiapoptopic signalling pathways, and therefore present as a new class of AT_1R-targeted therapeutics. The first, and most widely used biased ligand, Sar^1, Ile^4, Ile^8-AngII (SII), stimulates chemotaxis, promotes cellular proliferation, growth and protein synthesis, cardiac contractility and reduces apoptosis (Wei, Ahn, Shenoy et al., 2003). This has lead to the development of TRV120027, a β-arrestin-biased AT_1R ligand with increased potency and arrestin efficacy, and is currently in clinical trials for the treatment of acute heart failure (Felker, Butler, Collins et al., 2015; Violin, DeWire, Yamashita et al., 2010). However, this arrestin-dependent, G protein-independent ERK1/2 activation has been recently challenged. Grundmann et al., 2018 systematically dissected G protein- from arrestin-driven ERK1/2 phosphorylation for a broad set of GPCRs

(Grundmann, Merten, Malfacini et al., 2018). The study concluded that arrestins are still recruited to GPCRs in the absence of active G protein and that early stage and late phase ERK1/2 activity is driven by G proteins and not arrestins.

Like all GPCRs, the AT_1R lacks intrinsic kinase activity, yet following AT_1R stimulation it has been shown to induce cell growth, proliferation and differentiation, processes that are considered to be independent of traditional models of GPCR signalling (Eishingdrelo & Kongsamut, 2013). This has led to the discovery of signal transduction pathways that do not entirely involve G proteins; instead employing signalling intermediates to 'cross-talk' with receptor tyrosine kinases (RTKs) and subsequently lead to the rapid activation of MAPK (Liebmann, 2001). Arguably, the best-characterised AngII-mediated kinase pathway is ERK1/2. Since the discovery of cross-talk between AT_1R-RTK, many RTKs have been shown to be transactivated by the AT_1R, with the EGFR being the most widely studied. EGFR-stimulated growth promoting signalling pathways are well characterised in the literature, however what is less understood is GPCR-mediated, in particular AT_1R-mediated, mitogenic signalling pathways (Kim, Abraham, Williams et al., 2012).

1.4 EGFR Transactivation

1.4.1 Initial discovery of EGFR transactivation

30-40 years ago, it was thought that GPCRs and RTKs represent distinct and separate signalling pathways that converged on downstream effectors. This theory was disputed in the early to mid 1990's following observations that GPCR- and RTK-mediated signalling pathways are not mutually independent, with studies showing that GPCRs have the capacity to activate RTKs and mitogenic signalling (Faure, Voynoyasenetskaya & Bourne, 1994). However, the defining transactivation experiment did not occur until 1996 when Ullrich and colleagues first published the phenomenon of transactivation between GPCRs and the EGFR (Daub, Weiss, Wallasch et al., 1996). The seminal study (Daub, Weiss, Wallasch et al., 1996) showed that a variety of GPCR agonists: endothelin-1 (ET-1), lysophosphatidic acid (LPA) and thrombin induced rapid phosphorylation and subsequent activation of EGFR and HER2/neu in Rat-1 cells. GPCR activation induced transactivation and subsequently coupled GPCRs to ERK1/2 activation, *c-fos* gene expression and DNA synthesis. These effects were

blocked using a selective EGFR inhibitor, AG1478, and a dominant-negative EGFR. For the first time, activation of RTKs by GPCRs was revealed as the mechanism of mitogenic signalling and proliferation, countering prevailing views of GPCR signal transmission.

The observation of transactivation was subsequently substantiated by numerous independent groups; what was initially reported in Rat-1 fibroblasts was shown to extend in other more general cell types including the liver (Olivares-Reyes, Shah, Hernandez-Aranda et al., 2005; Shah & Catt, 2002; Shah, Yesilkaya, Olivares-Reyes et al., 2004) kidney (Chen, Chen, Neilson et al., 2006), cardiac fibroblasts (Murasawa, Mori, Nozawa et al., 1998), vascular smooth muscle cells (VSMC) (Eguchi, Numaguchi, Iwasaki et al., 1998), prostate (Lin & Freeman, 2003), anterior pituitary (Suarez, Diaz-Torga, Gonzalez-Iglesias et al., 2003), breast cancer (Greco, Muscella, Elia et al., 2003) and neuronal cultures (Huang, Richards & Sumners, 1996).

1.4.2 EGFR family of receptor tyrosine kinases signal transduction

EGFR, also designated HER1, is a 170-KDa glycoprotein and is one of four members of the HER family of ligand-activated RTK (Wieduwilt & Moasser, 2008). The four receptors belonging to the HER family are EGFR (HER1 or ErbB1), HER2 (neu or ErbB2), HER3 (ErbB3) and HER4 (ErbB4). HER receptor proteins are expressed in cells of mesodermal and ectodermal origins, with all four receptors expressed in the heart (Jorissen, Walker, Pouliot et al., 2003; Roskoski, 2014; Yarden & Sliwkowski, 2001). All HER receptors share conserved structural features, including an extracellular ligand-binding region, single membrane spanning domain and lastly, an intracellular cytoplasmic kinase domain, that contains autophosphorylation sites that flank the cytoplasmic kinase domain (Bogdan & Klambt, 2001). Of the twenty tyrosine residues capable of phosphorylation within the cytoplasmic domain, seven of these are identified as autophosphorylation sites (Bishayee, Beguinot & Bishayee, 1999).

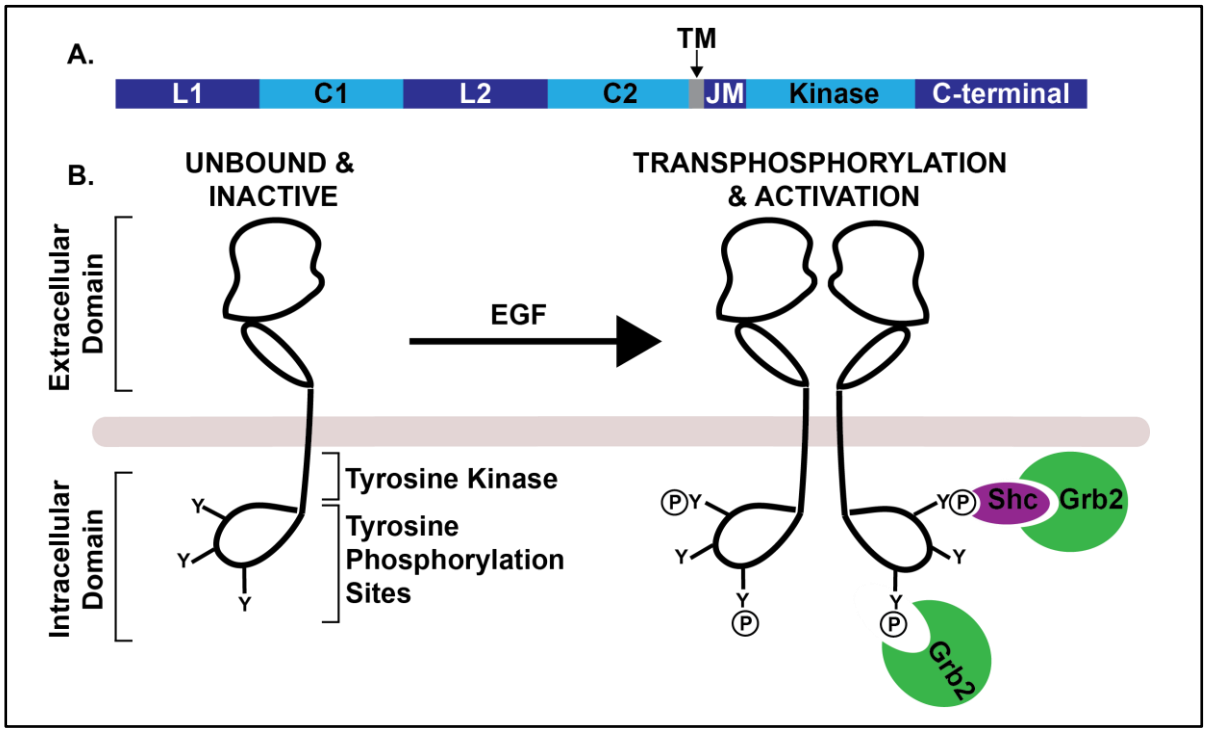


Figure 1.02: EGFR Activation
Schematic of EGFR domains, structure and dimerisation. **(A)** EGFR consists of an extracellular domain containing two leucine-rich ligand-binding domains (L1 and L2) and two cysteine-rich regulatory domains (C1 and C2). The transmembrane (TM) domain connects to a short juxtamembrane (JM) sequence followed by the tyrosine kinase domain (Kinase). The C-terminal comprises of the carboxyl terminal region and contains sites of phosphorylation. **(B)** EGF binding to EGFR monomer allows receptor activation and dimerisation of the EGFR, allowing EGFR autophosphorylation at distinct tyrosine residues. This phosphorylation then becomes a platform for binding adaptor proteins, Shc and Grb2.

Prevailing models identified that EGFR exists in the monomeric form at the cell surface with ligand binding inducing HER homo- or hetero-dimerisation (Purba, Saita & Maruyama, 2017). Upon ligand activation, the receptor sites become occupied by monomeric growth factor providing two stable 1:1 EGF:EGFR intermediate complexes, allowing the intracellular kinase domains into close proximity for trans-autophosphorylation (Böni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987). However, subsequent studies have shown that a subset of RTKs can exist in an inactive dimeric form in the absence of ligand (~30-40% of total receptors) (Clayton, Orchard, Nice et al., 2008; Clayton, Walker, Orchard et al., 2005; Hofman, Bader, Voortman et al., 2010; Yu, Sharma, Takahashi et al., 2002). EGFR, present as preformed dimers, are auto-inhibited and are kept in an inactive state through the intracellular domain and the transmembrane domain of the receptor (Hofman, Bader, Voortman et al., 2010; Kozer, Barua, Orchard et al., 2013; Purba, Saita & Maruyama,

2017). A specific region located in the extracellular module is essential for the multimerisation of the EGFR, (but not required for pre-formed receptor dimerisation), allowing dimers to self-associate and form higher-order structures (Huang, Bharill, Karandur et al., 2016). The current consensus is that following EGF stimulation, these preformed complexes allow for the full activation of receptor to occur more efficiently.

Although there are ten identified possible conformations of HER dimers, HER2 functions as a ligand-less receptor and relies on heterodimerisation with other HER members for signalling functionality. It is recognised that HER2 is the preferred co-receptor due to an intrinsically extended interaction loop, which renders it constitutively active (Graus-Porta, Beerli, Daly et al., 1997; Maruyama, 2014; Wilson, Gilmore, Foley et al., 2009). Additionally, while HER3, retains the ability to form an active signalling complex with remaining members of the HER family of receptors, it is generally considered to be kinase defective as it lacks the ability to phosphorylate exogenous peptides due to the absence of several key functional residues, although this has been recently challenged with ErbB3 shown to bind ATP and catalyse autophosphorylation (Ma, Lyu, Huang et al., 2014; Shi, Telesco, Liu et al., 2010; Steinkamp, Low-Nam, Yang et al., 2014).

Following autophosphorylation of key tyrosine phosphorylation sites, the activated receptor functions as a binding site for Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains (Kaneko, Joshi, Feller et al., 2012; Sakaguchi, Okabayashi, Kido et al., 1998). SH2 and PTB domains recognise phosphorylated tyrosine residues within the context of a specific amino acid sequence, allowing for protein-protein interaction (Pawson & Nash, 2000). Accordingly, the activated EGFR becomes a platform for the assembly of signalling molecules and adaptor molecules that possess site-specific phosphorylation docking sites (Wagner, Stacey, Liu et al., 2013). Adaptor proteins Shc and Grb2 lack enzymatic activity and assist signalling by acting as a platform upon which downstream signalling molecules are recruited and activated (Burke, Schooler & Wiley, 2001). Shc is tyrosine phosphorylated by the receptor and allows the binding of Grb2 via its SH2 domains. Grb2 interaction with the EGFR recruits the guanine nucleotide exchange factor, Sos, to the plasma membrane, allowing it to be in close vicinity to its target protein Ras, a small GTP-binding protein (Rojas, Oliva & Santos, 2011). The membrane-anchored Ras exchanges GDP for GTP and recruits Raf, a serine-threonine kinase upstream of the MAPK cascade. What follows, is the

sequential activation of the MAPK signalling cascade (Figure 1.03). Raf phosphorylates and activates the dual-specificity MEK which in turn is activated and phosphorylates MAPK, also denoted as ERK1/2. Phosphorylation of ERK1/2 initiates its translocation to the nucleus, where it activates transcription factors and alters gene expression.

MAPKs, a family of protein kinases, have remained functionally conserved throughout eukaryotes, and are a key players in a variety of fundamental cellular processes, including proliferation, growth, differentiation, migration, transformation and apoptosis (Mordret, 1993; Osaki & Gama, 2013). There are three major MAPK families – classical ERK1/2, Jun Kinase (JNK) and p38 MAPK, with the ERK1/2 pathway the best characterised mammalian MAPK pathway (Zhang & Liu, 2002), and is dysregulated in approximately one-third of all cancers (Dhillon, Hagan, Rath et al., 2007). ERK1/2 activation can be dependent or independent of RTK, however growing evidence supports the notion that EGFR is an important mediator of ERK1/2 activation.

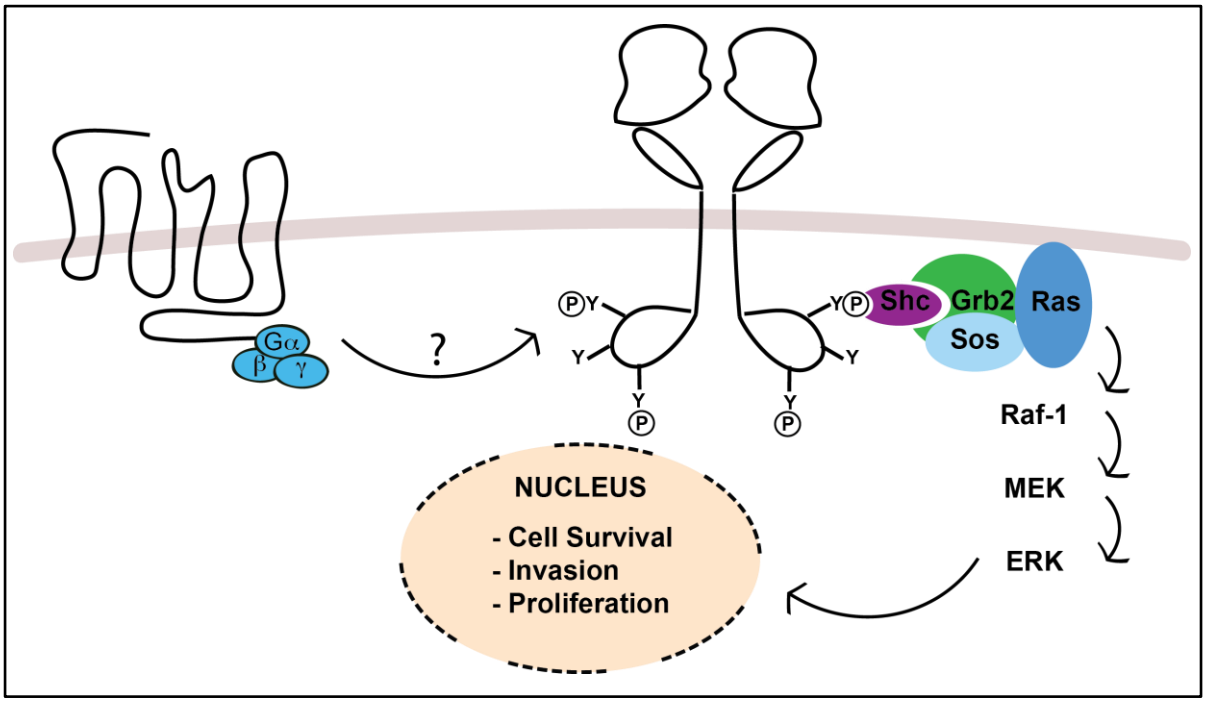


Figure 1.03: GPCR-activated MAP Kinase signalling pathway
Following GPCR activation, transactivation and dimerisation of the EGFR, the EGFR autophosphorylates distinct tyrosine residues. This then becomes a platform for adaptor proteins, Shc and Grb2, initiating the recruitment of Sos and allowing it to be in close vicinity to membrane-bound Ras. Ras recruits Raf-1, initiating the sequential activation of the MAPK signalling pathway, concluding with the phosphorylation of ERK1/2. ERK1/2 translocates to the nucleus, activating transcription factors and altering gene expression in favour of growth, mitosis and differentiation.

1.4.3 Triple Membrane Passing Signal

Ullrich's group proposed a model of GPCR-EGFR transactivation now referred to as the 'Triple Membrane Passing Signal' (TMPS). A large proportion of HER family receptor ligands are expressed as membrane-anchored proteins that require cleavage. In this TMPS model, inactive membrane-bound EGF-ligands are released, enabling these ligands to bind and activate the EGFR (Figure 1.04) (Prenzel, Zwick, Daub et al., 1999). Matrix metalloprotease (MMP) belong to the endopeptidase family of enzymes that are involved in ectodomain shedding of inactive membrane-bound precursors and produce the complementary active form (Heparin-binding-EGF (HB-EGF), neuregulins & EGF) (Manso, Elsherif, Kang et al., 2006; Ohtsu, Dempsey & Eguchi, 2006). The initial study suggested that GPCR activation induces the activation of a transmembrane MMP, leading to the cleavage of proHB-EGF and the binding of its mature form, HB-EGF, to activate the EGFR.

The process was designated TMPS as it involves three traversing signalling phases across the plasma membrane (Wetzker & Bohmer, 2003). A plethora of MMPs have since been shown to be involved in the transactivation of a variety of RTKs following GPCR activation (Cattaneo, Guerra, Parisi et al., 2014). As studies progressed, agonist bound GPCRs were also shown to activate another family of metalloproteases, A Disintegrin and Metalloproteases (the ADAMs) (Carpenter, 2000; Izumi, Hirata, Hasuwa et al., 1998). As molecular tools developed, a wide range of MMPs and ADAMs have since been implicated in the TMPS model (Asakura, Kitakaze, Takashima et al., 2002; Itabashi, Maesawa, Oikawa et al., 2008; Mifune, Ohtsu, Suzuki et al., 2005; Yan, Shirakabe & Werb, 2002; Zhang, Jiang, Black et al., 2000). Limitations however still exist, the exact identity of MMP/ADAMs and intracellular mechanisms contributing to their activation has not been fully elucidated, and reproducibility has been a major limiting factor. Given the wide range of EGFR ligands, in addition to the multiple GPCRs and RTKs shown to transactivate, to this day, no uniform mechanism explains transactivation. In the ensuing 20 years, it has become increasingly clear that other key mediators are involved, suggesting a more complex system involving non-receptor tyrosine kinases and the activation of additional second messengers.

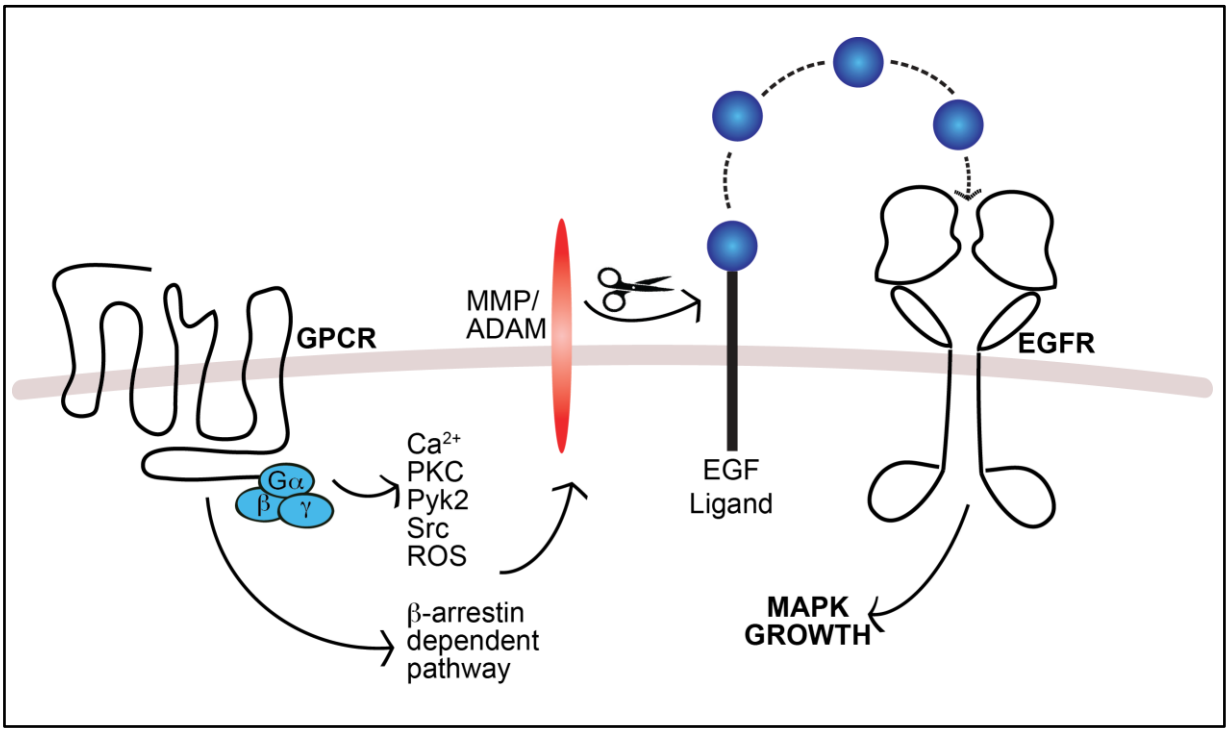


Figure 1.04: Triple membrane passing signal
Ligand activated GPCRs can signal via their cognate heterotrimeric G protein, leading to the activation of various second messengers including Ca^{2+}, PKC, Pyk2, Src and ROS, initiating the subsequent phosphorylation and activation of the EGFR. Alternatively, the 'TMPS' paradigm has also been widely studied, whereby second messengers activate membrane-bound MMPs/ADAMs that cleave latent growth factors and promote ligand shedding into the extracellular space. The activated EGFR dimerises and activates subsequent MAPK signalling pathways, leading to a cellular response. β-arrestins have also been shown to promote EGFR transactivation, mediating a G protein-independent response.

1.4.4 Key players in AT_1R-EGFR Transactivation

Many candidate proteins that potentially link GPCRs with mitogenic signalling have been heavily studied, particularly in the heart, VSMC, kidney and liver systems. Several intermediate signalling molecules including, PKC, Ca^{2+}, Pyk2, Src, reactive oxygen species (ROS) and β-arrestin have been heavily implicated throughout the literature. The extent to which these identified molecules participate in AngII-stimulated mitogenic signalling depends on several factors including largely the cell-type, the receptors involved and the cellular environment (healthy vs. disease state) (Gschwind, Zwick, Prenzel et al., 2001; Sadoshima, 1998; Zou, Komuro, Yamazaki et al., 1998).

1.4.4.1 Evidence for EGFR transactivation in VSMC

VSMCs are perhaps the best-studied tissue for AT_1R-EGFR transactivation, with EGFR transactivation critical in mediating pathophysiological vascular remodelling and subsequent end-organ damage and cardiovascular disease. All members of HER/ErbB family, as well as most of their ligands, are expressed by VSMC (Makki, Thiel & Miller, 2013). Accumulating evidence indicates that AngII induces $G\alpha_q/Ca^{2+}$-dependent EGFR activation, allowing the EGFR to serve as a 'scaffold' for the assembly of Src and Pyk2, which, in turn, leads to the activation of downstream kinases (ERK1/2, Akt, p38 MAPK) (Eguchi, Dempsey, Frank et al., 2001; Eguchi, Numaguchi, Iwasaki et al., 1998; Ohtsu, Higuchi, Shirai et al., 2008). VSMC contraction is regulated via activation of MLCK or inhibition of myosin light chain phosphatase (MLCP) (Mehta & Griendling, 2007; Nguyen Dinh Cat & Touyz, 2011). Src family kinases attenuate myosin light chain phosphorylation and contraction in AngII-infused mice, regulating vascular contraction via MCLP (Qin & Zhou, 2015).

AT_1R agonist stimulation transiently increases intracellular ROS concentration and protein synthesis in VSMC, causing hypertrophy, hyperplasia and migration of VSMC (Frank & Eguchi, 2003; Frank, Eguchi, Inagami et al., 2001; Seshiah, Weber, Rocic et al., 2002; Touyz, Cruzado, Tabet et al., 2003). Although the mechanism by which ROS activate tyrosine kinase can vary (Cattaneo, Guerra, Parisi et al., 2014), the predominant generation of ROS is via the membrane-bound NADPH oxidase complex. Following AT_1R stimulation and activation of NADPH oxidase, ROS production is thought to occur in a biphasic manner. The initial phase occurs quickly (within a minute) and is thought to be dependent on PKC activation. What follows is the second phase of ROS generation that is of greater magnitude (Seshiah, Weber, Rocic et al., 2002; Shah, Yesilkaya, Olivares-Reyes et al., 2004). ROS generation contributes to the metalloprotease-dependent shedding of HB-EGF (Frank & Eguchi, 2003).

Studies using adenoviral-mediated RNA interference showed ADAM17 to be the major sheddase for HB-EGF generation and further revealed its requirement for ERK1/2 activation and hypertrophy induction by AngII in VSMC (Elliott, Bourne, Takayanagi et al., 2013). The same group has previously shown that overexpression of a dominant-negative ADAM17 also completely abolished EGFR phosphorylation and EGFR-mediated protein synthesis

(Ohtsu, Dempsey, Frank et al., 2006), an effect not seen with overexpression of a dominant-negative ADAM10.

Furthermore, Caveolin-1 silencing prevented AngII-induced ADAM17 activation, vascular remodelling and abdominal aortic aneurysm (AAA) (Takayanagi, Crawford, Kobayashi et al., 2014). Caveolin, a structural component of caveolae, has been implicated as a major functional microdomain that is essential for AngII-mediated EGFR transactivation. It is thought that localised signalling domains formed in caveolae are essential for the dispersion and organisation of the AT_1R signalling repertoire in VSMC (Umesalma, Houwen, Baumbach et al., 2016). Furthermore, caveolin-1 is essential in AngII-stimulated inward remodelling and hypertrophy, with MMP9 involved exclusively in inward remodelling, but not hypertrophy (Umesalma, Houwen, Baumbach et al., 2016). However, infection of VSMC with adenovirus encoding caveolin-1 inhibits AngII-mediated HB-EGF shedding, EGFR transactivation, ERK1/2 activation, hypertrophy and migration (Takaguri, Shirai, Kimura et al., 2011), suggesting that ADAM17 co-localises with caveolin in lipid raft fractions and caveolin interferes with AngII-induced ADAM17 activation and EGFR transactivation.

1.4.4.2 Evidence for EGFR transactivation in the heart

EGFR transactivation plays an essential role in the physiology as well as the pathophysiology of cardiac myocytes and fibroblasts. Accumulating evidence suggests EGFR is involved in cardiac development, regulation of myocardial function and adaption/remodelling in response to physiological and pathological loads (Forrester, Kawai, O'Brien et al., 2016). Numerous GPCRs have been linked to EGFR transactivation in cardiac cells including the AT_1R (Smith, Chan, Qian et al., 2011; Thomas, Brandenburger, Autelitano et al., 2002), adrenergic (Kim, Eckhart, Eguchi et al., 2002; Turner, 2003), muscarinic (Krieg, Qin, McIntosh et al., 2002; Miao, Bi, Zhao et al., 2015), adenosine (Williams-Pritchard, Knight, Hoe et al., 2011), endothelin (Clerk & Sugden, 1999; Kodama, Fukuda, Takahashi et al., 2002), bradykinin (Methner, Donat, Felix et al., 2009), opioid (Cao, Liu & Van Winkle, 2005; Förster, Kuno, Solenkova et al., 2007) and the protease activated receptor (Sabri, 2002). G protein-dependent signalling in the heart has been studied for decades, revealing significant roles for $G\alpha_{q/11}$ in the increased production of protein synthesis and hypertrophic responses in the heart.

The use of neonatal cardiomyocyte model, whereby the AT_1R is reintroduced by adenoviral infection, demonstrated that a dominant negative $G\alpha_q$ inhibited AngII-induced hypertrophy and ERK1/2 activation, suggesting G_q is involved in EGFR transactivation and cardiomyocyte hypertrophy (Thomas, Brandenburger, Autelitano et al., 2002). Using the same neonatal cardiomyocyte model, cardiomyocyte hypertrophy required G protein coupling and ADAM-mediated HB-EGF production (Seta & Sadoshima, 2003; Smith, Chan, Qian et al., 2011). More recently, studies have also begun to comprehensively characterise transcriptional regulation and the relative contribution of $G\alpha_{q/11}$ dependent and independent signalling following AngII stimulation. Unlike β-arrestin signalling, direct induction of gene expression by AT_1R is dependent on $G\alpha_q$ activation (Christensen, Knudsen, Schneider et al., 2011). Seta and Sadoshima subsequently proposed the role of AT_1R tyrosine 319 (Y319) as the mechanism by which the EGFR is transactivated (Seta & Sadoshima, 2003). The authors showed that overexpression of the Y319F AT_1R mutant abrogated AngII-induced EGFR transactivation and AngII-induced cell proliferation in cardiac fibroblasts. It was suggested that phosphorylated Y319 acts as a scaffold for the adaptor protein, SHP-2, and mediated AT_1R-EGFR interaction and transactivation of the EGFR. However, this G protein independent mode of AT_1R-mediated transactivation has been challenged, with others reporting that Y319 phosphorylation is not required for AngII-mediated EGFR transactivation (Smith, Chan, Qian et al., 2011).

Many studies support a role of cardiac EGFR transactivation in pathological cardiac hypertrophy; however increasing evidence suggests that selective engagement of G protein-independent mechanisms, namely β-arrestin-mediated pathways, promotes cardioprotective signalling (Kim, Abraham, Williams et al., 2012; Rajagopal, Whalen, Violin et al., 2006; Tilley, Nguyen & Rockman, 2010; Violin, DeWire, Yamashita et al., 2010). In response to an *in vivo* cardiac injury, β-arrestin biased AT_1R ligand promoted cell survival during cardiac injury, cardiac contractility and enhanced ERK1/2 and Akt signalling (Kim, Abraham, Williams et al., 2012). Notably, the β-arrestin biased agonist of the AT_1R, SII, promotes positive ionotrophic effects on adult cardiomyocytes and increased cardiomyocyte contractility, identifying β-arrestin2 and GRK6 as the mediator of this $G\alpha_{q/11}$-independent response (Rajagopal, Whalen, Violin et al., 2006). This was confirmed in a more recent study showing that a β-arrestin biased agonist increases cardiomyocyte contractility (Tilley,

Nguyen & Rockman, 2010; Violin, DeWire, Yamashita et al., 2010). Interestingly, membrane stretch induces β-arrestin biased signalling of AT_1R in the absence of ligand or G protein activation. Hearts lacking β-arrestin2 fail to induce a response to mechanical stretch, as well as impaired EGFR transactivation and an increased myocyte apoptosis, overall revealing an inhibition of pro-survival signalling (Rakesh, Yoo, Kim et al., 2010).

1.5 New insights into AT_1R-EGFR transactivation

Since the discovery of GPCR-EGFR transactivation in the mid 1990's, hypothesis-driven candidate approaches have identified a number of proteins involved in receptor 'cross-talk.' Although this approach has been informative, the complete mechanism is still lacking with recent progress in the field stalling. A major requirement of the field is to identify key molecules that are critical in linking GPCR activation to EGFR transactivation. This lead the Thomas group to perform an unbiased functional genomics approach to identify potential proteins involved (George, Purdue, Gould et al., 2013). One major advantage of employing a functional genomic screen is the unbiased screening approach to identify novel genes involved in the transactivation process, which may potentially serve as therapeutic targets for the treatment of diseases commonly associated with AT_1R-induced mitogenic signalling.

Since the development of functional genomics in the early 2000's, technological advancements have enabled researchers to perform high-throughput methods to study gene and protein expression using large-scale screens. One of the key developments was the introduction of RNA interference (RNAi) technology. Although shown to occur endogenously (Martineau & Pyrah, 2007; Milhavet, Gary & Mattson, 2003), functional genomics has harnessed RNAi technology as a powerful molecular tool to better understand the function of specific gene products. RNAi inhibits gene expression through the use of RNA molecules: microRNA (miRNA) and small interfering RNA (siRNA), that bind to target messenger RNA (mRNA) and consequently initiate its degradation and/or decrease its translation (MacFarlane & Murphy, 2010). What has become increasingly popular in the scientific community is employing large-scale genome-wide RNAi screens combined with target genes 'knock-down' (Boutros & Ahringer, 2008; Simpson, Davis & Boag, 2012). This provides further insight into a gene's function, meaning an individual gene can be interrogated within a certain biological concept. Assuming that kinases are likely involved in

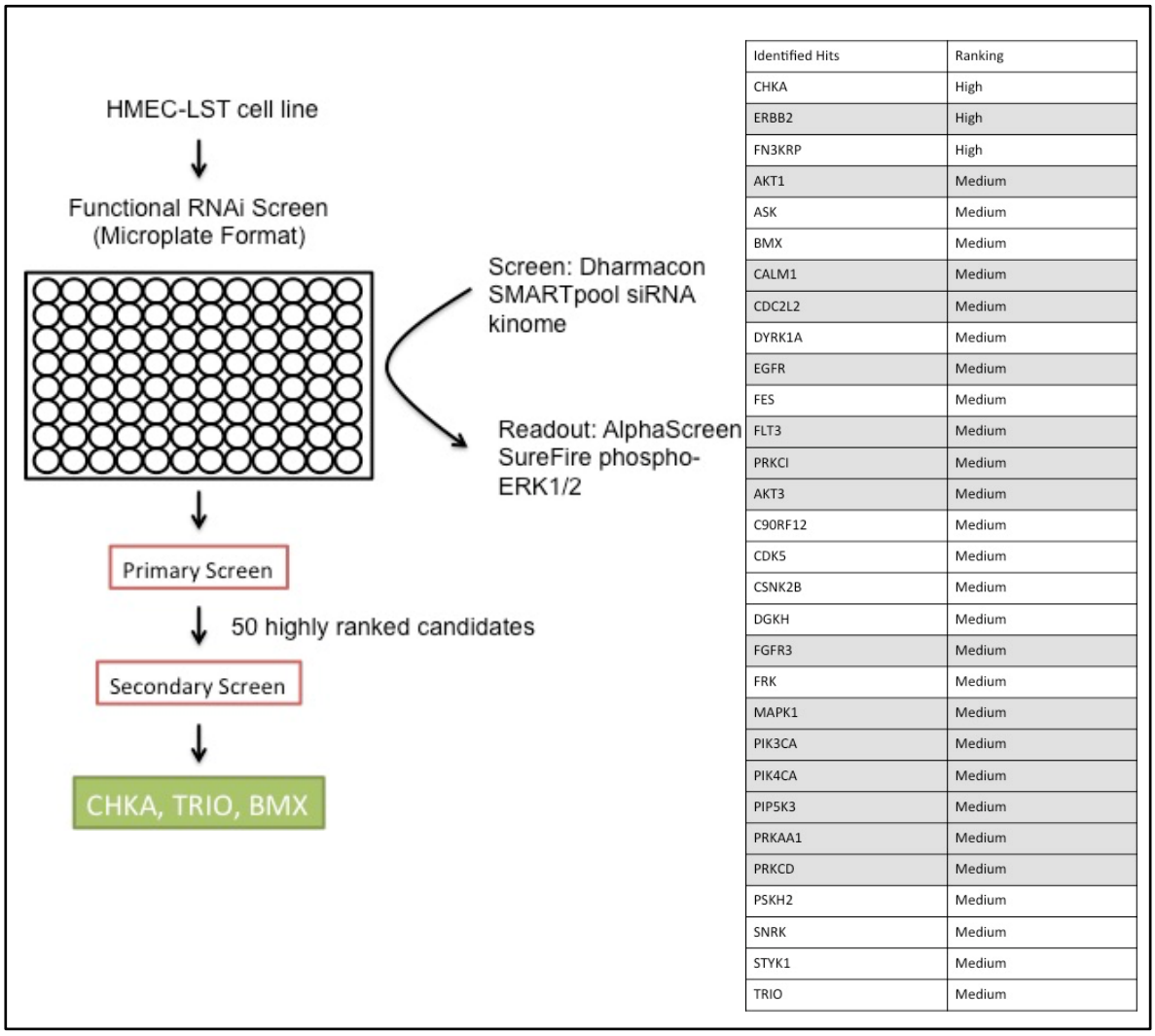
mediating AT_1R-EGFR transactivation, the Thomas lab incorporated these recent developments and used a cellular model to perform a functional RNAi screen that identified novel proteins that are involved in AT_1R-EGFR transactivation (George, Purdue, Gould et al., 2013).

Firstly, a human cellular model was generated by introducing the AT_1R into a human mammary epithelial cell (HMEC) line using retroviral delivery. The HMEC cell line robustly activated the EGFR and ERK1/2 in response to AngII, confirming a transactivation process. siRNA knockdown of known targets – AT_1R, its cognate G protein ($G_{q/11}$) and EGFR revealed a significant decrease in phosphorylation of both the EGFR and ERK1/2 following AngII stimulation, confirming the cell lines appropriateness. Following this, a transactivation assay was optimised in a microplate format, which used the AlphaScreen SureFire phospho-ERK1/2 kit as the readout.

The functional RNAi screen comprised of two separate screens – a primary and a secondary screen (Figure 1.05). The initial primary screen encompassed 720 kinase genes from the Dharmacon SMARTpool siRNA kinome library. Each kinase gene was then ranked on their ability to modulate AT_1R-EGFR transactivation. From this, 50 highly ranked candidates were identified as genes that either positively or negatively regulated transactivation and were further selected to be verified in the secondary screen, and were ranked as 'high', 'medium', or 'low' hits. When considering potential candidate genes, the primary interest was genes that have not been previously associated with HER function, although it is reassuring that many of the hits had already been identified in the literature (highlighted in grey Figure 1.05 table). From the screen, three novel genes were identified, as they have not been previously implicated in the literature to participate in GPCR-EGFR transactivation: BMX (Bone marrow kinase X-linked kinase), CHKA (Choline kinase alpha) and lastly TRIO (triple function domain protein).

Individual knockdown of BMX, CHKA or TRIO attenuated tyrosine phosphorylation of the EGFR by AngII stimulation and appear to function downstream of the activated AT_1R and upstream of the EGFR. BMX is a member of the Tec family of non-receptor tyrosine kinases (Tamagnone, Lahtinen, Mustonen et al., 1994). CHKA phosphorylates choline to produce phosphocholine, an important intermediate in the generation of the key membrane phospholipid, phosphatidylcholine (Aoyama, Liao & Ishidate, 2004), Lastly, TRIO is a large

multidomain protein with two functional guanine nucleotide exchange factor (GEF) domains and a serine/threonine kinase domain (Debant, Serra-Pagès, Seipel et al., 1996). None of these candidates have been specifically or mechanistically linked to GPCR-EGFR transactivation, although, as we will outline in subsequent sections of this thesis, there is prevailing literature that provides important clues as to how they might be acting.



Identified Hits	Ranking
CHKA	High
ERBB2	High
FN3KRP	High
AKT1	Medium
ASK	Medium
BMX	Medium
CALM1	Medium
CDC2L2	Medium
DYRK1A	Medium
EGFR	Medium
FES	Medium
FLT3	Medium
PRKCI	Medium
AKT3	Medium
C90RF12	Medium
CDK5	Medium
CSNK2B	Medium
DGKH	Medium
FGFR3	Medium
FRK	Medium
MAPK1	Medium
PIK3CA	Medium
PIK4CA	Medium
PIP5K3	Medium
PRKAA1	Medium
PRKCD	Medium
PSKH2	Medium
SNRK	Medium
STYK1	Medium
TRIO	Medium

Figure 1.05: Kinome siRNA screen used to identify three novel genes involved in AT_1R-EGFR transactivation – CHKA, TRIO & BMX

Using the HMEC-LST cell line, the functional screen identified potential kinase genes (using the Dharmacon SMARTpool siRNA kinome library) involved in ERK1/2 activation following AngII stimulation. The ERK1/2 readout was performed using the AlphaScreen SureFire phospho-ERK1/2. The primary screen ranked all tested kinases on their ability to modulate AT_1R-EGFR transactivation. The secondary screen validated 50 of the highest ranked candidates. Following this, the functional screen identified three novel genes – CHKA, TRIO and BMX.

1.6 Where to next?

In order to improve our understanding of GPCR-EGFR transactivation, adequate technology is required to better interrogate the molecular mechanisms governing EGFR transactivation. A common growth target of both the AT_1R and EGFR is the phosphorylation and activation of ERK1/2, which is widely used as a surrogate marker for AT_1R-EGFR cross-talk. EGFR transactivation is subsequently defined as the ability of AG1478, a moderately selective inhibitor of EGFR, to prevent AT_1R-mediated ERK1/2 phosphorylation. Although the activation of ERK1/2 culminates in the activation of growth promoting signals, it represents a kinase distally downstream of the EGFR (as shown in Figure 1.03). Arguably, a more direct readout of EGFR transactivation is the phosphorylation and activation of the EGFR, the convergence point for many signalling pathways, including that of transactivation. However, phospho-EGFR assays, through the use of western blots, are often laborious, finicky, time consuming, insensitive and inconsistent.

Despite the utility of ERK1/2 phosphorylation assays using western blotting, this approach has several well-known limitations. These include the inability to detect the activation and recruitment of proteins in live cells and in real-time. Over the last decade, the development of Resonance Energy Transfer (RET) assays has enabled the study of dynamic cellular processes in living cells and has been widely used in the field of GPCR and RTK biology (Kocan & Pfleger, 2011; Stoddart, Kilpatrick & Hill, 2017). RET technology measures protein-protein interactions in which energy transfer occurs between a luminescent donor complex (e.g., *Renilla* luciferase; Rluc) attached to a protein of interest and a second protein labelled with a complementary fluorescent acceptor molecule (e.g., yellow fluorescent molecule; Venus) (Figure 1.06). Light is emitted following the oxidation of an external substrate (coelenterazine h) by the donor luciferase. If the donor- and acceptor-tagged proteins are in close proximity (no more than 10nm apart), non-radiative transfer of energy occurs between the two molecules, which in turn excites the acceptor fluorescent moiety and enables the acceptor molecule to emit light at a longer wavelength (Figure 1.06). Two main types of RET-based methods exist, fluorescent-RET (FRET) and bioluminescent-RET (BRET) based assays.

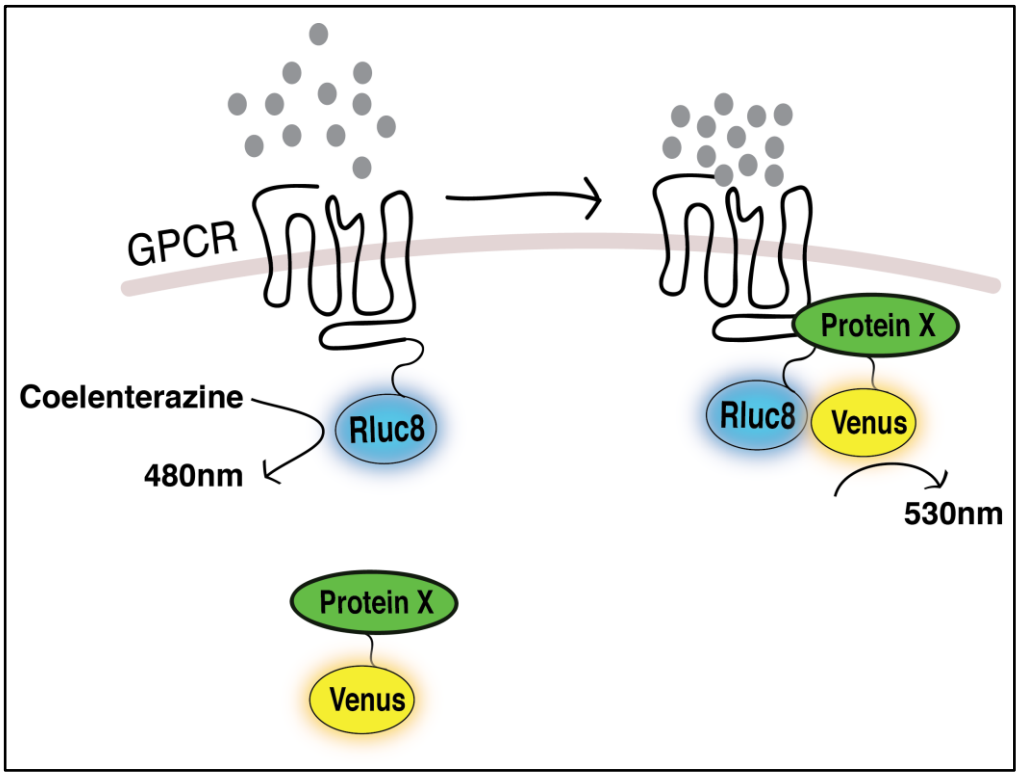


Figure 1.06: The principle of BRET[1] – studying protein-protein interactions
BRET allows researchers to study the interactions of GPCR (fused alongside a donor luminescent, Rluc8) with a protein of interest (fused alongside a complementary acceptor fluorophore, Venus). Following the addition of the Luciferase substrate coelenterazine to the system, if there is no interaction between the co-transfected donor- and acceptor-tagged proteins, light energy from the oxidation of coelenterazine by the luciferase will be emitted at 480nm. If the GPCR and the protein of interest are within 100Å, resonance energy transfer from the luminescent donor to the complementary acceptor fluorophore occurs, which in turn emits light energy at a longer wavelength of 520nm.

One of the first such studies to utilise a BRET-based approach, monitored GPCRs interaction with its associated G protein, specifically β2-adrenergic receptor (β_2AR) interaction with $G\alpha_s\beta_1\gamma_2$ (Galés, Rebois, Hogue et al., 2005). Following this seminal study, BRET assays have been used to identify effector proteins downstream of GPCRs, GPCR conformational changes (Hudson, 2016) and GPCR homo- and hetero-dimerisation (Porrello, Pfleger, Seeber et al., 2011). Detection of β-arrestin recruitment using BRET has been reported in more than 50 manuscripts and for a wide range of GPCRs, including time courses, dose responses and ligand dependency, all accessed in live cells and in real-time (Ayoub & Pin, 2013; Namkung, Radresa, Armando et al., 2016). More recently, FRET and BRET techniques have shown that many GPCRs, including the AT_1R, exist as homo- or hetero-dimers with other GPCRs (Sleno, Devost, Pétrin et al., 2017; Tóth, Gyombolai, Szalai et al., 2017), with receptor-

receptor complexes shown to alter GPCR pharmacology, coupling as well as receptor trafficking. BRET-based approaches have also revealed that RTKs have the capacity to homo- and hetero-dimerise, demonstrating that the EGFR and HER3 heteromerise in a ligand-dependent manner (Ayoub, See, Seeber et al., 2013). The main limitation of RET-based assays is that it requires the exogenous expression of the receptor/protein of interest genetically fused with the luminescent donor or acceptor fluorophore. The recent development of the NanoLuc variant results in a significantly stable and smaller luciferase molecule (19kDa, compared to Rluc8 38kDa) that produces a significantly brighter and sustained luminescent signal (Hall, Unch, Binkowski et al., 2012). The development of NanoLuc enables the expression of tagged-proteins of interest at near physiological expression, however more promising is the ability to express NanoLuc-GPCR fusions under an endogenous promotion using a CRISPR/Cas9 system (White, Vanyai, See et al., 2017).

Using a combination of BRET and FRET technologies, the existence of GPCR-RTK heteromer complexes have been reported for a number of receptors (Jensen, Godfrey, Niklas et al., 2013; Maudsley, Pierce, Zamah et al., 2000). Most notably, Bhattacharya and colleagues showed that through the use of FRET, G protein-coupled receptor 54 (GPCR54) interacts directly with EGFR, with this association further enhanced following kisspeptin treatment, GPR54 endogenous ligand (Zajac, Law, Cvetkovic et al., 2011). Similarly, FRET studies have shown that isoprenaline treatment induces β1-adrenergic receptor (β_1AR) association with the EGFR, whereas in contrast, EGF stimulation significantly disrupts this association (Tilley, Kim, Patel et al., 2009). These higher order receptor complexes create new alternatives in term of physiological function, from ligand binding to signal transduction, trafficking, desensitisation and downregulation. To date, such approaches have not been used to interrogate transactivation, and while BRET/FRET approaches have the potential to report on the most proximal events in ligand-mediated receptor activation, they can also provide information about where and when these interactions occur in the cell.

1.7 Project Rationale

Although a new light was shone on the process of mitogenic signalling via GPCRs when Ullrich's group published their pioneer study in 1996 (Daub, Weiss, Wallasch et al., 1996), a gap still exists in the current knowledge. There is an ever present need to digress from conventional western-based approaches that study signalling proteins in solution and/or isolated membranes, to instead measure dynamic protein-protein interactions in live cells and in real-time. The use of BRET has proven beneficial in monitoring receptor-specific events, and can offer tremendous potential in better understanding the most proximal events in ligand-mediated RTK activation and its sensitivity to chemical inhibitors of their function. Therefore, in this thesis, the aims of my project are split into three different sections with the following goals:

1) To use a BRET-based approach to identify and characterise the molecular basis of transactivation, examining the spatio- and temporal-requirements of AT_1R-mediated EGFR transactivation.
2) To further interrogate the role of BMX, CHKA and TRIO in AT_1R-EGFR transactivation.
3) To investigate the structural and functional characteristics of a phenomenon that I have titled 'reverse transactivation', a novel concept I have identified during my canditure whereby the AT_1R is activated by the EGFR.

Chapter Two

Methods.

2.0 Methods

2.1 Materials

This Chapter describes the general methods that have been used throughout this thesis. These methods are described here in detail. The sources of drugs and plasmids are indicated in individual chapters.

2.2 Bacterial Methods

2.2.1Transformation of 5-alpha Heat Shock competent cells

Transformation of New England Biolabs (NEB) High Efficiency 5-alpha Competent E.Coli cells (NEB Cat# C2987H) were performed according to manufacturers instructions. Briefly, 1-100ng of plasmid DNA was mixed with 50μL of 5-alpha Competent E.Coli cells and incubated on ice for 30 minutes before being heat shocked at 42°C for 30 seconds and placed on ice for 5 minutes. SOC medium was added and incubated at 37°C for 1 hour with shaking at ~250 revolutions per minute (rpm). Approximately 50-100μL of SOC media/competent cells were spread on agar plates containing the appropriate antibiotics, and left overnight for 12-16 hours at 37°C. Surviving colonies were used to inoculate 3-4mL of LB media containing the relevant antibiotic and left overnight shaking (250rpm) for 12-16 hours. This was then used as a starter culture for mini- and maxi-prep plasmid DNA preparation.

2.2.2 Glycerol Stocks

A glycerol stock was created for long-term storage of positive clones. 2mL of bacterial culture was centrifuged and pelleted at 12,000 x g for 5minutes at room temperature. The pellet was resuspended in a 1:1 ratio of sterile lysogeny broth (LB) media (Appendix A) and glycerol. All glycerol stocks were stored at -80°C.

2.3 DNA Methods

2.3.1 Mini-prep plasmid DNA preparation

Small-scale plasmid preparation was carried out using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Cat# K210010) as per the manufacturer's instructions. Briefly, 2mL of starter cultures described as per section 2.2.1 was centrifuged at 12, 000 x g for 5 minutes and resuspended, lysed and precipitated. The supernatant was resuspended onto a spin column and centrifuged at 12,000 x g for 1 minute. A variety of wash steps were performed prior to DNA elution with TE Buffer (supplied in the kit). Plasmid DNA was stored at -20°C.

2.3.2 Maxi-prep plasmid DNA preparation

Large-scale plasmid preparation was carried out using PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Cat# K210016) as per the manufacturer's instructions. LB media (300mL) was inoculated with 300μL of starter culture and transformed overnight shaking (250rpm) for 12-16 hours at 37°C. The overnight culture was pelleted by centrifugation (4, 000 x g for 10 minutes), resuspended and lysed, and DNA was then transferred to an equilibrated HiPure Filter Column. The column containing the DNA was washed and eluted before precipitating the DNA with isopropanol and washed with ethanol. The pellet was air-dried and resuspended in 500μL of TE Buffer (supplied in the kit). Plasmid DNA was stored at -20°C.

2.3.3 DNA Quantification

DNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies). DNA concentrations were estimated by measuring an OD reading of 1 at 260nm absorbance.

2.4 Cell Culture

2.4.1 Immortalised Cell Lines

Mouse embryo fibroblast (NIH-3T3) cells, monkey fibroblast (COS-7) cells, human colorectal adenocarcinoma (HT-29) cells, human embryonic kidney 293 (HEK293), HEK293FT cells, HEK-adherent-293 cell line, which is a derivative of HEK293 with increased adherence and human cervical cancer (HeLa) cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Life Technologies Cat# 11995065) supplemented with 10% Foetal Bovine Serum (FBS, Life Technologies) and antibiotic-antimycotic solution with HEK293FT cells containing an additional 0.3mg/mL glutamine. Stably expressing NHA-AT_1R-HEK293 and HEK293FT cells were maintained as above in DMEM/10%FBS with the addition of 200μg/mL Geneticin (Gibco, Cat# G-148). Chinese hamster ovary (CHO-K1) cells were cultured in α-Modified Eagle Media (α-MEM, Life Technologies Cat# 12571063) supplemented with 10% FBS and antibiotic-antimycotic solution. All cells were maintained at 37°C at 5% CO_2 and passaged when confluent. HEK293, HeLa and CHO-K1 were purchased from American Type Tissue Culture (ATCC). HEK293FT and COS-7 cells were kindly provided by Professor Kevin Pfleger (Harry Perkins Institute, University of Western Australia, Perth, AUS) and NIH-3T3, HT-29 cells a kind gift from Dr Daniel Croker (University of Queensland, Brisbane, AUS). All cells were grown in 75cm^2 or 175cm^2 cell culture flasks (Corning) under sterile conditions. Cells were subcultured by washing once in sterile phosphate buffer saline (PBS, Life Technologies, Cat# 21600010) before 5 minutes incubation with 0.25% trypsin (Gibco, Cat# 25200072). Cells were counted with a haemocytometer prior to plating.

2.4.2 DNA Transfection – Immortalised cell lines

Lipid phosphate transfection using FuGene (Promega, Cat# E2311) was used to introduce exogenous DNA for protein expression. Cells were seeded onto a 6-well tissue culture plate the day prior to transfection to achieve 70-80% confluence. DNA transfection was performed according to manufacturer's protocol and was scaled up or down in accordance with cell culture plate dimensions. Briefly, for one well of a 6-well plate, 100μL of Opti-MEM (Life Technologies, Cat# 31985070) was mixed with 3μL of FuGene and incubated at room temperature for 5 minutes. No more than 1μg of DNA total was added and incubated at room temperature for 10 minutes to enable lipid:DNA complex to form. DNA/lipid mixture was

then added directly to the cells and left overnight to incubate at 37°C at 5% CO_2. Typically, experiments were performed 24 hours following transfection.

2.4.3 Neonatal cardiomyocyte isolation and purification

Approximately 24-hour-old neonatal Sprague Dawley pups (approximately 40-100 per preparation) were killed by decapitation before an incision along the thoracic wall and the extraction of the intact heart. The atrium was removed and the remaining ventricles were cut into approximately 6-8 pieces to allow for increased surface area for the enzymatic digestion. Heart tissue was then transferred to a 50mL Wheaton Celstir water-jacketed double side-arm spinner flask pre-warmed to 37°C. Heart tissue was incubated at slow speed with Enzyme Digestion Buffer (Appendix A; 0.3mL buffer per whole heart). Supernatant, which included cell debris, red blood cells and extracellular matrix, was excluded by disposing of the first 20-minute dispersion. The remaining 20 minute dispersion was repeated 4-8 times, with each supernatant carefully transferred to a sterile 50mL falcon tube containing 5mL newborn calf serum (NBCS, Gibco Cat# 16010159) and placed on ice. After the final dispersion, the cell suspension/NBCS mixture was centrifuged at 1000 x g for 6 minutes to pellet cells. The pellet was carefully resuspended in 1 x ADS buffer (Appendix A; 2mL per gradient, 1 gradient per 8-10 pups).

Cardiomyocytes were purified using a discontinuous Percoll gradient (1.060/1.086 g/mL) diluted in 1 x ADS. Carefully, 2mL of cell suspension/1 x ADS mixture was gently overlaid a freshly prepared gradient before centrifugation at 1400 x g for 30 minutes (slowest acceleration, no deceleration). The non-myocyte portion, which included fibroblasts, appeared at the top interface between the top Percoll layer and the ADS buffer. This fibroblast portion is either collected and seeded into a 175cm^2 cell culture flask with 10% FBS and antibiotic (1 gradient/175cm^2 cell culture flask) or discarded. The second interface between the top and bottom Percoll densities contained neonatal myocytes and were carefully removed and transferred to a new 50mL falcon tube. The cells were resuspended in 1 x ADS buffer to remove any remaining Percoll gradient, and washed twice. Pelleted cardiomyocytes were resuspended in a final volume of 20mL plating medium (Minimal Essential Medium supplemented with 10% NBCS; Appendix A). To prevent the proliferation of any remaining

fibroblasts, plating media was supplemented with the anti-miotic agent 5-Bromo-2'deoxyuridine (BrdU; 0.5mM).

Resuspended cells were counted using a haemocytometer and plated on 0.1% gelatine-coated tissue culture dishes. Cells were seeded at a high density (1250 cell/mm2) for Westerns. If cells were to be used for nucleofector transfection, $5.0x10^6$ cells were used/cuvette (refer to Nucleofector section 2.4.5). Following overnight incubation, cells were washed twice with PBS and media was replaced with defined myocyte media (DMEM $CCTSS_2$ with 50mM KCl; Appendix A). The Percoll Isolation process usually resulted in a 95-98% pure cardiomyocyte population.

2.4.4 Isolation of vascular smooth muscle cells

Purified VSMC cultures were performed by Dr Tatsuo Kawai (Lewis Katz School of Medicine, Temple University, Philadelphia, USA). Animals were supplied by Charles River Breeding Laboratory Inc., Wilmington, MA, USA. The thoracic aorta of a male Sprague Dawley rat removed and placed into DMEM (4.5g/L glucose) supplemented with 10% FBS and antibiotic. Longitudinally, the isolated aorta was cut and then endothelium was removed using a dull scissor. The aorta was then cut into 3mm square sections with each section placed intimal side down in a well of a 6-well plate. Carefully, 100-200μL of DMEM (4.5g/L glucose) supplemented with 10% FBS and antibiotic was added to the tissue and cultured at 37°C in 5% CO_2. 24-48 hours post-explant, the tissue was then monitored for cell growth and additional media was added to avoid drying of the tissue.

2.4.5 Nucleofector

For nucleofector delivery of plasmid DNA into isolated primary cells, a 4D-Nucleofector (Lonza) was used to transfect cardiomycoytes and cardiac fibroblasts; meanwhile an Amaxa Nucleofector (Lonza) was used to transfect VSMC.

2.4.5.1 4D-Nuclefector: Cardiac myocytes and fibroblasts

Cardiomyocyte and fibroblasts were transfected using the 4D-Nucleofector system (Lonza) using the primary cell 4D nucleofector kit (Cat# VX4P-3012) according to the manufacturer's protocol. In brief, following cardiomyocyte isolation and purification, both cardiac myocytes and fibroblasts ($5.0x10^6$ cells/cuvette) were resuspended in 100µL of room temperature Nucleofector Buffer solution™ containing a 3:1 ratio of receptor:protein of interest (0.15µg receptor and 0.05µg protein of interest) and transfected using the 4D-Nucleofector program CM-150. 400µL of RPMI (Life Technologies, Cat# 61870036) supplemented with 10% horse serum was added to each cuvette containing transfected cells and then plated on 0.1% gelatine-coated white F96 MicroWell plate (Nunc, Cat# 136101). Following overnight incubation, cells were washed twice with PBS and media was replaced with defined myocyte media (DMEM $CCTSS_2$ with 50mM KCl; Appendix A) or DMEM supplemented with 10% FBS and antibiotic for fibroblasts. Typically, experiments were performed 48 hours following transfection.

2.4.5.2 Amaxa Nucleofector: VSMC

VSMC were transfected using the Amaxa nucleofector kit (Lonza, Cat# V4XP-1024) according to the manufacturer's protocol. In brief, following VSMC isolation, VSMC ($1.0x10^6$) were transfected using Amaxa program U-25 in 100µL room temperature smooth muscle cells (SMC) nucleofector solution™ containing 3:1 ratio of receptor:protein of interest (0.15µg receptor and 0.05µg protein of interest). 500µL of DMEM supplemented with 10% FBS was added to each cuvette containing transfected cells and then plated on white F96 MicroWell plate. Following overnight incubation, cells were washed twice with PBS and media was replaced with serum-free DMEM. Typically, experiments were performed 48 hours following transfection.

2.5 Plasmid DNA Cloning

2.5.1 Restriction Digest

Each restriction enzyme was used to digest DNA as specified by the manufacturer. In general, 1 U of restriction enzyme (NEB) was used to digest 1μg of substrate DNA in appropriate 1 x NEBuffer, and incubated at 37°C for 1 hour. Digested DNA was resolved (Section 2.5.2), excised (Section 2.5.3) and quantified (Section 2.3.3) before cloning into vectors.

2.5.2 Agarose gel electrophoresis

Digested DNA and DNA fragments were separated using a 1% agarose gels in 1 x Tris-Acetate-EDTA (TAE) buffer (Appendix A) containing 1 x SYBR® Safe DNA gel stain (1:10,000 dilution) (Invitrogen, Cat# S33102). DNA samples were resuspended with 1 x Gel purple or blue loading dye (NEB) and loaded alongside a 1kb DNA ladder (NEB, Cat# 161-0374). Digested DNA was resolved at 100V for ~40-60 minutes or until the bands had sufficiently separated. DNA fragments were visualised under UV illumination using a UVP BioDoc-It™ System UV Transilluminator (UVP).

2.5.3 Recovery of DNA fragments from agarose gels

DNA was extracted from 1% agarose gels using the QIAquick® Gel Extraction Kit (Qiagen Pty. Ltd., Cat# 20200531) according to manufacturer's protocol. In brief, SYBR® Safe DNA gel stained bands of interest were excised from gels using a scalpel blade. DNA from gel fragments was dissolved and extracted using QIAquick spin columns and eluted in 20μL of TE buffer. Excised DNA was stored at -20°C.

2.5.4 DNA ligation

DNA ligation was performed using a Quick Ligation™ Kit (NEB, Cat# M2200) as per manufacturer's protocol. In general, a 3:1 vecotr:insert (as per online NEBioCalculator™) was mixed with Quick Ligase Reaction Buffer and Quick Ligase. The mixture was incubated

at room temperature for 5 minutes and then placed on ice before being transformed using competent cells (Section 2.2.1).

2.5.5 DNA sequencing

Sequencing was performed at the Australian Genome Research Facility (AGRF) (University of Queensland, Brisbane, AUS). All samples submitted contained a total amount of 0.6μg-1.5μg of plasmid DNA and 10pmol of desired primer. All sequencing was performed using a high throughput Sanger sequencing using Applied Biosystems 3730 and 3730xl capillary sequencers.

2.6 Assays

2.6.1 Ligand-induced BRET assays and Receptor-Heteromer Identification Technology assay

Briefly, BRET[1] quantifies interactions between receptor (fused with a donor luminescent, *Renilla* luciferase (Rluc8)) and a protein of interest (fused with a complementary acceptor fluorophore, Venus) (Kocan & Pfleger, 2011; Kocan, See, Seeber et al., 2008). Following the addition of the Luciferase substrate coelenterazine, if there is no interaction between the co-transfected donor- and acceptor-tagged proteins, light energy from the oxidation of coelenterazine by the luciferase will be emitted at 480nm. If the receptor and the protein of interest are within 100Å, resonance energy transfer from the luminescent donor to the complementary acceptor fluorophore occurs, which in turn emits light energy at a longer wavelength of 520nm. Therefore, the degree of BRET signal or protein-protein interaction is quantified as a ratio of light emitted at 520nm over 480nm.

Cells were transiently transfected with proteins of interest as per FuGene's protocol (Section 2.4.2). 24h post transfection, cells were washed with PBS and detached using 0.25% trypsin, resuspended in FluoroBrite™ DMEM (Life Technologies, Cat# A1896701) phenol red-free media supplemented with 5% FBS + 10% L-Glutatmine or phenol-red free DMEM containing 25mM HEPES, 0.3mg/mL glutamine, 100IU/mL penicillin and 100μg/mL

streptomycin supplemented with 5% FBS and added to poly-D-lysine-coated white F96 MicroWell plate at 100,000 cells/well. Early the following day, cells were serum starved with FluoroBrite™ DMEM phenol red-free media supplemented with 10% L-Glutatmine or 25mM HEPES with 0.3mg/mL glutamine, 100IU/mL penicillin and 100μg/mL streptomycin for at least 6 hours. 48 hours post-transfection, media was replaced with PBS or Hanks' balanced salt solution (HBSS) containing coelenterazine-h (Promega) at a final concentration of 5μM. BRET[1] measurements were taken at 37°C every 30 seconds using: a M1000 PRO Tecan plate reader (Tecan, Life Sciences) with 430-485 nm and 505-590 nm filters; a CLARIOstar Microplate Reader (BMG Labtech) with 420-480 nm and 520-620 nm filters; or a LUMIstar Omega Microplate Reader (BMG Labtech) with 450-480 nm and 520-550 nm filters. All BRET experiments were performed using the Tecan M1000 PRO plate reader unless otherwise specified in the figure legend. Following baseline readings, cells were treated with vehicle, 10μM AngII (Auspep, Cat# 2079) or 1μM EGF (R&D Systems, Cat# 236-EG). The BRET signal observed between interacting proteins was normalised by subtracting the background BRET ratio as follows: the long wavelength emission over the short wavelength emission for a cell sample treated with vehicle was subtracted from the same ratio for a second aliquot of the same cell sample treated with 10μM AngII or 1μM EGF (Porrello, Pfleger, Seeber et al., 2011). For these BRET kinetic assays, the arrow indicates the first BRET reading following treatment of agonist/vehicle (time of ligand/vehicle addition). Ligand and vehicle treated wells were assayed in triplicate wells in each independent experiment.

2.6.2 Bimolecular Fluorescence Complementation Assay

CHO-K1 cells were plated on a collagen-coated 35mm glass bottom dishes (MatTek, Cat# P35GCol-1.5-14-C) at a density of approximately 400,000 cells/dish. 24h post-seeding, cells were transiently transfected with 150ng DNA of Vn-tagged protein and 150ng DNA of Vc-tagged protein, to a total of 300ng of protein (compensated with empty vector when expressing DNA constructs individually) using FuGene. 24 hours post transfection, cells were serum starved using FluoroBrite phenol red-free media + 10%L-Glutamine for 24 hours. Cells were assayed 48 hours after transfection. Localisation of complemented Venus

fluorescence was viewed with an Olympus FV1000 inverted confocal (FV1000, Olympus, Tokyo, Japan) 60x oil immersion objective on a heated (37°C) chamber stage.

2.6.3 [^{125}I]- AngII binding assay

HEK-adherent-293 were transfected with wild type and mutant AT_1R: [TK325]AT_1R or [Y215F]AT_1R. Twenty-four hours post-transfection, cells were serum starved for 12-16 hours. Cells were washed twice in ice-cold PBS and incubated at 4°C for four hours in serum-free DMEM + 0.1% BSA and [^{125}I]-AngII (60μL containing 60,000 counts per minute, equivalent to approximately 50pM AngII) with or without unlabelled 1μM AngII to define non-specific binding. Each condition was tested in triplicate. Following incubation, to remove unbound [^{125}I]-AngII, cells were washed with PBS and lysed in 1mL 0.25M NaOH/0.25% SDS lysis buffer for 10 minutes at room temperature. Radioactivity was determined using a Wizard2 Gamma Counter 2470 (Perkin Elmer)

2.6.4 Conformational Biosensor Assay

HEK293 cells were transfected with 1μg with each fluoresceinarsenical hairpin binder (FlAsH)-labelled AT_1R as per FuGene's protocol (Section 2.4.2). 24h post transfection, cells were washed with PBS and de-attached using 0.25% trypsin, resuspended in FluoroBrite DMEM phenol red-free media supplemented with 5% FBS + 10% L-Glutatmine and added to poly-D-lysine-coated white F96 MicroWell plate at 100,000 cells/well. 48 hours post-transfection, 25mM solution of 1,2-ethanedithiol (EDT; Sigma Aldrich, Cat# 02390) was prepared in dimethyl sulfoxide (DMSO; Sigma Aldrich, Cat# D4540). One volume of 2mM FlAsH-EDT_2 (Invitrogen, Cat# T34561) labelling reagent (1.87μL) was added to two volumes of EDT (3.73μL) to make a 667μM FlAsH solution, this was incubated at room temperature and protected from light for 10 minutes. 100μL of phenol red free HBSS was added and incubated for 5 minutes at room temperature, followed by 4.9mL of HBSS. In parallel, cells were washed with 150μL of HBSS and then labelled with 50μL of FlAsH-EDT_2 solution described above at 37°C for 1 hour, protected from light. Following incubation, cells were washed with 100μL1M 2,3-Dimercapto-1-propanol (BAL; Invitrogen, supplied with FlAsH) diluted in HBSS buffer and incubated at 37°C for 10 minutes. The

cells were washed again with 1M BAL followed by 150μL of Krebs buffer, with no incubation. Lastly, 80μL of Krebs buffer was added to the cells and incubated at 37°C for 2 hours protected from light. 10μL of coelenterazine-h was added to each well to give a final concentration of 5μM and incubated for 5 minutes in a pre-warmed plate reader at 37°C. BRET[1] measurements were taken at 37°C every 10 milliseconds using a M1000 PRO Tecan plate reader. Filtered light emissions were sequentially measured at 430-485 and 505-590. Following base line readings, cells were treated with PBS/HBSS, 10μM AngII or 1μM EGF for the remaining assay. The change in BRET ratio due to the addition of agonist (or the Δ BRET) was calculated by subtracting the average BRET across all reads pre-treatment from the average BRET of the last 30 seconds post-treatment.

2.6.5 CHKA Activity Assay

HEK293 cells were seeded in a 6-well plate at a density of approximately 700,000 cells/well. 24h after seeding, 1 well was left untreated, the remaining wells were transiently transfected with 1μg of mock vector (pcNDA3), pcDNA3-Venus, Venus-CHKA, CHKA-Venus or pcDNA3-CHKA. 24h post-transfection, cells were washed with PBS, and labelled with 1μCi/mL methyl-(^{14}C)-choline chloride. Cells were maintained for 14 additional hours in the presence of radioactivity. Cells were washed with ice-cold TB buffer (137mM NaCl, 5mM KCl, 20mM Tris, pH 7.4). Cells were then fixed with 16% ice-cold trichloroacetic acid. Trichloroacetic acid-soluble material, containing choline and phosphocholine, were washed three times with four volumes of diethylehter, dried under vacuum and resuspended in 50μL of water. 30μL of each sample was resolved on thin layer chromatography plates using 0.9% NaCl:methanol:ammonium hydroxide (50:70:5; V:V:V) as liquid phase.

2.7 Protein Methods

2.7.1 Signalling inhibition/stimulation and preparation of extracts

Cells were serum starved for a minimum of 8 hours prior to inhibition with antagonists and/or stimulation with agonists. Unless otherwise specified, stimulation of cell was performed at 37°C and terminated on ice. Following termination, cells were washed twice with ice-cold

PBS before lysis with radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor cocktail table (PhosSTOP™, Roche Cat# 04906837001) and protease inhibitor cocktail tablet (Roche, Cat# 11836170001) (Appendix A). Cells containing RIPA lysis buffer (200µL/6-well) were rocked for 1 hour at 4°C and then transferred to a fresh 1.5mL eppendorf tube and centrifuged at 10, 000 x g at 4°C for 15 minutes. The supernatant was collected and placed in a fresh 1.5mL eppendorf tube and assayed for protein concentration.

2.7.2 Protein Estimation

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Cat# 23225) as per the manufacturer's protocol. Briefly, standards containing diluted albumin was prepared as well as the Working Reagent (WR), 50 parts of BCA reagent A with 1 part of BCA reagent B. 10µL of each unknown sample was loaded in triplicate into a 96-well plate followed by 200µL of WR reagent and mixed on a plate shaker for 30 seconds. The plate was incubated at 37°C for 30 minutes and absorbance was measured at 562nm using a plate reader. For the resolution of all proteins, protein samples were mixed with Laemmli sample buffer (BioRad, Cat# 161-0747) containing 8% β-mercaptoethanol (Sigma Aldrich, Cat# M6250) and heated at 95°C for 5 minutes.

2.7.3 SDS-PAGE and Western Blotting

Samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples and a molecular weight standard (Precision Plus Protein™ Dual Color standards, BIO-RAD, Cat# 161-0374) were electrophoresed at 100V for approximately 2 hours. Proteins were transferred electrophoretically onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Merck Millipore, Cat# IPFL00010). Membranes were incubated with blocking buffer (Odyssey, Cat# 927-50000) at room temperature for 1 hour and then with primary rabbit polyclonal anti-p44/42 ERK1/2 MAPK and primary mouse monoclonal anti phospho-p44/42 MAPK P-ERK1/2 (Cell Signaling, Cat #4695 and #9106 respectively) (1:1000 dilution in blocking buffer) at 4°C overnight. Membranes were washed with washing buffer (PBS with 0.1% Tween20) 4 times for 5 minutes each and incubated

with a goat anti-mouse IRDye 800CW (LI-COR Biosciences, Cat# 926-32210) and donkey-anti rabbit IRDye 680RD (LI-COR Biosciences, Cat# 926-32222) for 1 hour at room temperature (1:10, 000 dilution in blocking buffer). After further washing, membranes were scanned using an Odyssey Licor Scanner (LI-COR Biosciences). Densitometry analysis of membrane was performed using Image Studio Version 5.2 software.

2.7.4 Calcium Mobilisation Assay

HEK293 cells were seeded in 6-well plates at a density of approximately 700,000 cells/well. 24 hours after seeding, cells were transiently transfected with 500ng GCaMP and 500ng of receptor using FuGene (Section 2.4.2). 24 hours post-transfection, cells were washed with PBS, and detached using 0.25% trypsin, resuspended in FluoroBrite phenol red-free Complete media containing 5% FBS and added to poly-D-Lysine-coated clear bottom black polystyrene 96-well microplate at 100,000 cells/well (Corning). Early the following day, cells were serum starved with FluoroBrite phenol red-free media + 10% L-Glutamine for at least 6 hours. Prior to assay, cells were loaded with calcium buffer (1xHBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4). If using inhibitors, inhibitors were diluted in calcium buffer and left to incubate for 30minutes at 37°C and 5% CO_2. Prior to stimulation with AngII, background fluorescence of cells was imaged for 10 seconds on the FLIPR Tetra (Molecular Devices, Sunnyvale, CA, USA). AngII was added using the FLIPR Tetra and fluorescence was measured for a total of 250 seconds/well. Data was analysed using the FLIPR Tetra Software (Screenworks 3.1.0.3, Molecular Devices) to calculate Max-Min (10–250 seconds) values, then data plotted into GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) to fit curves using non-linear regression. AngII was assayed in triplicate wells in each independent experiment.

2.8 Statistical Analysis

Data was analysed in GraphPad Prism 7.0 and expressed as mean ± standard error of mean (SEM) of at least two independent experiments. Unless indicated, p values including $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*) and not significant (ns) between data sets was determined by one-way ANOVA and Bonferroni's Multiple Comparisons Test.

Chapter Three

Characterisation of a BRET-based model.

3.0 Characterisation of a BRET based model as a readout of AT_1R-EGFR transactivation

3.1 Introduction

Previous studies link the AT_1R and its transactivation of the EGFR to remodelling of the heart, blood vessels and kidney. Hence, elucidating the mechanism of AngII-mediated transactivation may lead to important therapeutic strategies for treating CVD. Ullrich and colleagues first published the phenomenon of GPCR-mediated transactivation, and proposed a model of EGFR transactivation now referred to as the TMPS (Daub, Weiss, Wallasch et al., 1996). According to this model, activated GPCRs stimulate MMPs, including ADAMs, to cleave inactive membrane-bound EGF ligands to activate EGFR (Carpenter, 2000; Cattaneo, Guerra, Parisi et al., 2014; Manso, Elsherif, Kang et al., 2006; Prenzel, Zwick, Daub et al., 1999). This model has been broadly accepted, although others have suggested a more complex mechanism (perhaps cell-specific) involving non-receptor tyrosine kinases and the activation of additional second messengers. Definitive resolution of the precise mechanism of GPCR-EGFR transactivation requires approaches to monitor this process in living cells, in real-time, as well as the capacity to identify key proteins and interactions involved. While some progress has been made on the latter problem – a siRNA screen was recently used to identify novel mediators of AT_1R-EGFR transactivation (George, Purdue, Gould et al., 2013) – to date most readouts of EGFR transactivation have been biochemical, end-point assays that do not capture live cell dynamics or kinetics.

Examination of AT_1R-mediated EGFR transactivation has generally been defined as the activation of ERK1/2 that is inhibited by the small molecular antagonist of the EGFR kinase domain (i.e., AG1478). However, AG1478 can also inhibit the kinase activity of additional members of the HER receptors, raising the possibility that HER2, HER3 and HER4 may contribute to AT_1R transactivation (Chan, Jenkins, Pipolo et al., 2006; Levitzki & Gazit, 1995). Furthermore, EGFR signal transduction is complex with ERK1/2 phosphorylation representing a signal quite distal from the initial step of EGFR activation. Arguably, a more direct readout would be to examine upstream events, including the activation and autophosphorylation of the EGFR. Although a direct readout of phosphpo-EGFR may mitigate the limitations of the ERK1/2 assay, both phospho-EGFR and phospho-ERK1/2

assays are endpoint approaches and only provide a snapshot of cellular events. More recently, proximity-based assays, such as RET assays and fluorescein arsenical hairpin (FlAsH) biosensors have evolved to enable dynamic analysis of protein-protein as well as intra-molecular interactions in real-time (Bourque, Pétrin, Sleno et al., 2017; Devost, Sleno, Pétrin et al., 2017). The development and validation of proximity-based assays (particularly BRET- and FRET-based assays) have been widely used to characterise receptor interactions and have become extremely useful tools for studying GPCR biology, specifically the interaction of GPCRs with G proteins and arrestins (Adjobo-Hermans, Goedhart, van Weeren et al., 2011; Shukla, Violin, Whalen et al., 2008; Tang, Strachan, Lefkowitz et al., 2014; Turu, Szidonya, Gaborik et al., 2006).

A major aim of this thesis was to validate a BRET-based assay that can quantitatively measure, AT_1R-mediated EGFR transactivation, in live cells and in real-time. The present chapter describes the initial validation of a BRET based assay as a suitable readout of AT_1R-EGFR transactivation.

3.2 Materials & Methods

Methods used throughout this chapter are detailed at length in Chapter 2.0, this includes cell culture (Section 2.4), isolation of VSMC (Section 2.4.4), nucleofector delivery of plasmid DNA (Section 2.4.5.2), ligand-induced BRET assays (Section 2.6.1) and confocal imaging (Section 2.6.2)

3.2.1 Materials

HEK293, CHO-K1, NIH-3T3, HeLa cells were obtained from American Type Culture Collection. HEK293FT were obtained by Thermo Fisher Scientific. HT-29 cell line was a generous gift from Dr Daniel Croker, University of Queensland, AUS. Amaxa nucleofector kit (Lonza, Cat# V4XP-1024) was used to transfect isolated VSMC. AngII was obtained from Auspep or Sigma Aldrich and EGF from R&D Systems or Peprotech. Inhibitors were obtained from Wako Pure Chemical Industries (YM-254890), AstraZeneca (Candesartan), Merck Millipore (AG1478), Sigma Aldrich (PP2) and Calbiochem (BAPTA).

3.2.2 DNA Constructs

EGFR-Rluc8 and Grb2-Venus were generously provided by Professor Kevin Pfleger, University of Western Australia, Australia and have been described previously (Ayoub, See, Seeber et al., 2013). AT_1R was generated as previously described (Porrello, Pfleger, Seeber et al., 2011; Smith, Chan, Qian et al., 2011). HER2-Rluc8 and HER3-Rluc8 were generated by inserting EGFR, HER2, and HER3, obtained from Origene (Rockville, MD, USA) into pcDNA3-Rluc8. Control green fluorescent protein (GFP) plasmid DNA, used to assess transfection efficiency in isolated VSMC, was provided with the Lonza Nucleofector kit (Cat #V4XP-3032, Lonza). HA-V_2R was provided from Dr Thierry Durroux (Functional Genomics Institute, Montpellier, France). HA-β_1AR was generated by GeneArt (Thermo Fisher Scientific, Regensburg, Germany) and α_{1b}AR, α_{2a}AR, β_2AR, HA-B_2R and HA-D_1R cDNAs were obtained from the Missouri S&T cDNA Resource Centre (www.cdna.org).

3.3 Results

3.3.1 Optimising a BRET-based AT_1R-EGFR transactivation assay

3.3.1.1 DNA construct stoichiometry

In this study, we used a BRET-based assay to monitor the most proximal event in EGFR activation, namely the recruitment of the EGFR adaptor protein, growth factor receptor-bound protein 2 (Grb2), which associates with the EGFR in an activation- and phosphorylation-dependent manner (Ayoub, See, Seeber et al., 2013). My co-supervisor Professor Kevin Pfleger, who is a specialist in the area, kindly provided several control constructs, including EGFR-Rluc8 and Grb-2Venus, which have been published previously (Ayoub, See, Seeber et al., 2013). Plasmid DNA constructs were confirmed by Sanger sequencing and all experiments were designed to promote optimal BRET assay conditions (e.g., titration of AT_1R, EGFR-Rluc8 and Grb2-Venus plasmid constructs to obtain optimal stoichiometry).

Initially, receptor/adaptor molecule DNA was titrated in order to achieve optimal ligand-dependent BRET signal in cells expressing EGFR-Rluc8 and Grb2-Venus. Both HEK293 and COS cells were transfected with a fixed amount of Grb2-Venus (0.2μg) with increasing amounts of receptor EGFR-Rluc8 DNA (0.15μg, 0.3μg, 0,6μg & 0.9μg). EGF-stimulation produced a robust and sustained ligand-dependent BRET signal in transfected HEK293 cells and COS cells respectively, indicating Grb2 translocation and interaction with the activated EGFR (Figure 3.01A and 3.01B respectively), validating that both receptor and adaptor molecule were functional. Both cell lines demonstrated that 0.2μg of Grb2-Venus and 0.15μg of EGFR-Rluc8 maximally recruited Grb2 to the EGFR. All remaining experiments were transfected with the aforementioned amounts, unless otherwise specified.

Previously, EGFR activation (and Grb2 interaction) has been shown to be dependent on the small tyrosine kinase inhibitor, AG1478 (Ayoub, See, Seeber et al., 2013; Lanzerstorfer, Borgmann, Schütz et al., 2014). Consistent with previous literature, EGF-stimulated recruitment of Grb2 to the EGFR was completely inhibited with pre-treatment of AG1478 in both HEK293 and COS cells (Figure 3.01C & 3.01D, respectively). EGF-treatment also resulted in a dose-dependent increase in BRET signal in HEK293 cells (Figure 3.01E).

Lastly, in an attempt to refine the BRET response in COS cells, different cell seeding densities were compared. Figure 3.01F shows that a cell density of 40,000 cells/well seeded into a 96-well plate gave the maximal BRET response with the smallest error.

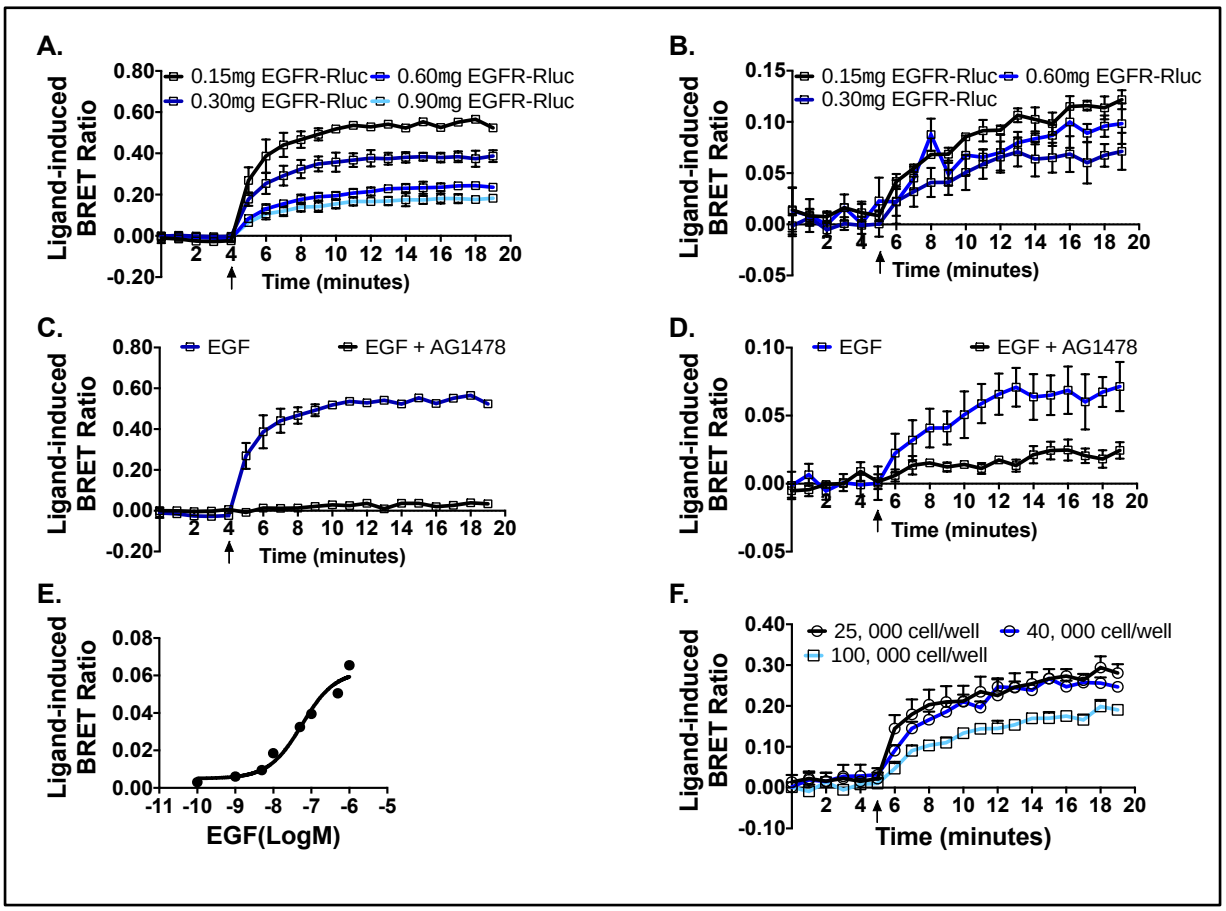


Figure 3.01: Kinetic analysis of EGF-induced recruitment of Grb2 to EGFR in HEK293 and COS cells.

(A) HEK293 cells (100,000 cells/well) and **(B)** COS cells (70,000 cells/well) were transfected with 0.2µg of Grb2-Venus alongside increasing amounts of EGFR-Rluc8 (0.15µg, 0.3µg, 0,6µg & 0.9µg) and were stimulated with 1µM EGF or vehicle. Similarly, **(C)** HEK293 cells and **(D)** COS cells co-expressing 0.2µg of Grb2-Venus and 0.15µg EGFR-Rluc8 were pre-treated with 1µM AG1478 (EGFR inhibitor) for 30 minutes prior to stimulation with 1µM EGF or vehicle. **(E)** HEK293 cells expressing the aforementioned ratio of EGFR-Rluc8 and Grb2-Venus were treated with increasing concentration of EGF and measured 3 minutes following EGF-stimulation. **(F)** COS cells expressing the aforementioned amount of EGFR-Rluc8 and Grb2-Venus were seeded at various cell densities in a 96-well plate (25,000, 40,000 and 100,000 cells/well). BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 2-4 independent experiments with triplicates within.

3.3.1.2 Establishing AT_1R-mediated EGFR transactivation

To investigate AT_1R-mediated EGFR transactivation, EGFR-Rluc8 & Grb2-Venus plasmids were co-transfected with increasing amounts of AT_1R DNA (0.15μg, 0.3μg, 0,6μg & 0.9μg). In HEK293 cells, Figure 3.02A demonstrates that 0.3μg of transfected AT_1R produced ligand-dependent Grb2 translocation to the activated EGFR following AngII-treatment. Comparably, AT_1R-mediated transactivation was shown to extend to COS cells, with 0.3μg of transfected AT_1R also shown to mediate a sustained ligand-dependent BRET signal (Figure 3.02B). Consistent with the idea that GPCR-mediated transactivation engages only a small percentage of the total EGFR signalling capacity, the AngII-mediated Grb2 recruitment and the downstream activation of ERK1/2 represent approximately 20% of that stimulated by the EGF ligand. We next compared cell-seeding density required to achieve optimal AngII-mediated BRET response, with 100,000 and 40,000 cells/well displaying maximal BRET response for HEK293 and COS cells, respectively (Figure 3.02C and 3.02D).

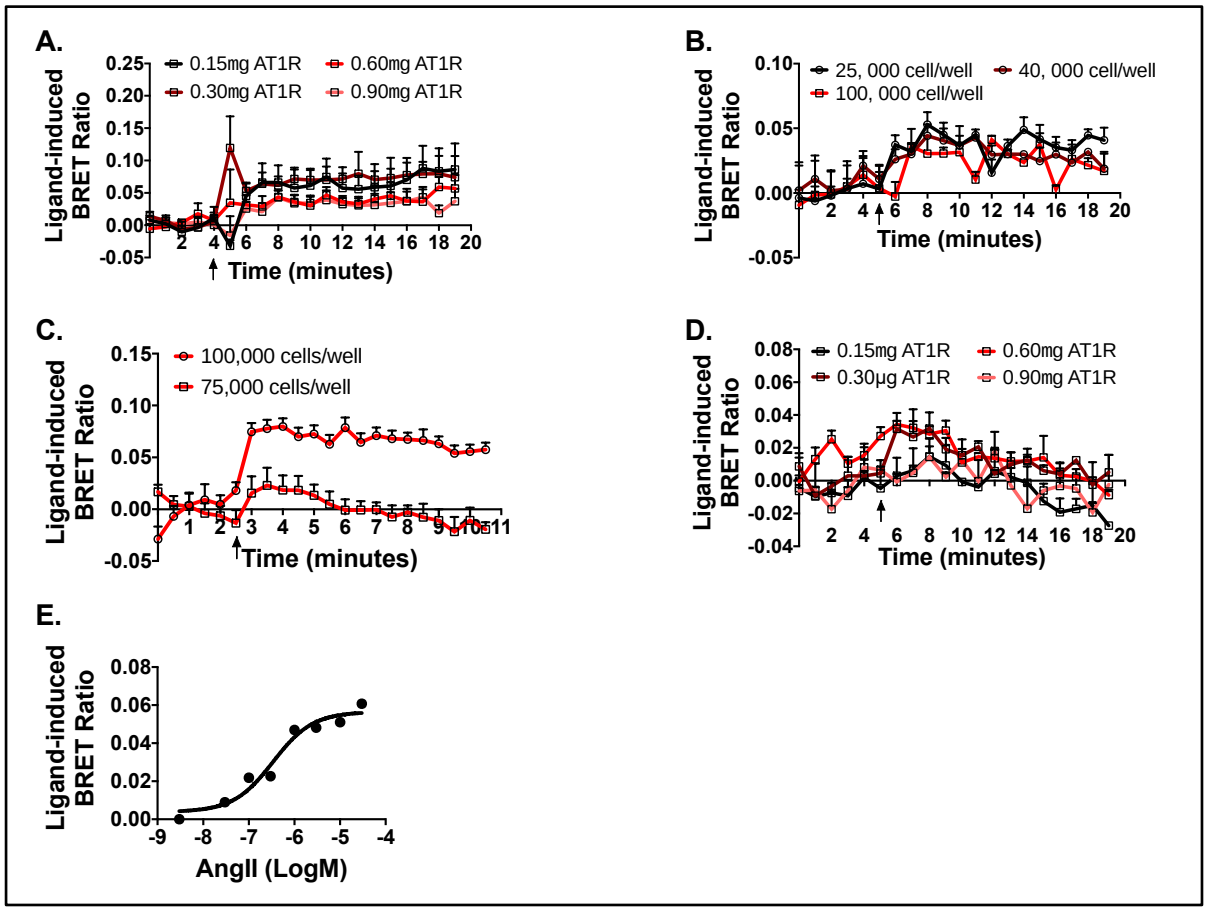


Figure 3.02: Development of a BRET assay to study AT_1R-mediated EGFR transactivation.

(A) HEK293 cells and **(B)** COS cells transfected with 0.2µg of Grb2-Venus, 0.15µg EGFR-Rluc8 and increasing amount of AT_1R (0.15µg, 0.3µg, 0.6µg & 0.9µg) were treated with 10µM AngII or vehicle. Similarly, HEK293 cells **(C)** and COS cells **(D)** were transfected with 0.2µg of Grb2-Venus, 0.15µg EGFR-Rluc8 and 0.3µg AT_1R were seeded at various cell densities (25,000 – 100,000 cells/well) and treated with 10µM AngII or vehicle. **(E)** HEK293 cells expressing the aforementioned ratio of EGFR-Rluc8, Grb2-Venus and AT_1R were treated with increasing concentrations of AngII and measured 3 minutes following AngII-stimulation. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 2-4 independent experiments with triplicates within.

3.3.1.3 Examination of plate readers

A final optimisation related to the plate reader used to quantitatively measure AngII-mediated transactivation, with a total of three plate readers tested: BMG LumiStar, Tecan M1000 Pro and a VictorLight. All plate readers showed a sustained EGF-mediated BRET response (Figure 3.03A-C), however only the BMG and Tecan plate readers showed an AngII-mediated response (Figure 3.03A & 3.03B respectively). All remaining experiments used either a Tecan M1000 Pro or BMG LumiStar plate reader.

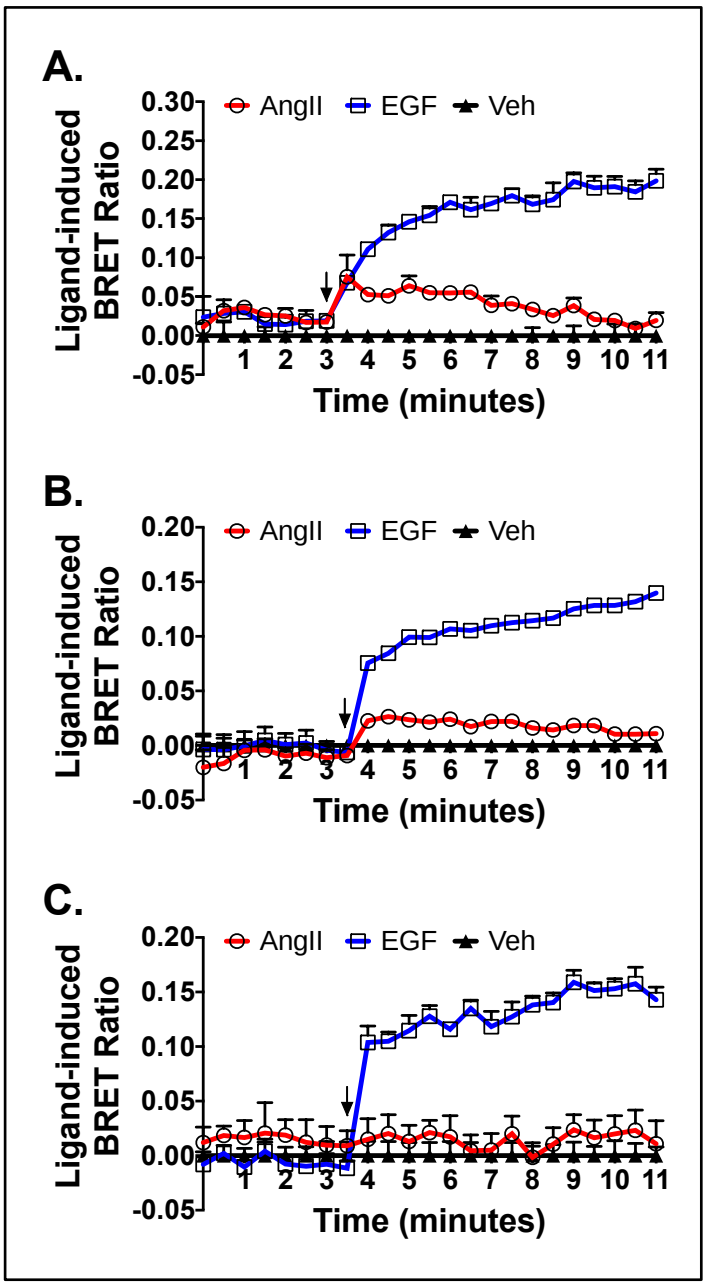


Figure 3.03: AT_1R-EGFR transactivation BRET assay using different plate readers.
HEK293 cells transfected with 0.2µg of Grb2-Venus, 0.15µg EGFR-Rluc8 and 0.3µg AT_1R were stimulated with 10µM AngII, 1µM EGF or vehicle. BRET measurements were taken using **(A)** BMG LumiStar, **(B)** Tecan M1000 Pro or **(C)** VICTOR Light. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 2 independent experiments with triplicates within.

3.3.2 Examination of a variety of immortalised cell lines to transactivate EGFR following AngII stimulation.

Over the past 18 years, several cell lines have been show to mediate EGFR transactivation and ERK1/2 activation following AngII application. While HEK293 cells and COS cells have been extensively used by others to characterise EGFR transactivation, we wanted to next determine whether this phenomenon occurs in a variety of immortalised cell lines using the BRET-based assay (Shah, Yesilkaya, Olivares-Reyes et al., 2004). Four additional immortalised cell lines were tested, these included HT-29 cells (Figure 3.04A, E, L, M), NIH-3T3 cells (Figure 3.04D, F, J, N), CHO-K1 cells (Figure 3.04C, G, K, O) and HeLa cells (Figure 3.04D, H, L, P). A total of 12 conditions were tested for each cell line, with each cell line transfected with increasing amounts of Grb2-Venus (0.05μg, 0.2μg and 0.4μg) alongside increasing amounts of EGFR-Rluc8 DNA (0.15μg, 0.3μg, 0.6μg and 0.9μg). The optimal ratio of receptor:adaptor molecule was determined for each cell line (Table 3.01) and then co-transfected with increasing amount of AT_1R to determine if the cell line has the capacity to transactivate EGFR following AT_1R-activation. As shown in Figure 3.04, NIH-3T3 and CHO-K1 cell lines demonstrated an increase in EGFR-Grb2 BRET ratio following AT_1R activation, however HT-29 and HeLa cell lines did not. Thus, in addition to HEK293 cells and COS cells, NIH-3T3 and CHO-K1 cells appear to be appropriate models for interrogating AT_1R-mediated EGFR transactivation.

Cell Line	Optimal EGFR-RLuc8:Grb2-Venus ratio	AT_1R-mediated transactivation
HT-29	0.3μg EGFR-Rluc8:0.4μg Grb2-Venus	×
NIH-3T3	0.3μg EGFR-Rluc8:0.4μg Grb2-Venus	✓
CHO-K1	0.9μg EGFR-Rluc8:0.2μg Grb2-Venus	✓
HeLa	0.15μg EGFR-Rluc8:0.6μg Grb2-Venus	×

Table 3.01: Optimal ratio of EGFR-Rluc8 and Grb2-Venus DNA transfected for each immortalised cell line.

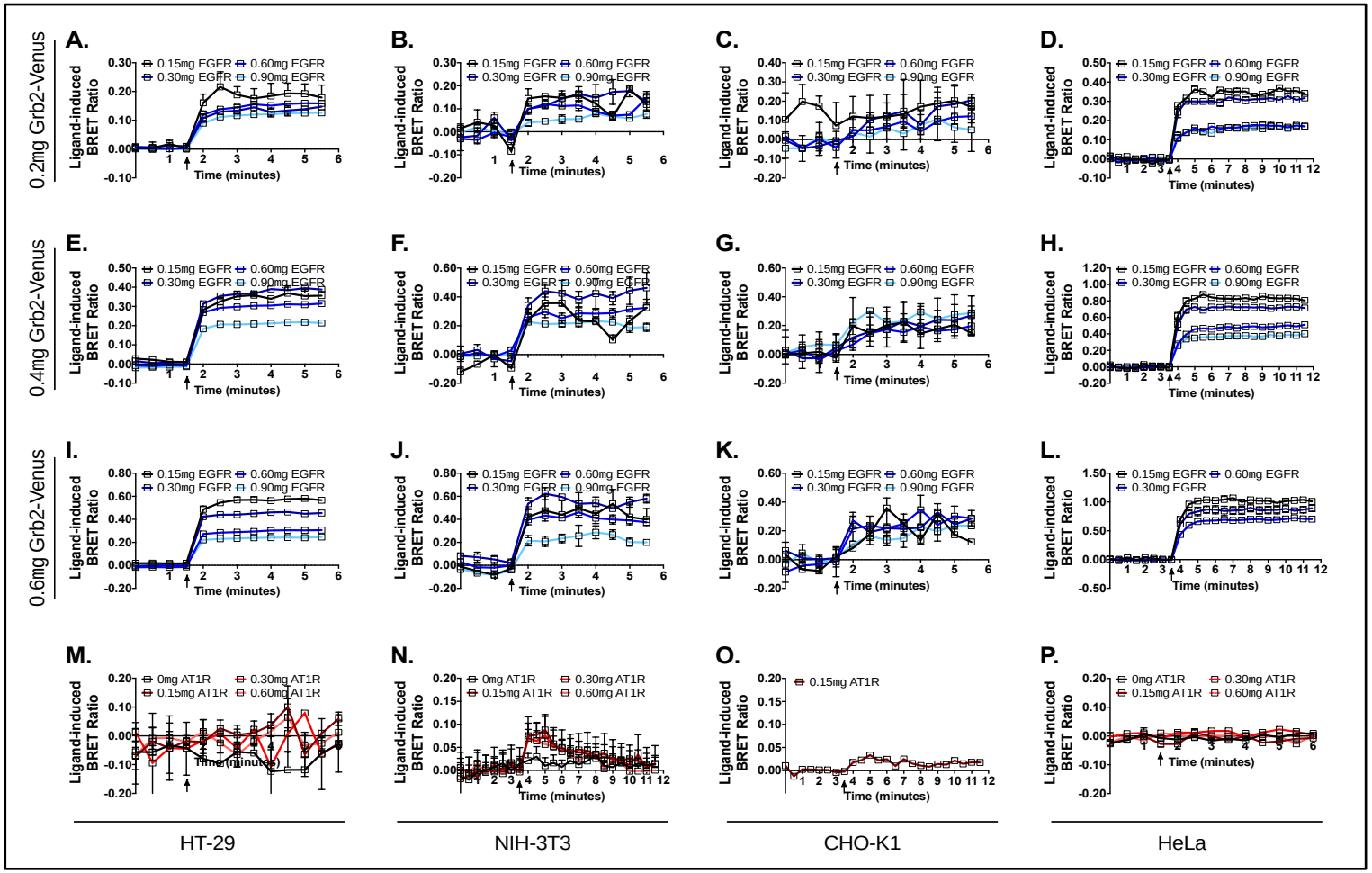


Figure 3.04: Adaptability of a BRET-based approach to measure AT_1R-EGFR transactivation in a variety of immortalised cell lines.
As per the title of each panel, increasing amounts of EGFR-Rluc8 and Grb2-Venus were co-transfected into **(A,E,I)** HT-29, **(B,F,J)** NIH-3T3, **(C,G,K)** CHO-K1 and **(D,H,L)** HeLa cells and stimulated with 1μM EGF or vehicle. The optimal EGFR-Rluc8 and Grb2-Venus ratio for each cell line was co-transfected with increasing amounts of AT_1R in **(M-P)** and stimulated with 10μM AngII or vehicle. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 2-4 independent experiments with triplicates within.

3.3.3 AT_1R-EGFR transactivation in primary VSMC

A final optimisation was to examine the effects of AT_1R-EGFR BRET sensors in isolated VSMCs. There is strong evidence demonstrating that EGFR transactivation is critical in mediating vascular remodelling, hypertrophy and migration, and is therefore considered to be important in the development of cardiovascular diseases (Higuchi, Ohtsu, Suzuki et al., 2007; Montezano, Cat, Rios et al., 2014; Touyz, Wu, He et al., 2002). Many of these seminal studies have used downstream ERK1/2 activation as a readout of EGFR transactivation. A final optimisation was to assess whether the BRET sensors were portable to isolated primary cells and also to establish their value in a more physiologically relevant context.

Electroporation based-transfection, using an Amaxa nucleofector, was used to deliver plasmid DNA to primary isolated VSMCs. Firstly, plasmid DNA encoding alkaline phosphatase (ALP) was transfected into VSMCs using electroporation, and used as a positive control for delivery and expression of exogenous DNA. VSMCs that expressed ALP showed increased ALP activity compared to VSMC that were not transfected with ALP (Figure 3.05A). Furthermore, cells were transfected with a GFP reporter plasmid to assess transfection efficiency. We were able to detect GFP positive VSMC while maintaining cell viability (Figure 3.05B and 3.05C). We next sought to transfect isolated primary cells with the BRET constructs. Using an already optimised 3:1 ratio of EGFR-Rluc8 and Grb2-Venus DNA (data not included), EGF-stimulation mediated a robust and ligand-dependent Grb2 translocation to the EGFR (Figure 3.05D). Furthermore, upon transfection of the AT_1R, we were able to detect Grb2 recruitment following AngII-treatment, indicative of EGFR transactivation (Figure 3.05D)

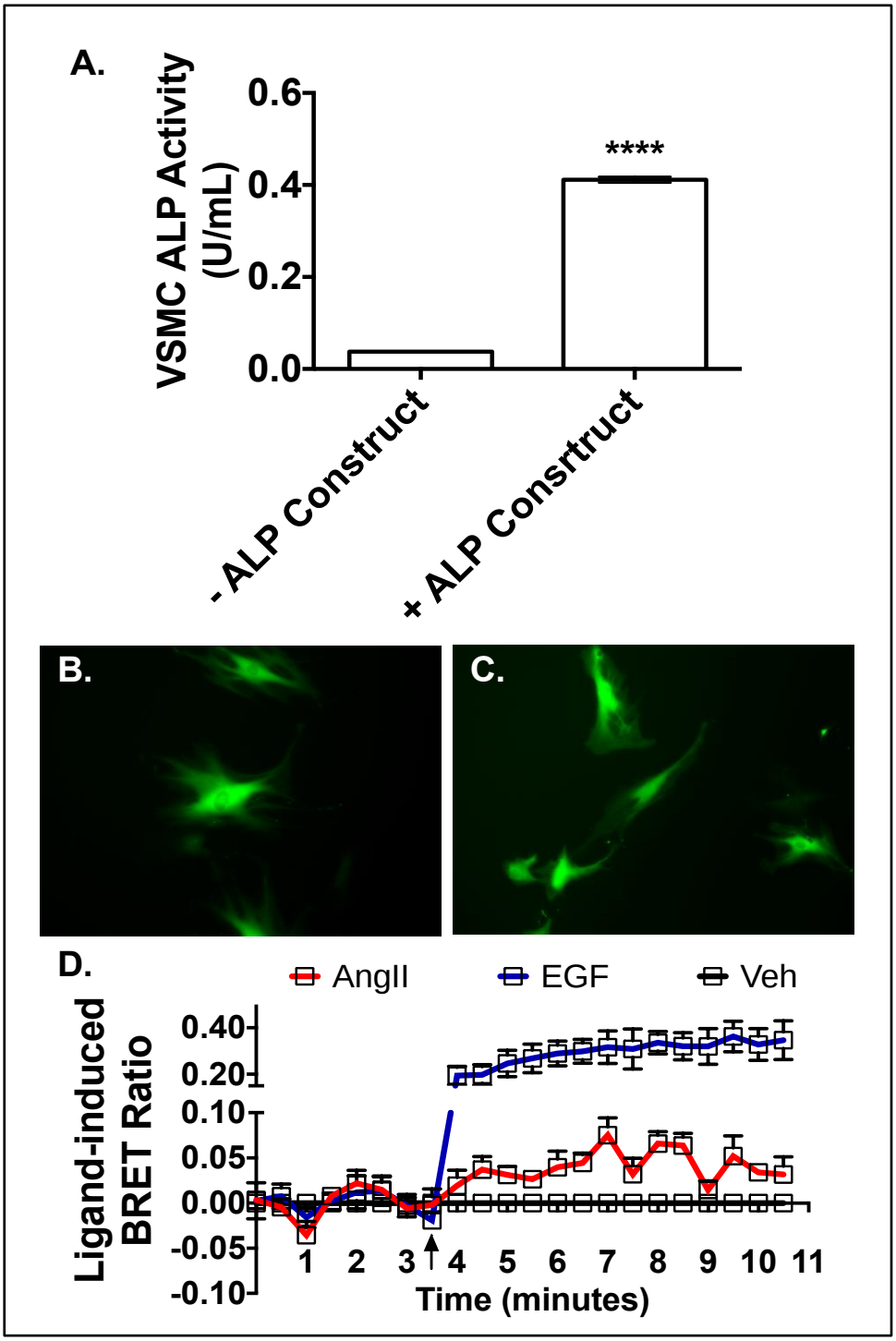


Figure 3.05 Adaptability of BRET in isolated VSMC
(A) Alkaline phosphatase activity measured in isolated VSMC transfected with and without ALP plasmid. **** $p<0.0001$ compared to cells not expressing ALP construct. **(B, C)** Confocal imaging of isolated VSMC expressing GFP. **(D)** Rat isolated primary VSMC transfected with Grb2-Venus, EGFR-Rluc8 and AT_1R were stimulated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 2-4 independent experiment with triplicates within.

3.3.4 AngII capacity to transactivate additional RTK members

Having demonstrated the universality of the BRET-based approach to measure EGFR transactivation, a final consideration was to test if this technology could be used to screen a range of GPCRs and interrogate their capacity to transactivate EGFR, as well as assess if AngII can transactivate other members of the RTK family. In addition to the EGFR, other members of the RTK family have been shown to transactivate following AngII-stimulation (Lin & Freeman, 2003; Negro, Brar, Gu et al., 2006). We investigated whether AngII-stimulation recruited Grb2 to other members of the EGFR family, specifically HER2 (preferred dimer partner for EGFR, which is not ligand-activated) and HER3 (which is ligand-activated, but is kinase-deficient). As shown in Figure 3.06, EGFR and HER2, but not HER3, strongly complexes with Grb2 following AngII-stimulation.

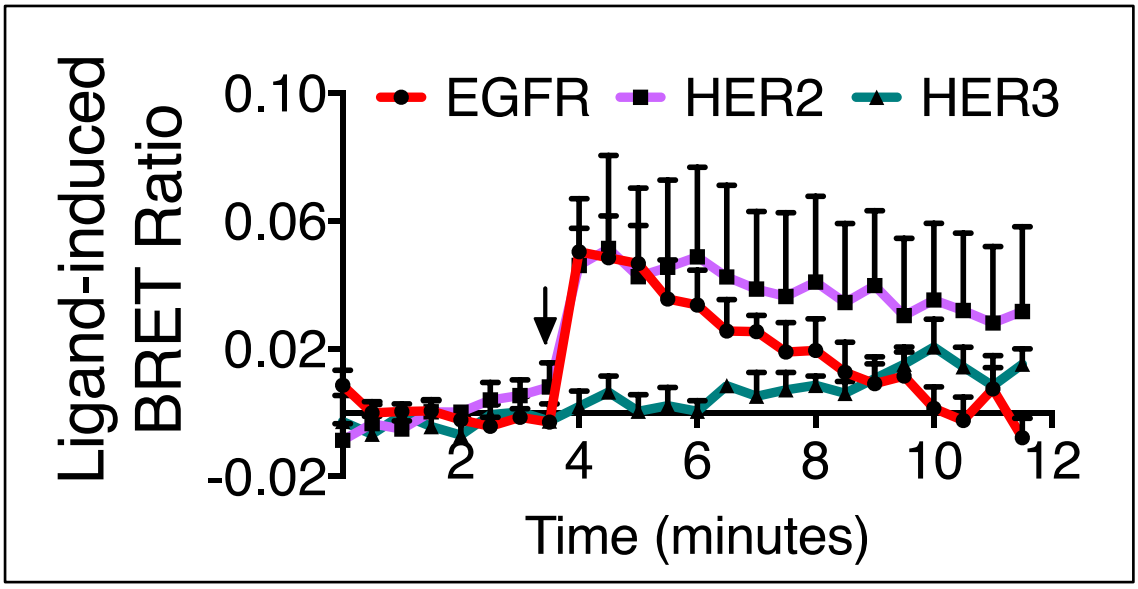


Figure 3.06: AngII-mediated transactivation of additional members of the EGFR RTK family
HEK293 cells expressing AT_1R, Grb2-Venus and either of EGFR-Rluc8, HER2-Rluc8 or HER3-Rluc8 were stimulated with 10μM AngII or vehicle. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 4 independent experiments with triplicates within.

3.3.5 Screening for EGFR transactivation by multiple GPCRs

A number of prominent GPCRs have been reported to transactivate the EGFR, including the adrenergic (Kim, Eckhart, Eguchi et al., 2002; Turner, 2003), dopamine (Yoon & Baik, 2013), bradykinin (Methner, Donat, Felix et al., 2009), vasopressin (Fuentes, Reyes, Sarmiento et al., 2008; Zhang, Wang, Cao et al., 2016) and adenosine receptors (Williams-Pritchard, Knight, Hoe et al., 2011), although the exact mechanisms remain unresolved. We next examined whether the BRET-based Grb2 recruitment assay could be used to screen multiple GPCRs and measure their capacity to transactivate the EGFR. Shown in Figure 3.07 is a focused screen of 19 separate GPCRs and their capacity to recruit Grb2 to the EGFR. All assays included EGF activation of the EGFR as an internal control. Interestingly, we observed consistent increases in the EGFR-Grb2 BRET ratio for the AT_1R and the Vasopressin 1b receptor ($V_{1b}R$). For a number of other GPCRs, their activation led to a decrease (or no change) in the EGFR-Grb2 BRET ratio. A BRET-based arrestin trafficking assay was used for these receptors to substantiate their expression and activation following ligand stimulation (Figure 3.08).

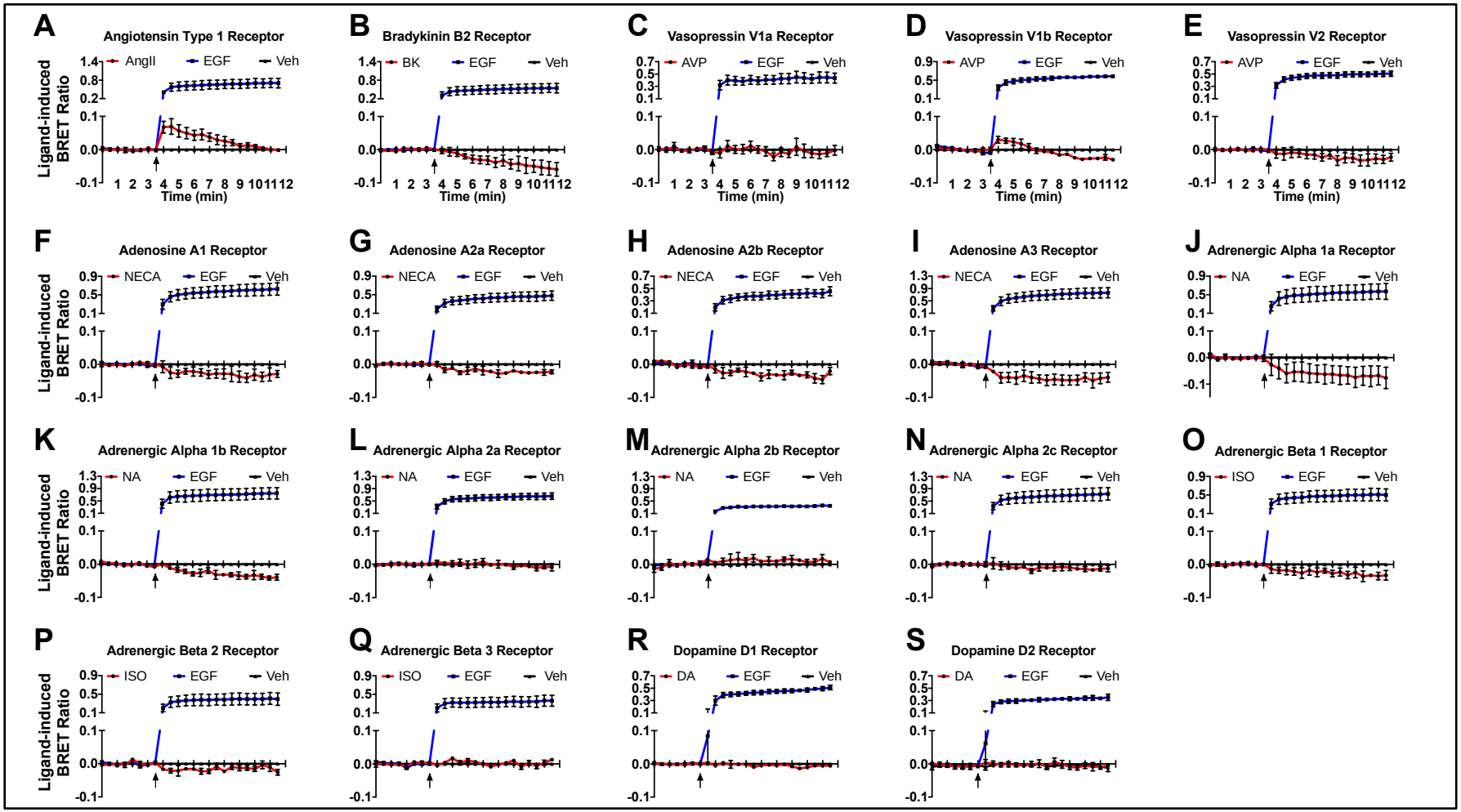


Figure 3.07: Screen of multiple GPCRs using the BRET-based transactivation assay.
As per the title of each graph, parts **(A-S)**, 19 different GPCRs expressed in HEK293FT cells and their ability to induce Grb2-Venus interaction with EGFR-Rluc8 following stimulation with 1μM EGF, 10μM GPCR agonist-stimulation or vehicle. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represents mean ± SEM of 3-5 independent experiments.

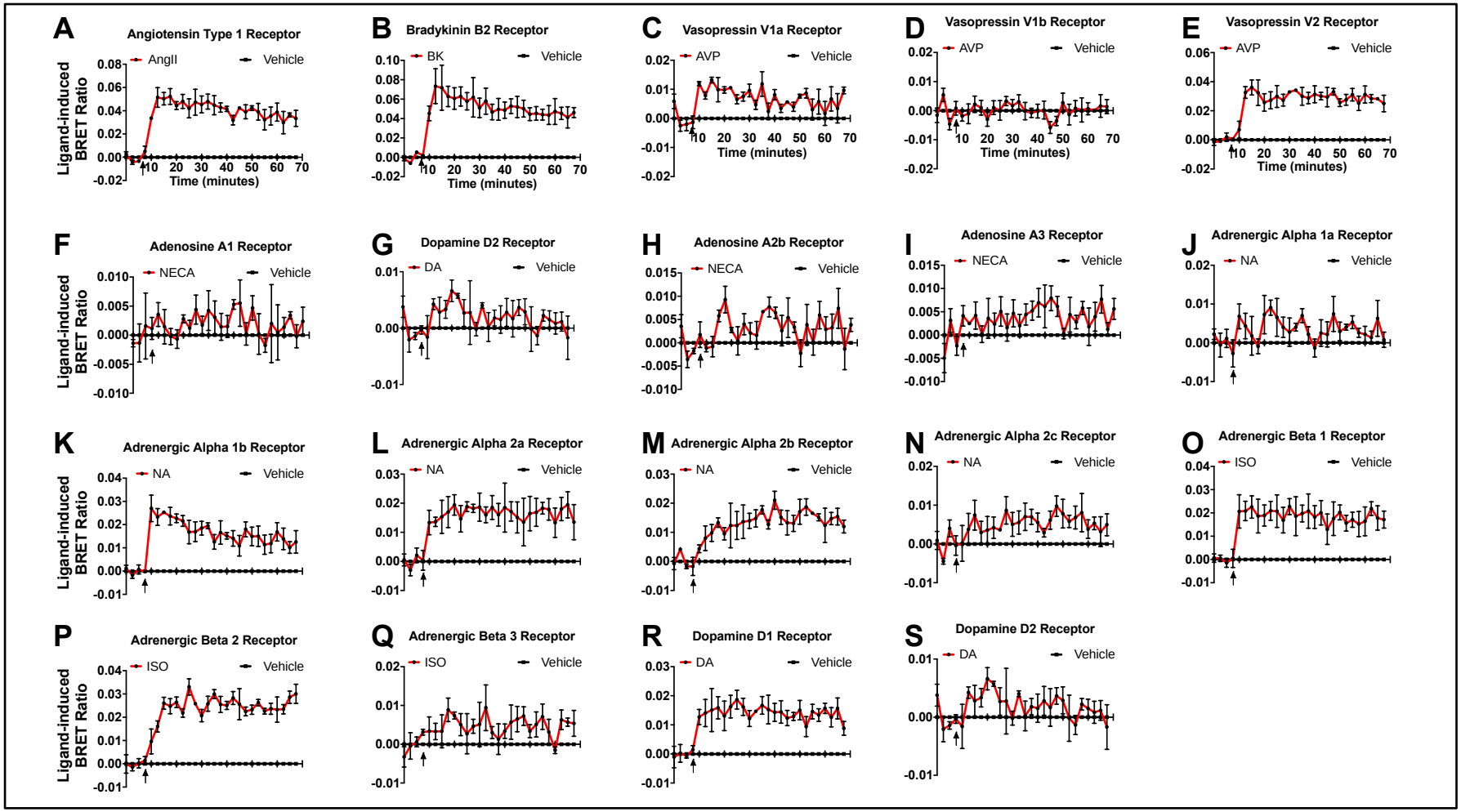


Figure 3.08: Validation of GPCR functionality

As per the title of each graph, parts **(A-S)**, show 19 different GPCRs expressed in HEK293FT cells, and their ability to induce β-arrestin2-Rluc8 interaction with membrane marker, K-ras-Venus, following 10μM GPCR agonist-stimulation or vehicle. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represents mean ± SEM of 3-5 independent experiments.

3.4 Discussion

GPCR-mediated EGFR transactivation is commonly defined as the activation of ERK1/2, which although informative, is an indirect and distal readout. Herein, I describe a BRET assay, based on the proximal recruitment of Grb2 to the EGFR to quantitatively monitor, in living cells and in real-time, the direct activation of EGFR. The development of novel approaches to study GPCR-mediated transactivation in live cells is important, but to our knowledge BRET-based quantification of Grb2 recruitment to the EGFR has not been used as a direct readout of GPCR-mediated transactivation. Pfleger and colleagues have previously used the recruitment of Grb2 to RTKs (EGFR and HER3) as a measure of EGFR activation and heterodimerisation in HEK293 cells following stimulation with EGF and Heregulin (Ayoub, See, Seeber et al., 2013). Moreover, others have also identified such BRET approaches as *bona fide* readouts of EGFR activation (Schiffer, Reding, Fuhs et al., 2007; Tan, Wang, Littler et al., 2007). In this chapter, I initially aimed to determine whether stimulation of the AT_1R could promote Grb2 translocation and interaction with EGFR in a ligand-dependent manner in live cells. AT_1R-mediated EGFR transactivation was readily demonstrable, but was consistently lower in magnitude compared to EGF-stimulation. The selective activation of EGFR by the AT_1R is consistent with many observations that the degree of signalling elicited by GPCRs appears to be only a subset of that obtained following full activation by EGF ligands (Daub, Weiss, Wallasch et al., 1996; Eguchi, Numaguchi, Iwasaki et al., 1998; Shah, Yesilkaya, Olivares-Reyes et al., 2004; Tilley, Kim, Patel et al., 2009).

EGFR transactivation was initially shown to occur in HEK293 and COS cells. There is considerable debate as to which immortalised cell lines have the capacity to transactivate, with HEK293 previously considered incapable of transactivation due to their lack of MMPs, an essential protein in the transactivation process (Shah, Yesilkaya, Olivares-Reyes et al., 2004). Catt and colleagues showed in contrast to HEK293 cells, following AngII-stimulation COS cells activate EGFR and subsequently phosphorylate ERK1/2 through the release of HB-EGF. This led to the theory that there may be alternative cells lines that show a more robust transactivation response. The use of BRET as a readout of AT_1R-EGFR transactivation was extended to several immortalised cell lines, with CHO-K1 and NIH-3T3

cells shown to mediate EGFR activation following AngII stimulation, indicting the versality of this approach to measure EGFR transactivation. In addition, it is possible that by creating an artificial environment where EGFR-Rluc8 & Grb2-Venus are co-transfected alongside the AT_1R, that transactivation is artificially introduced into a cell that does not normally have the capability. Our laboratory has previously demonstrated that HeLa cells do not activate ERK1/2 phosphorylation in an AG1478-dependent manner, therefore it was reassuring that I was unable to demonstrate AT_1R-EGFR transactivation in HeLa cells.

We next investigated whether AT_1R-mediated transactivation extends to isolated primary cells. AngII has been shown to mediate VSMC remodelling through the transactivation of the EGFR (Chan, Umesalma & Baumbach, 2015; Eguchi, Dempsey, Frank et al., 2001). Previous studies have shown that AngII induces G_q/Ca^{2+}-dependent and/or Src-dependent transactivation of the EGFR, leading to downstream activation of the MAPK pathways (ERK1/2, Akt, p38) and subsequent ROS generation, NADPH oxidase activation and protein synthesis, resulting in VSMC migration, hypertrophy and hyperplasia. Using nucleofector delivery, I demonstrated EGF-mediated Grb2 recruitment to the activated EGFR. Upon AngII-stimulation we could also report Grb2 recruitment, indicative of EGFR transactivation.

A final consideration was if the BRET-based assay could be used to screen multiple GPCRs and interrogate their capacity to transactivate the EGFR. Of the panel of 19 GPCRs tested, only the AT_1R and $V_{1b}R$ showed consistent EGFR transactivation, as measured by increased BRET-based Grb2 recruitment. An apparent lack of EGFR transactivation for a number of these receptors was surprising because they have been reported previously to promote EGFR transactivation as measured by activation of MAPK signalling pathways (ERK1/2, p38, PI3K/Akt), phospho-EGFR, metalloprotease activation, EGF ligand shedding and transcriptional responses (Fuentes, Reyes, Sarmiento et al., 2008; Huang, Benaich, Tape et al., 2014; Inoue, Ishiguro, Kitamura et al., 2012; Matus, Ehrenfeld, Pavicic et al., 2016; Volpi, Liu & Aguilera, 2006).

It is important to note that a negative response in our assay does not necessarily mean that the GPCR is not transactivating nor should it call into question the veracity of our assay. There are a number of plausible explanations: firstly, a trivial argument might be that these 'non-transactivating' GPCRs were non-functional – however, we confirmed their activation by

using a readout of ligand-dependent β-arrestin trafficking. Secondly, these GPCRs may not be recruiting Grb2 as part of their activation process or, alternatively, there is an association but it is different in nature to that generated by the AT_1R and $V_{1b}R$. Indeed, for a number of the GPCRs that did not show enhanced EGFR-Grb2 association, we actually observed decreased BRET ratios following ligand stimulation. It could be reasonably argued that this reflects dynamic changes in an equilibrium between association, dissociation and reconfiguration of putative EGFR-Grb2 complexes – where the balance may favour dissociation or reconfiguration that leads to an increased distance between the donor and acceptor moieties.

Thirdly, as our assay only reports on the Grb2 recruitment to the EGFR, the possibility exists that receptors other than the EGFR (HER2, HER3 or HER4) and or combinations of dimers between those receptors, are selectively used by these other GPCRs for transactivation. In this regard, it is important to acknowledge that most studies of EGFR transactivation typically use end-point assays, where specificity is inferred via inhibition by the small molecule inhibitor of the EGFR kinase, AG1478. However, high micromolar concentrations of AG1478 (as commonly used) inhibit receptors other than the EGFR (Chan, Jenkins, Pipolo et al., 2006; Levitzki & Gazit, 1995). Additionally, we report the recruitment of Grb2 to HER2, but not HER3, in response to AngII stimulation. Whether a specific GPCR is able to transactivate EGFR, HER2, HER3 or HER4 will require further investigation, using the approaches we have developed. It will be important to consider that HER2 is the preferred dimerisation partner for other EGFR family members, and therefore potentially acting in *cis* to modulate other HER receptors. Indeed, following AngII-stimulation, HER2 has been shown to be transactivated and/or modulate transactivation (Lin & Freeman, 2003; Negro, Brar, Gu et al., 2006).

3.5 Summary

We report an alternative approach to monitoring AT_1R-EGFR transactivation in live cells. The use of a BRET-based assay to assess recruitment of the EGFR adaptor protein, Grb2, to the EGFR, was shown in a variety of variety of immortalised cell lines as well as primary isolated VSMCs. Both EGF and AngII stimulated Grb2 recruitment to EGFR, indicative of

EGFR transactivation. The BRET assay was used to screen a panel of 19 GPCRs and further developed for other EGFR family members (HER2 and HER3); the AT_1R was able to transactivate HER2, but not HER3. The adaptability of the BRET-based approach provides a novel platform to better interrogate the molecular mechanisms underpinning AT_1R-EGFR transactivation.

Chapter Four

BRET-based assay to monitor EGFR transactivation by the AT_1R reveals $G_{q/11}$ protein-independent activation and AT_1R-EGFR complexes.

This chapter is incorporated into the following publication:

O'Brien, S.L., Johnstone, E.K., Devost, D., Conroy, J., Reichelt, M.E., Purdue, B.W., Ayoub, M.A., Kawai, T., Inoue, A., Eguchi, S., Hébert, T.E., Pfleger, K.D.G., Thomas, W.G (2018). BRET-Based assay to monitor EGFR transactivation by the AT_1R reveals $G_{q/11}$ protein-independent activation and AT_1R-EGFR complexes. *Biochemical Pharmacology*

Contributor	Statement of Contribution
O'Brien, S.L	Conception and design (20%) Performing experiments (70%) Analysis and interpretation (25%) Drafting and production (30%)
Johnstone E.K	Conception and design (10%) Performing experiments (20%) Analysis and interpretation (15%) Drafting and production (10%)
Devost, D	Conception and design (5%) Performing experiments (5%) Analysis and interpretation (5%) Drafting and production (5%)
Conroy, J	Conception and design (0%) Performing experiments (0%) Analysis and interpretation (5%) Drafting and production (0%)
Reichelt, M.E	Conception and design (0%) Performing experiments (0%) Analysis and interpretation (5%) Drafting and production (0%)
Purdue, B.W	Conception and design (0%) Performing experiments (0%)

	Analysis and interpretation (5%) Drafting and production (0%)
Ayoub, M.A	Conception and design (0%) Performing experiments (0%) Analysis and interpretation (0%) Drafting and production (5%)
Kawai, T	Conception and design (0%) Performing experiments (5%) Analysis and interpretation (5%) Drafting and production (0%)
Inoue, A	Conception and design (0%) Performing experiments (0%) Analysis and interpretation (5%) Drafting and production (0%)
Eguchi, S	Conception and design (0%) Performing experiments (0%) Analysis and interpretation (5%) Drafting and production (0%)
Hébert, T.E	Conception and design (5%) Performing experiments (0%) Analysis and interpretation (5%) Drafting and production (5%)
Pfleger, K.D.G	Conception and design (30%) Performing experiments (0%) Analysis and interpretation (10%) Drafting and production (15%)
Thomas, W.G	Conception and design (30%) Performing experiments (0%) Analysis and interpretation (10%) Drafting and production (30%)

BRET-based assay to monitor EGFR transactivation by the AT_1R reveals $G_{q/11}$ protein-independent activation and AT_1R-EGFR complexes

Running Title

BRET assay for AT_1R-EGFR transactivation

Shannon L. O'Brien[a], Elizabeth K.M. Johnstone[b], Dominic Devost[c], Jacinta Conroy[a], Melissa E. Reichelt[a], Brooke W. Purdue[a], Mohammed A. Ayoub[b#], Tatsuo Kawai[e], Asuka Inoue[d], Satoru Eguchi[e], Terence E. Hébert[c], Kevin D.G. Pfleger[b, f], Walter G. Thomas[a, g]

[a] Receptor Biology Group, The School of Biomedical Sciences, Faculty of Medicine, The University of Queensland, St Lucia 4072, Queensland, Australia

[b] Molecular Endocrinology and Pharmacology, Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands and Centre for Medical Research, The University of Western Australia, Crawley, Western Australia, 6009, Australia

[c] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada

[d] Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan

[e] Cardiovascular Research Centre, Department of Physiology, Lewis Katz School of Medicine at Temple University, Philadelphia, PA

[f] Dimerix Limited, Nedlands, Western Australia, 6009, Australia

[g] Centre for Cardiac and Vasculature Biology, The University of Queensland, St Lucia 4072, Queensland, Australia

[#] Present address: Biology Department, College of Sciences, United Arab Emirates University, Al Ain, UAE

Corresponding Author

Professor Walter G Thomas

The School of Biomedical Sciences

Faculty of Medicine

The University of Queensland, St Lucia, Queensland, 4072 Australia

w.thomas@uq.edu.au

Tel: 61-7-3365-1074; Fax: 61-7-3365-1766

4.0 BRET-based assay to monitor EGFR transactivation by the AT_1R reveals $G_{q/11}$ protein-independent activation and AT_1R-EGFR complexes

4.1 Introduction

GPCRs can employ a range of mechanisms to transactivate EGFR and activate downstream ERK1/2, leading to spatially and functionally distinct pools. For example, others have reported that the classical second messenger, Ca^{2+}, as well as ROS mediate AT_1R transactivation in COS-7 cells (Mifune, Ohtsu, Suzuki et al., 2005b). Our own group has previously shown that EGFR transactivation in neonatal cardiomyocytes requires G protein coupling, however inhibition of PKC or Ca^{2+} had no effect on AngII-mediated ERK1/2 activation (Smith, Chan, Qian et al., 2011). It is clear that the second messengers required for EGFR transactivation is dependent upon cellular background, as is transactivation itself.

Despite the implied role of $G_{q/11}$ in AngII-mediated ERK1/2 activation, several studies have suggested $G_{q/11}$-independent mechanisms for this process (Grisanti, Guo & Tilley, 2017). β-arrestin scaffolding signalling has been shown to mediate prosurvival pathways in the heart. β_1AR recruits GRK5/6 and β-arrestin, leading to EGFR transactivation and the activation of prosurvival-antiapoptopic signalling pathways including Akt and ERK1/2 (Noma, Lemaire, Prasad et al., 2007). This 'secondary signalling' has lead to the development of drugs that antagonise overactive G protein-signalling, while simultaneously activating β-arrestin mediated cardioprotective pathways.

In this chapter, the major aim of this project was to determine the functional and structural requirements of AT_1R-EGFR transactivation. Having established and optimised a BRET-based approach that measures the most proximal event in EGFR activation, I extended the technology to investigate the molecular processes involved.

4.2 Materials & Methods

Methods used throughout this chapter are detailed in length at Chapter 2.0, this includes cell culture (Section 2.4), ligand-induced BRET assays & Receptor-HIT assays (Section 2.6.1) BiFC (Section 2.6.2), [125^{I}]-AngII binding assays (Section 2.6.3), Western blot (Section 2.7.1-2.7.3) and calcium mobilisation assay (Section 2.7.4).

4.2.1 Materials

HEK293 and CHO-K1 cells were obtained from American Type Culture Collection. A HEK293 cell line that lacks both β-arrestin1 and β-arrestin2 was used as previously described (Alvarez-Curto, Inoue, Jenkins et al., 2016). [^{125}I]-Labelled AngII was obtained from ProSearch. AngII was obtained from Auspep or Sigma Aldrich and EGF from R&D Systems or Peprotech. Inhibitors were obtained from Wako Pure Chemical Industries (YM-254890), AstraZeneca (Candesartan), Merck Millipore (AG1478), Sigma Aldrich (PP2) and Calbiochem (BAPTA).

4.2.2 DNA Constructs

Preparation of the following cDNA constructs has been previously described: Grb2-Venus (Ayoub, See, Seeber et al., 2013), β-arrestin2-Venus (Kocan, See, Sampaio et al., 2009), AT_1R-Vn β-arrestin2-Rluc8 (Porrello, Pfleger, Seeber et al., 2011), pcDNA3.1 β-arrestin1-Flag and β-arrestin2-Flag (Manglik, Kim, Masureel et al., 2015), and AT_1R, [TK325]AT_1R and [Y215F]AT_1R (Smith, Chan, Qian et al., 2011). EGFR-Vc was supplied from Ichi N. Maruyama (Okinawa Institute of Science and Technology, Okinawa, Japan) (Tao & Maruyama, 2008). The following cDNA constructs were gifts from collaborators: Venus-K-ras from Prof Nevin Lambert (Georgia Regents University). AT_1R-Rluc8 was generated by inserting AT_1R from AT_1R-Venus into pcDNA3-Rluc8, prepared previously from cDNA kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, California, USA. EGFR-Rluc8, was generated by inserting EGFR obtained from Origene (Rockville, MD, USA) into pcDNA3-Rluc8. EGFR-Venus was subsequently generated from EGFR-Rluc8 by replacing the Rluc8 coding region with Venus cDNA from pcDNA3-Venus prepared previously from pcC2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan). Internally EE-tagged $G\alpha_q$ and $G\alpha_{12}$ and untagged DN

$G\alpha_q$ Q209L/D277N mutant, $G\alpha_{11}$ and $G\alpha_{13}$ were from UMR cDNA resource centre (University of Missouri-Rolla (www.cdna.org)).

4.3 Results

4.3.1 Pharmacological Characterisation of GPCR-EGFR transactivation

In order to examine the molecular mechanisms underlying AT_1R-EGFR transactivation, we first examined ERK1/2 activation as an indirect readout. HEK293 cells expressing the AT_1R were stimulated with AngII, leading to the robust and reproducible activation of ERK1/2 (Figure 4.01A). To confirm previous observations by us that ERK1/2 activation by AngII was due to EGFR transactivation (Smith, Chan, Qian et al., 2011; Thomas, 2001), cells were pre-treated with the EGFR kinase inhibitor AG1478 (Figure 4.01A-B), and also the $G_{q/11}$ inhibitor YM-254890, the AT_1R inverse agonist candesartan, the Src inhibitor PP2 or the Ca^{2+} chelator BAPTA (Figure 4.01C and 4.01D). Consistent with previous studies (George, Purdue, Gould et al., 2013; Ohtsu, Higuchi, Shirai et al., 2008), AngII-induced ERK1/2 activation was attenuated by antagonism of the AT_1R, inhibition of the EGFR kinase as well as the inhibition of $G_{q/11}$ (Figure 4.01C and 4.01D). Furthermore, AT_1R-EGFR transactivation was blunted by pharmacological inhibition of Ca^{2+} signalling and Src activity, however to a lesser extent (Figure 4.01C and Figure 4.01D). Taken together, EGFR transactivation (as measured by ERK1/2 activation) by the AT_1R appears to be a $G_{q/11}$-mediated pathway and dependent on EGFR kinase activity.

We next examined whether this pharmacological profile was recapitulated when the BRET based Grb2 recruitment assay was used as a readout for EGFR transactivation. Similar to AngII-mediated ERK1/2 activation, candesartan completely inhibited AngII-stimulated Grb2 recruitment to the EGFR, indicating that transactivation of the EGFR is due to ligand activation of the AT_1R (Figure 4.01E). However, remarkably AngII-mediated recruitment of Grb2 to the EGFR was not inhibited by pre-treatment with the $G_{q/11}$ inhibitor, YM-254890, which is in stark contrast to the outcome of the ERK1/2-based readout mentioned above. Indeed, YM-254890 treatment led to a sustained BRET signal (Figure 4.01E). Additionally, Grb2 recruitment to the EGFR in response to AngII was only partially dependent upon the EGFR kinase activity, as well as Src and Ca^{2+} (Figure 4.01E and 4.01F).

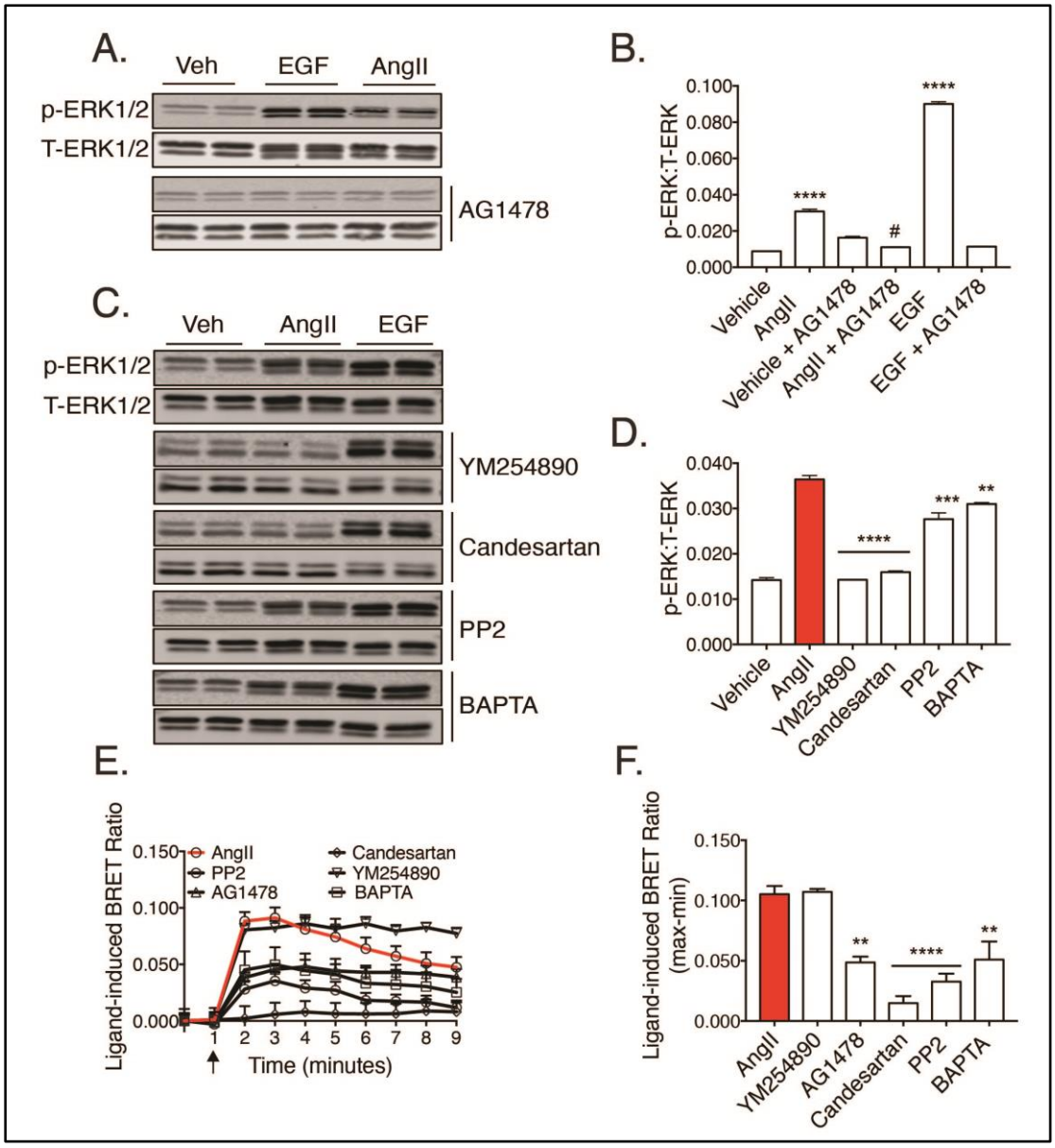


Figure 4.01 Analysis of AT_1R-EGFR transactivation when comparing ERK1/2 phosphorylation and Grb2 recruitment to the EGFR.
(A-B) HEK293 cells stably expressing AT_1R were treated with 500nM AG1478 (EGFR inhibitor) for 30 minutes prior to stimulation with 100nM AngII, 10nM EGF or vehicle for 5 minutes before processing for phospho-ERK1/2:total-ERK1/2 (p-ERK:T-ERK) western blots. Blots are representative of 3 independent experiments **(B)** Quantification of western blot data using densitometry analysis (ImageJ) illustrates p-ERK1/2 changes. **** $p<0.001$ vs. vehicle treated control; # $p<0.001$ vs. AngII treated cells. **(C-D)** HEK293 stably expressing AT_1R were treated with 1μM YM-254890 ($G_{q/11}$ inhibitor), 1μM Candesartan (AT_1R inverse agonist), 10μM BAPTA (Ca^{2+} chelator) and 5μM PP2 (Src inhibitor) for 30 minutes prior to stimulation with 100nM AngII, 10nM EGF or vehicle for 5 minutes before processing for ERK/p-ERK1/2 western blots. Blots are representative of 3 independent experiments. **(D)** Quantification of western blot data using densitometry analysis (ImageJ) illustrates phospho-ERK1/2 changes. **** $p<0.001$, *** $p<0.005$, ** $p<0.01$ vs. AngII treated cells. **(E-F)** HEK293 cells transiently expressing AT_1R, EGFR-Rluc8 and Grb2-Venus were treated with 1μM AG1478, 1μM YM-254890, 1μM Candesartan, 10μM BAPTA and 5μM PP2 for 30 minutes prior to stimulation with 10μM AngII, 1μM EGF or vehicle. **(F)**

Quantification of ligand-induced BRET ratio (maximum-minimum) between EGFR-Rluc8 and Grb2-Venus following AngII-treatment and in the presence of inhibitors. Agonist stimulation is indicated by arrows. Data represents mean ± SEM of 3 independent experiments. Statistical analysis by a one-way ANOVA with a Dunnett's post-test for multiple comparisons; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$ vs. AngII treated cells.

4.3.2 The role of $G_{q/11}$ and β-arrestin in AT_1R-EGFR transactivation

Given, the disparity in the apparent contribution of $G_{q/11}$ to EGFR transactivation, we next sought to interrogate a single point mutant of the AT_1R, [Y215F]AT_1R, previously reported to retain high affinity for AngII, but an inability to couple to $G_{q/11}$ mediated signalling (Hunyady, Bor, Balla et al., 1995). [Y215F]AT_1R had equivalent AngII affinity compared to wild type (Figure 4.02C), however it possessed a significant decrease in AngII-stimulated Ca^{2+} production (Figure 4.02A), confirming that [Y215F]AT_1R is G protein-uncoupled. Comparably, [TK325]AT_1R, a truncated AT_1R lacking 34 carboxyl terminal amino acids and deficient in ligand-mediated phosphorylation and recruitment of β-arrestins (Qian, Pipolo & Thomas, 2001; Thomas, Motel, Kule et al., 1998) demonstrated a significant reduction in β-arrestin2 recruitment (Figure 4.02B) while still maintaining wild type AngII affinity (Figure 4.02C).

Stimulation of the [Y215F]AT_1R promoted Grb2 recruitment to the EGFR approximately equivalent to the wild type AT_1R (Figure 4.03B). Similarly, the truncated mutant of the AT_1R, [TK325]AT_1R, also showed similar levels of Grb2 recruitment as the wild type receptor. To extend our observations that AT_1R-EGFR transactivation may be independent of $G_{q/11}$ and β-arrestin, I also performed EGFR-Grb2 BRET assays in CRISPR-mediated $G_{q/11}$ and β-arrestin knockout HEK293 cell lines (Devost, Sleno, Pétrin et al., 2017). The BRET-based sensors were transfected with the wild type AT_1R into a HEK293 parental (PL) cell line, a $G_{q/11}$ knockout ($\Delta G_{q/11}$) cell line or a β-arrestin1/2 knockout (Δβ-arr1/2) cell line. Although comparable AngII-induced EGFR-Grb2 BRET ratios were observed in all three cell lines (Figure 4.03C), the use of the G protein-uncoupled mutant [Y215F] AT_1R and the $G_{q/11}$ knockout cell line show a sustained, yet sub-maximal, EGFR-Grb2 recruitment following AngII-stimulation, suggesting that $G_{q/11}$ coupling may influence EGFR downstream signalling transmission.

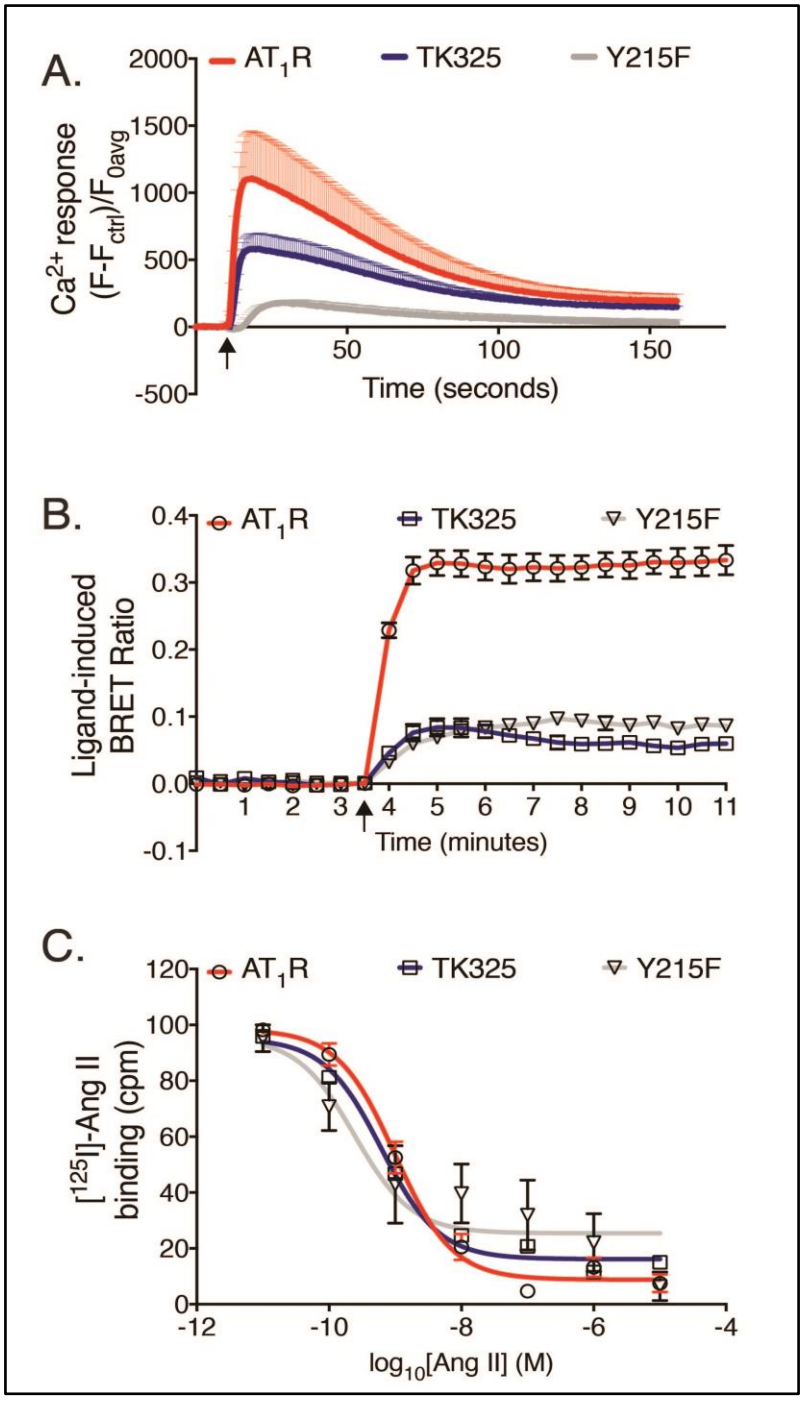


Figure 4.02 Validation of mutant AT_1Rs.

(A) HEK293 cells transiently expressed wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII and their receptor-dependent Ca^{2+} responses were measured. **(B)** HEK293 cells expressing β-arrestin2-Venus alongside Rluc8-tagged wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII and BRET ratios determined. Agonist stimulation is indicated by an arrow. **(C)** Wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R were transfected into a HEK-adherent-293 cells, with receptor binding assessed by $[^{125}I]$-AngII radioligand binding assay. Data expressed as a percentage of maximal $[^{125}I]$-AngII binding (i.e., in the absence of competing ligand). Curves were fitted to one-site binding equations. Data represent mean ± SEM of 3 independent experiments.

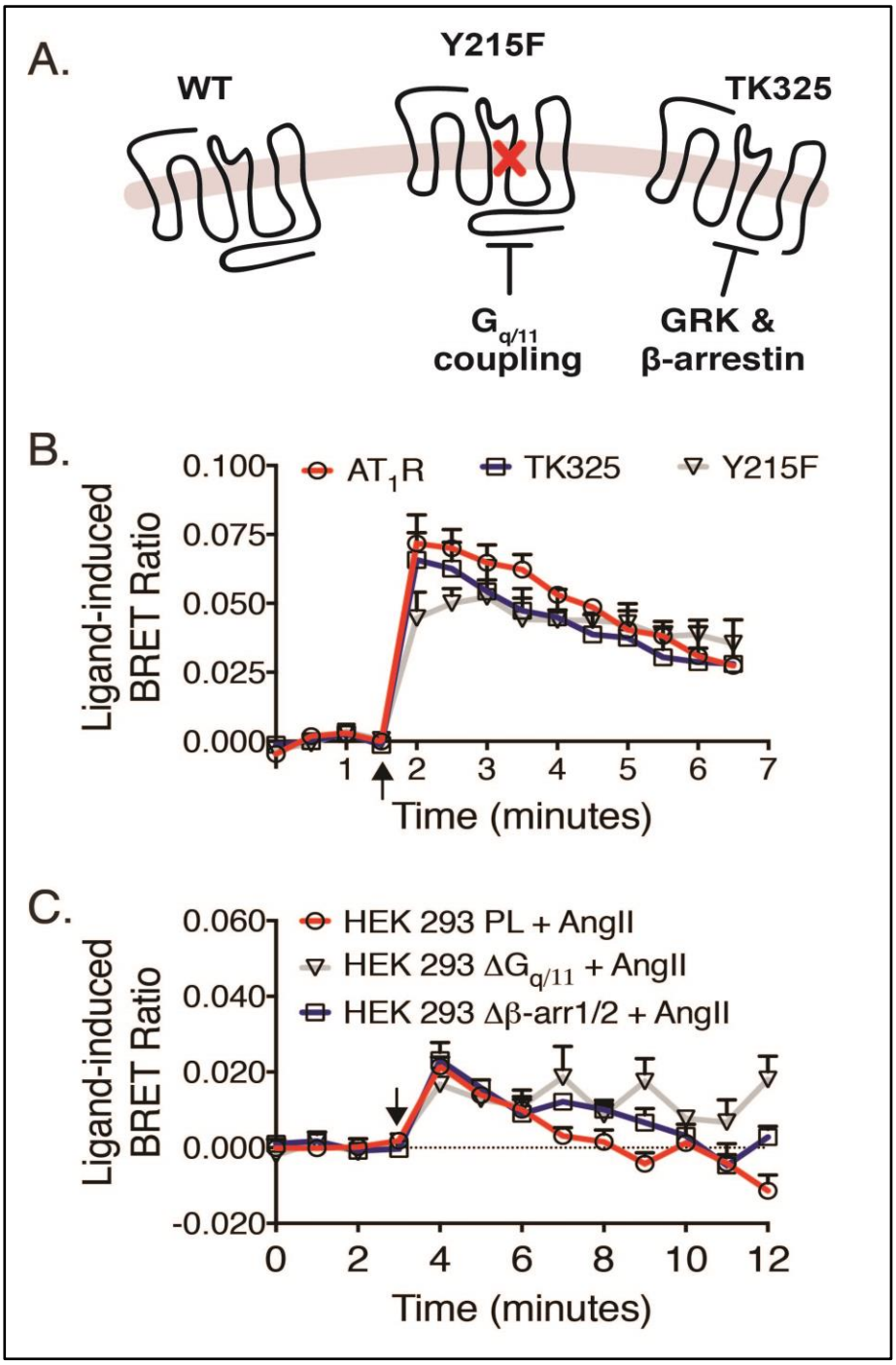


Figure 4.03 Grb2 recruitment to the EGFR is independent of $G_{q/11}$ and β-arrestin.
(A) Schematic of wild type AT_1R and mutant AT_1Rs - [TK325]AT_1R (truncated AT_1R deficient in recruiting β-arrestins) and [Y215F]AT_1R (inability to couple to $G_{q/11}$ signalling pathways). **(B)** HEK293 cells expressing EGFR-Rluc8 and Grb2-Venus, alongside wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII or vehicle. **(C)** EGFR-Rluc8, Grb2-Venus and AT_1R were transfected into the HEK293 parental cell line, as well as $G_{q/11}$- and β-arrestin1/2-knockout CRISPR cell lines. BRET measurements were taken using the Victor-X-light (Perkin Elmer). All cell lines were treated with 10μM AngII. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3 independent experiments.

Rescue experiments (i.e., transfection separately of either G_q, G_{11} or a dominant negative G_q back into the $\Delta G_{q/11}$ cells); or β-arrestin1/2 (transfected separately or together) into the Δβ-arr1/2 cell line were also performed (Figure 4.04A-F). When comparing ligand-induced BRET ratio (AUC), restoration of $G_{q/11}$ proteins inhibited AngII-mediated transactivation (Figure 4.04C), however had no significant effect on ligand-induced BRET ration (max-min) (Figure 4.04E) suggesting, if anything, that $G_{q/11}$ coupling increases the rate of EGFR signalling termination and desensitisation (Figure 4.04A, 4.04C, 4.04E). Reintroduction of β-arrestin1 or β-arrestin2 into the Δβ-arr HEK293 cells did not affect AngII-mediated EGFR transactivation (Figure 4.04B, 4.04D and 4.04F). Together, these data confirm the independence from $G_{q/11}$ and β-arrestin for AT_1R-EGFR transactivation.

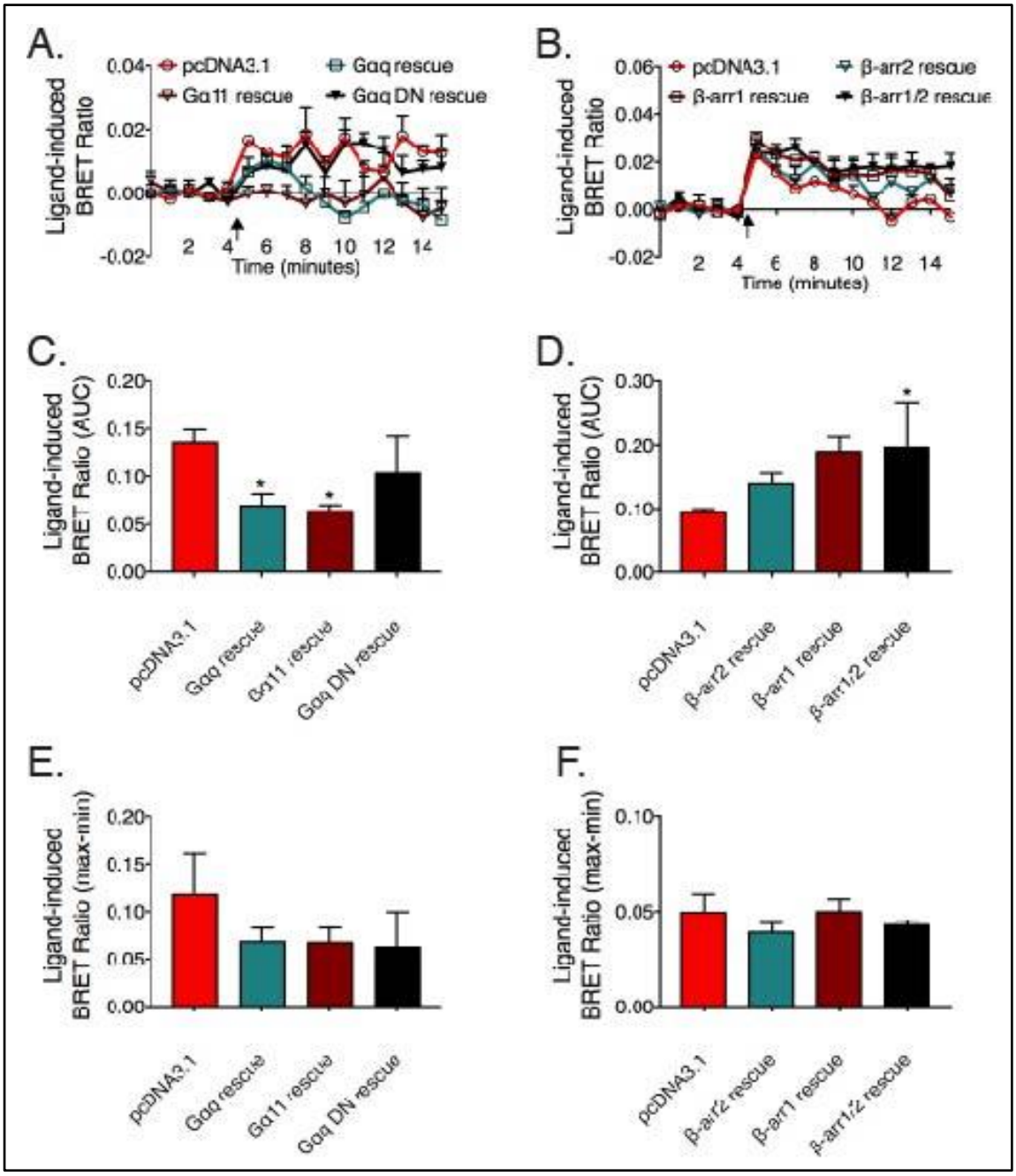


Figure 4.04: Effects of $G_{q/11}$ and β-arrestin1/2 deletion on Grb2 interaction with the EGFR.

EGFR-Rluc8, Grb2-Venus and AT_1R were transiently expressed in the $G_{q/11}$ knockout CRISPR cell line **(A-B)** or β-arrestin1/2 knockout CRISPR cell line **(C-D)**. **(A)** BRET constructs were expressed in the absence (pcDNA3.1) or presence of either G_{11}, G_q or G_q dominant-negative (DN) and were treated with 10μM AngII, **(B)** with the ligand-induced BRET ratio quantified using area under the curve (AUC). **(C)** BRET constructs were expressed in the absence (pcDNA3.1) or presence of either β-arrestin1, β-arrestin2 or β-arrestin1/2 and were treated with 10μM AngII, **(D)** with the ligand-induced BRET ratio quantified using area under the curve (AUC). BRET measurements were taken using the Victor-X-light (Perkin Elmer). Data represent mean ± SEM of 3 independent experiments. Statistical analysis by a one-way ANOVA with a Dunnett's post-test for multiple comparisons; * $p<0.05$

4.3.3 AT_1R and EGFR exist as complexes

We speculated as to whether the mechanism of GPCR-EGFR transactivation may involve heteromerisation of the receptors. In order to study this, we initially used a bimolecular fluorescence complementation (BiFC) assay, whereby complexing between AT_1R and EGFR would result in the complementation of yellow fluorescent protein (YFP) fragments attached separately to the AT_1R (AT_1R-Vn) and the EGFR (EGFR-Vc) (Figure 4.05A). AT_1R-Vn and EGFR-Vc were not fluorescent when expressed separately in cells (Figure 4.05B and 4.05C, respectively). Co-expression of AT_1R and EGFR lead to complemented fluorescence with clear membrane localisation (Figure 4.05D-F).

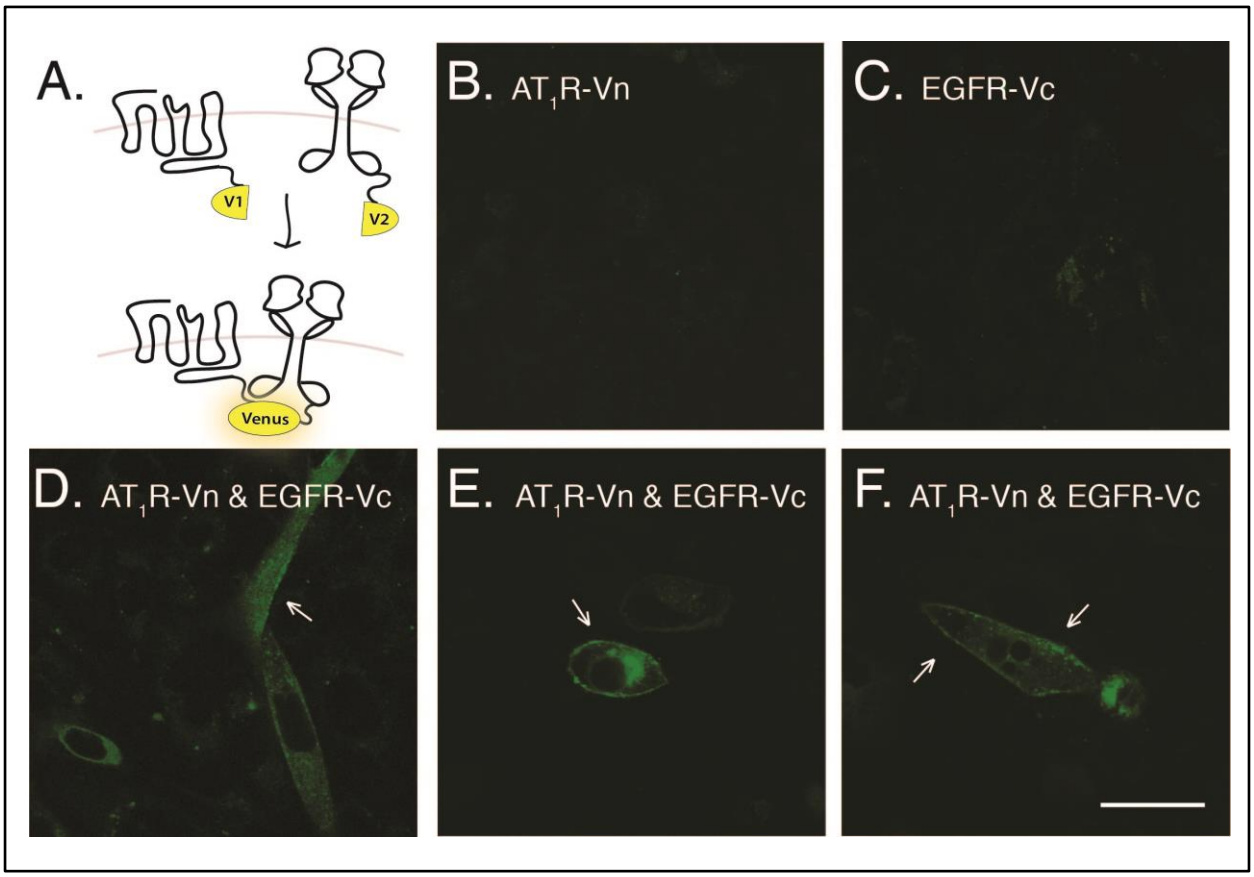


Figure 4.05: BiFC for studying AT_1R-EGFR heteromerisation.
(A) The Venus fluorophore is split into N-terminal (Vn) and C-terminal (Vc) fragments and genetically fused to AT_1R and EGFR, respectively. Therefore, if AT_1R and EGFR that bear C-terminal Vn and Vc tags form heteromers, fluorescence will be generated via the reconstitution of the Venus fluorophore. **(B, C)** CHO-K1 cells expressing AT_1R-Vn or EGFR-Vc constructs separately displayed very low levels of autofluorescence. **(D-F)** Co-expression of AT_1R-Vn and EGFR-Vc in CHO-K1 cells. Scale bar, 30μm.

To examine whether these complexes are ligand-dependent, we utilised Receptor-Heteromer Investigation Technology (Receptor-HIT). Receptor-HIT is a BRET technology that offers insights into specific ligand-dependent recruitment of proteins to heteromers (Johnstone & Pfleger, 2012; See, Seeber, Kocan et al., 2011). AngII-induced recruitment of β-arrestin2-Venus to unlabelled AT_1R, resulting in BRET with EGFR-Rluc8, is indicative of heteromerisation (Figure 4.06A). Upon AngII stimulation, an increase in BRET ratio between β-arrestin2-Venus and EGFR-Rluc8 was observed only when the AT_1R was co-expressed (Figure 4.06B). We also used a complementary variation of the Receptor-HIT assay, whereby AT_1R-Rluc8, unlabelled EGFR, and Grb2-Venus were co-expressed in living cells, as shown in Figure 4.06C. In contrast to the EGFR, the AT_1R does not interact with Grb2 in a ligand-dependent manner. However, if AT_1R and EGFR exist as a heteromer, AngII stimulation will transactivate the EGFR and initiate translocation of Grb2 to the activated EGFR, facilitating BRET between AT_1R-Rluc8 and Grb2-Venus**.** Even in the absence of exogenous EGFR transfection, some AngII-mediated BRET was observed, indicating the engagement of endogenous EGFR to recruit Grb2-Venus to AT_1R-Rluc8 (Figure 4.06D). This effect was enhanced by the ectopic expression of additional unlabelled EGFR (Figure 4.06D). As a final confirmation of ligand-dependent complexing between the AT_1R and the EGFR, we co-transfected cells with AT_1R-Rluc8 and EGFR-Venus, and stimulated with AngII or EGF. Both AngII and EGF appeared to modulate the proximity of the receptors, as shown in Figure 4.06E and 4.06F. Together, these experiments indicate that AT_1R and EGFR not only exist in preformed complexes, but that agonist stimulation can modulate their association and/or relative conformations.

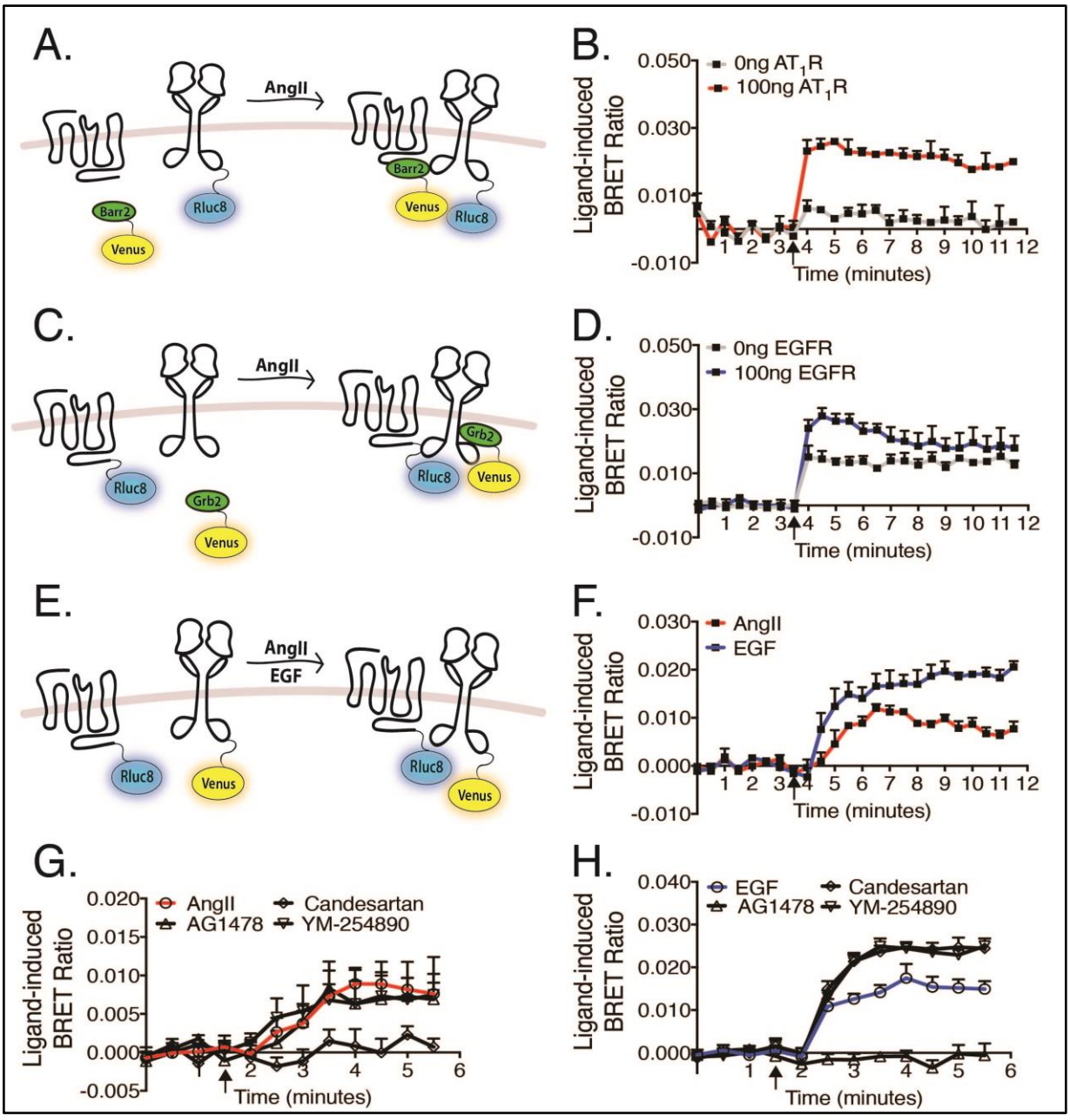


Fig. 4.06: Detection of ligand-driven AT_1R and EGFR receptor complexes using Receptor-HIT.

(A) If the AT_1R and EGFR exist as a heteromers, AngII-stimulation will activate β-arrestin2 translocation to the AT_1R, enabling BRET to occur between EGFR-Rluc8 and β-arrestin2-Venus. **(B)** HEK293 cells expressing β-arrestin2-Venus and EGFR-Rluc8 in the presence or absence of AT_1R were stimulated with 10μM AngII and the BRET ratio determined. **(C)** In contrast to EGFR, the AT_1R does not interact with Grb2 in a ligand-dependent manner. However, if the AT_1R and EGFR exist as a heteromers, AngII stimulation resulting in transactivation of the EGFR and initiation of Grb2 translocation to the activated EGFR should enable BRET to occur between AT_1R-Rluc8 and Grb2-Venus. **(D)** HEK293 cells expressing AT_1R-Rluc8 and Grb2-Venus in the presence or absence of transfected EGFR, was stimulated with 10μM AngII. **(E)** A direct readout of ligand-induced changes in AT_1R-Rluc8 proximity to EGFR-Venus. **(F)** Co-expression of AT_1R-Rluc8 and EGFR-Venus in HEK293 cells stimulated with 10μM of AngII or 1μM EGF. HEK293 cells expressing AT_1R-Rluc8 and EGFR-Venus were treated with 1μM YM-254890, 1μM Candesartan or 1μM

AG1478 for 30 minutes prior to stimulation with **(G)** 10μM AngII only or **(H)** 1μM EGF only. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3-4 independent experiments.

4.3.4 Pharmacological characterisation of AT_1R-EGFR complexes

Ligand-induced changes in proximity between AT_1R-Rluc8 and EGFR-Venus, following AngII- and EGF-stimulation, are shown in Figure 4.06G and 4.06H. AngII-mediated increases in BRET between AT_1R and EGFR were inhibited by candesartan, but not AG1478 or YM-254890, indicating that ligand-dependent modulation does not require $G_{q/11}$ coupling or EGFR kinase activity (Figure 4.06G). In contrast, EGF modulation of AT_1R-EGFR complexes was abrogated by pre-treatment with AG1478 (Figure 4.06H). Interestingly, pre-treatment with candesartan or YM-254890 further increased the EGF-mediated BRET between AT_1R-Rluc8 and EGFR-Venus (Figure 4.06H). These data provide evidence for distinct mechanisms controlling AngII- and EGF-induced modulation of the heteromer.

AT_1R-EGFR heteromers are unlikely to merely reflect agonist-induced clustering of these proteins in the membrane and/or their concentration and co-trafficking in endosomes, because the association of the EGFR with a membrane marker, K-ras, was decreased following EGF stimulation, but not AngII-treatment (Figure 4.07A)

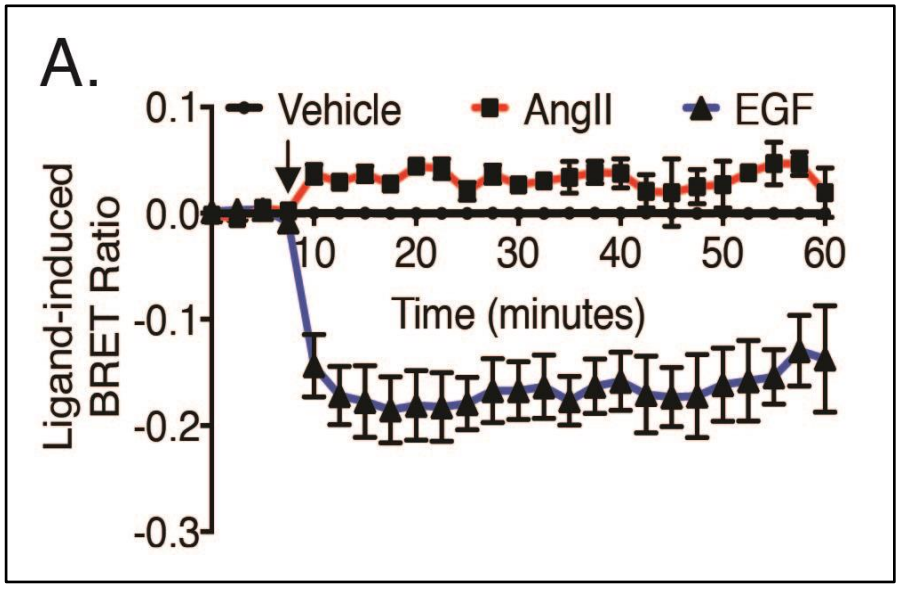


Fig 4.07: EGFR localisation and trafficking
(A) HEK293 cells expressing AT_1R, EGFR-Rluc8 and K-ras-Venus were treated with 10μM AngII, 1μM EGF and vehicle. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Agonist stimulation is indicated by arrows. Data represents mean ± SEM of 5 independent experiments.

4.4 Discussion:

GPCR-mediated EGFR transactivation is commonly defined as the activation of ERK1/2, which although informative, is an indirect and distal readout. Herein, I utilise a BRET assay, based on the recruitment of Grb2 to the EGFR to quantitatively monitor, in living cells and in real-time, the proximal activation of EGFR. Importantly, I observed that the molecular requirements for EGFR transactivation differed depending on the readout used. For the Grb2-EGFR assay, I showed that EGFR transactivation is independent of $G_{q/11}$ coupling and does not apparently involve the AT_1R carboxyl-terminal tail or interaction with arrestin. Using a series of additional BRET-based assays, I provide evidence for preformed complexes between the AT_1R and EGFR that show different attributes, depending on which receptor is activated. In summary, the capacity to interrogate proximal EGFR transactivation provides a platform to better define the complex molecular processes involved.

Another major observation of our study was the apparent disconnect between pharmacological inhibitors and their relative effect on the readout of transactivation vis-à-vis ERK1/2 versus BRET-based Grb2 recruitment assays. We clearly showed that candesartan completely inhibited AngII-induced ERK1/2 phosphorylation and recruitment of Grb2 to the EGFR, indicating that EGFR transactivation is due to ligand activation of the AT_1R in both systems. Interestingly, cells treated with AG1478 showed a complete inhibition of ERK1/2 activation in response to AngII, however only a partial reduction of AngII-mediated transactivation as measured by BRET-based Grb2 recruitment assay, indicating a possible disconnect between proximal recruitment of Grb2 and the eventual activation of ERK1/2. I would argue that Grb2 recruitment is necessary, but not in itself sufficient, to drive ERK1/2 activation following AT_1R activation. Importantly, EGF-mediated recruitment of Grb2 to the EGFR was completely inhibited with pre-treatment of AG1478, confirming the efficacy of AG1478.

Even more unambiguous was the relative contribution of $G_{q/11}$ signalling in AngII-mediated transactivation as measured by the ERK1/2 and the BRET-based readout. Although $G_{q/11}$ was absolutely required for ERK1/2 phosphorylation following AngII-stimulation, in contrast, we observed a sustained EGFR-Grb2 interaction when cells were

pre-treated with YM-254890. One potential confounding issue was that different concentrations of ligands were used to achieve maximal activation of ERK1/2 and BRET assays (refer to the legend of Figure 4.01 for detail) and this may have influenced the pharmacological inhibitory profile. However, we observed similar patterns of inhibition of the ERK1/2 readout when cells were stimulated with higher concentration (i.e., 10μM AngII and 1μM EGF, data not shown), indicating the differential contribution of $G_{q/11}$ is unlikely to be a mere artefact of ligand concentration. Previously, it was assumed that transactivation was a consequence of $G_{q/11}$ activation (Smith, Chan, Qian et al., 2011), but our data using the proximal assay suggests a re-evaluation of this paradigm might be required. We further adapted the BRET experiments to incorporate mutant versions of the AT_1R, specifically a G protein-uncoupled AT_1R, [Y215F]AT_1R, that does not couple efficiently to $G_{q/11}$ (Hunyady, Bor, Balla et al., 1995; Smith, Chan, Qian et al., 2011). Consistent with previous results, activation of [Y215F]AT_1R resulted in wild type-like Grb2 recruitment to the EGFR. Interestingly, others have reported β-arrestin-mediated ERK1/2 signalling, with an involvement in cardiomyocyte survival and hypertrophy (Aplin, Christensen, Schneider et al., 2007; Esposito, Perrino, Cannavo et al., 2011; Kim, Ahn, Rajagopal et al., 2009). Here, we used a carboxyl-terminal truncated AT_1R, [TK325]AT_1R, to demonstrate that AT_1R C-tail phosphorylation and β-arrestin interaction is not crucial for Grb2 recruitment. Furthermore, using CRISPR knockout HEK293 cell lines we were also able to show that transactivation (indicated by Grb2 recruitment to the EGFR) appears to be $G_{q/11}$- as well as β-arrestin1/2-independent. This disparity warrants further study and clearly emphasises the importance of interrogating EGFR transactivation directly at the most proximal point in the process. It is possible that other G proteins might be involved or that the cell background is critical, and this requires further study.

Based on the above observations, a final consideration for the current study was the possibility that GPCR-mediated EGFR transactivation involved receptor heteromerisation (Olivares-Reyes, Shah, Hernandez-Aranda et al., 2005). We initially used a BiFC assay to provide preliminary evidence for heteromers between the AT_1R and EGFR. Although positive, the limitations of this technique in defining ligand-driven heteromer formation meant that we then progressed to Receptor-HIT assays and a BRET-based assay measuring

direct association of AT_1R-EGFR. Using all three approaches, we provide evidence that AT_1R and EGFR can exist as preformed complexes that occur constitutively (i.e. in the absence of ligand) and are predominantly localised to the plasma membrane. In addition to preformed complexes, AT_1R-EGFR heteromers appear to be responsive to agonist stimulation and receptor activation. Ligand-induced modulation of the AT_1R-EGFR heteromer was validated using two complementary Receptor-HIT approaches, with both demonstrating that AngII leads to a rapid and robust increase in BRET that required both the AT_1R and EGFR. Lastly, we demonstrate a more 'direct' readout, tagging both receptors and monitoring ligand-induced changes in BRET due to modulation of donor-acceptor proximity. Together, these data strongly indicate that both AngII- and EGF-stimulation can induce the formation of multi-receptor complexes and/or alter the relative conformations of AT_1R and EGFR in pre-existing complexes. An alternative explanation (that these data merely reflect a bystander effect or the co-accumulation of the receptors in endosomes) is not supported by our observation (see Fig. 4.07) that the trafficking of the EGFR from the membrane occurs with EGF stimulation, but not with AngII.

The concept of GPCRs and EGFRs existing as heteromers is consistent with previous studies. Co-immunoprecipitation studies have shown that agonist stimulation (both AngII and EGF) promotes a multi-protein complex containing the AT_1R and EGFR, however these endpoint assays were not in live cells or in real-time (Olivares-Reyes, Shah, Hernandez-Aranda et al., 2005). The β_1- and β_2-adrenergic receptors associate with the EGFR (Maudsley, Pierce, Zamah et al., 2000; Tilley, Kim, Patel et al., 2009), and while isoprenaline enhances β_1-adrenergic receptor association with EGFR, this is disrupted by EGF-stimulation. Others have also used BRET to provide evidence for the bile acid receptor, TGR5, forming preassembled heterodimers with the EGFR in lipid rafts, however agonist stimulation showed no further enhancement in receptor association (Jensen, Godfrey, Niklas et al., 2013). Complementary to our studies, Bhattacharya and colleagues showed G protein-coupled receptor 54 interacts with EGFR at the plasma membrane in the absence of agonist. However, only stimulation with its endogenous ligand, kisspeptin, significantly increased their association, while EGF-treatment did not affect the receptor complex (Zajac, Law, Cvetkovic et al., 2011). Although it appears that many GPCRs can transactivate EGFRs, heteromerisation has only been indicated for a subset. It is still unknown how this interaction

affects signalling of either receptor, with the mechanism and/or binding domains that facilitate heteromer formation largely unknown.

Whether the complex subserves actual transactivation is difficult to reconcile because specific inhibitors that prevent complex formation are not available. Remarkably, we observed that the modulation of BRET signals associated with AT_1R-EGFR heteromerisation differed depending on whether the complex was modulated by activation of the AT_1R or the EGFR. The AngII-induced complex modulation is apparently independent of both $G_{q/11}$ and EGFR kinase activity. G protein-independent transactivation has been reported for β_1-adrenergic receptor and EGFR continued association (Tilley, Kim, Patel et al., 2009). In contrast, in our hands, EGF-mediated modulation of the AT_1R-EGFR complex was absolutely dependent on the EGFR kinase domain. Moreover, antagonism of the AT_1R or inhibition of $G_{q/11}$ did not inhibit complex modulation, but rather enhanced the EGF-mediated increase in BRET signal between AT_1R-Rluc8 and EGFR-Venus. Whether this enhancement plays any role in receptor activation is unclear, but it is interesting to note that traditional models of monomeric EGFR dimer formation as the active component have been superseded by models that suggest that up to 40% of EGFRs are present as inactive dimers that form higher order active receptor signalling complexes (Clayton, Orchard, Nice et al., 2008; Clayton, Walker, Orchard et al., 2005; Hofman, Bader, Voortman et al., 2010; Yu, Sharma, Takahashi et al., 2002). This negative modulation of EGFR may be mirrored by the AT_1R, whereby the AT_1R and its associated G protein negatively modulate monomeric/dimeric/oligomeric EGFR.

4.5 Summary

In summary, the combination of different BRET approaches emphasises the importance of monitoring EGFR activation proximally and in real-time. Using this approach, we propose a G protein-independent mechanism of EGFR transactivation, instead providing evidence of ligand-modulated AT_1R-EGFR complexes. The results of our studies need to be considered in terms of the broader literature regarding EGFR activation. The classical view that EGFR monomers form activated dimers following EGF ligand binding has been replaced by more sophisticated models suggesting preformed dimers and activated tetramers. In fact there is evidence that only a fraction of monomers and dimers recruit Grb2, with Grb2 associating

predominantly with EGFR tetramers (Kozer, Barua, Henderson et al., 2014). This begs the question whether GPCR-mediated transactivation, and its clear proclivity to activate a subset of the total EGFR, may reflect a selective and as yet unexplained transactivation of monomers vs. dimers vs. higher order structures.

Chapter Five

EGFR signalling involves contemporaneous activation of the type 1 Angiotensin receptor.

5.0 EGFR signalling involves contemporaneous activation of the type 1 Angiotensin receptor.

5.1 Introduction

RTKs and GPCRs are two major families of plasma membrane receptors, and drugs that target them are frontline therapies in cancer and cardiovascular disease (Li, Croce, Steensma et al., 2015; Wheeler-Jones, 2005). RTKs and GPCRs, and their signalling pathways, are closely integrated to control cellular physiology. One example is the capacity of GPCRs, including the AT_1R, to transactivate the EGFR, as a mechanism to usurp the EGFR growth promoting pathways. The capacity of GPCRs, like the AT_1R, to transactivate EGFR may explain the capacity of inhibitors of AngII production and/or actions at the AT_1R to mitigate to aberrant cell growth. It may also explain that some current cancer treatments e.g., Herceptin (an antibody that targets HER2), have apparently off target effects on tissues, such as the heart (Huszno, Leś, Sarzyczny-Słota et al., 2013; Keefe, 2002; Onitilo, Engel & Stankowski, 2014; Riccio, Coppola, Piscopo et al., 2016). Much of our current understanding of transactivation classically refers to the activation of RTK by GPCRs, however very little is known about the reciprocal process, whereby GPCR function is modulated by RTKs.

The EGFR (HER1/ErbB1) is a RTK that elicits potent mitogenic responses and plays a critical role in the pathogenesis of many cancers (Nicholson, Gee & Harper, 2001; Normanno, De Luca, Bianco et al., 2006; Yarden, 2001). As per the introduction, the four EGFR family members – HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) – possess an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic protein tyrosine kinase domain. Members of the EGF ligand family bind directly to the EGFR, leading to the homo- and/or hetero-dimerisation, with HER2 being the preferred dimer partner. What then follows is the auto- or trans-phosphorylation of several tyrosine residues and the activation of EGFR intrinsic tyrosine kinase activity. The activated EGFR is known to bind a number of secondary signalling proteins that subsequently leads to downstream signalling. The activated EGFR recruits and activates PLCγ, and leads to the cleavage of PIP2 generating IP3, which causes the release of Ca^{2+} from intracellular stores.

In addition to GPCRs transactivation of growth factor receptors, evidence exists that activation of growth factor receptors may also influence GPCR activity. The insulin receptor was the first RTK shown to engage G protein signalling (Luttrell, Kilgour, Larner et al., 1990; Luttrell, van Biesen, Hawes et al., 1995), with insulin-like growth factor 1 (IGF1)-mediated MAPK signalling shown to be sensitive to both pertussis toxin (G_i inhibitor) treatment or sequestration of G-protein βγ subunits (Luttrell, van Biesen, Hawes et al., 1995). Subsequently, other RTKs have also been shown to engage GPCR signalling molecules, including heterotrimeric G proteins (Poppleton, Sun, Fulgham et al., 1996; Shan, Chen, Wang et al., 2006) and β-arrestin (Lin, Daaka & Lefkowitz, 1998; Povsic, Kohout & Lefkowitz, 2003). Alternatively, others and I (see Chapter 4.0) have provided evidence that GPCR transactivation involves the formation of GPCR-RTK complexes, coinciding with the phosphorylation and activation of the transactivated GPCR (Akekawatchai, Holland, Kochetkova et al., 2005; Alderton, Rakhit, Kong et al., 2001; Delcourt, Thouvenot, Chanrion et al., 2007).

As part of my ongoing studies into AT_1R-EGFR transactivation, I observed (as controls for an experiment) that the EGF-mediated stimulation of the EGFR Ca^{2+} signalling was unexpectedly inhibited by the selective non-peptide AT_1R antagonist (candesartan) as well as a selective $G_{q/11}$ inhibitor (YM-254890). Based on this, we hypothesis that a proportion of EGF-stimulated activity may require the coincident activation of the AT_1R and its downstream signalling. Here I use a BRET-based arrestin trafficking to the AT_1R and conformation-sensitive AT_1R biosensors to confirm that EGFR stimulation is capable of activating the AT_1R, but in a mechanism distinct from the cognate AT_1R ligand, AngII.

5.2 Materials & Methods

Methods used throughout this chapter are detailed at length in Chapter 2.0, this includes cell culture (Section 2.4), isolation of VSMC (Section 2.4.4), nucleofector delivery of plasmid DNA (Section 2.4.5.2), ligand-induced BRET assays (Section 2.6.1) Conformational FlAsH Biosensor AT_1R assay (Section 2.6.4) and calcium mobilisation assay (Section 2.7.4).

5.2.1 Materials

HEK293 cells were obtained from American Type Culture Collection. HEK-adherent-293 cell line was a generous gift from Dr Dominic Ng, University of Queensland, Australia. Amaxa nucleofector kit (Lonza, Cat# V4XP-1024) was used to transfect isolated VSMC. AngII was obtained from Auspep or Sigma Aldrich and EGF from R&D Systems or Peprotech. Inhibitors were obtained from Wako Pure Chemical Industries (YM-254890), AstraZeneca (Candesartan), Merck Millipore (AG1478), Sigma Aldrich (PP2) and Calbiochem (BAPTA).

5.2.2 DNA Constructs

Preparation of the following cDNA constructs has been previously described: β-arrestin2-Venus (Kocan, See, Sampaio et al., 2009), AT_1R, [TK325]AT_1R and [Y215F]AT_1R (Smith, Chan, Qian et al., 2011), ICL2, ICL3 and C-tail intramolecular sensors (Devost, Sleno, Pétrin et al., 2017) . The following cDNA constructs were gifts from collaborators: The plasma membrane marker Venus/K-ras, as well as the subcellular compartment marker Rab GTPases (Rabs) Venus/Rab5a (early endosomes), Venus/Rab7a (late endosomes/lysosomes), and Venus/Rab11 (recycling endosomes), were kindly provided by Professor Nevin Lambert (Georgia Regents University). Rab1 (endoplasmic reticulum trafficking to the *cis*-Golgi), Rab4 (early endosome recycling), Rab6 (Golgi and *trans*-Golgi network), Rab8 (*trans*-Golgi network to the plasma membrane), and Rab9 (late endosome trafficking to the *trans*-Golgi network) (Sherwood & Roy, 2013; Wandinger-Ness & Zerial, 2014) were synthesised and subcloned by GeneArt into a pcDNA3 expression vector containing Venus without a stop codon and with a 20 residue linker of GGGAGGGAGGGAGGGAGGGA, to generate the

resulting fusion proteins with the Venus tag on the N terminus of the Rab protein. The Rab proteins have been described previously (Tiulpakov, White, Abhayawardana et al., 2016).

5.3 Results

5.3.1 EGFR activates pathways downstream of the AT_1R

Activated EGFR phosphorylates PLC-γ1 (Sekiya, Poulin, Kim et al., 2004), which translocates to the plasma membrane, allowing the hydrolysis of PIP2 to IP3, and the release of intracellular Ca^{2+} (Falasca, Logan, Lehto et al., 1998; Nishibe, Wahl, Hernandez-Sotomayor et al., 1990). As shown in Figure 5.01, EGF-stimulation leads to a rapid and robust increase in intracellular Ca^{2+} as determined by a GCaMP biosensor (a genetically encoded Ca^{2+} sensor). This Ca^{2+} transient is completely inhibited by the EGFR kinase antagonist AG1478 (Figure 5.01A). Pre-treatment of cells with candesartan or YM-254890, lead to an approximate 50% decrease in maximal EGF-stimulated Ca^{2+} (Figure 5.01B and 5.01C). This data indicates that, in part, EGFR signalling relies on AT_1R activation and its capacity to couple to $G_{q/11}$.

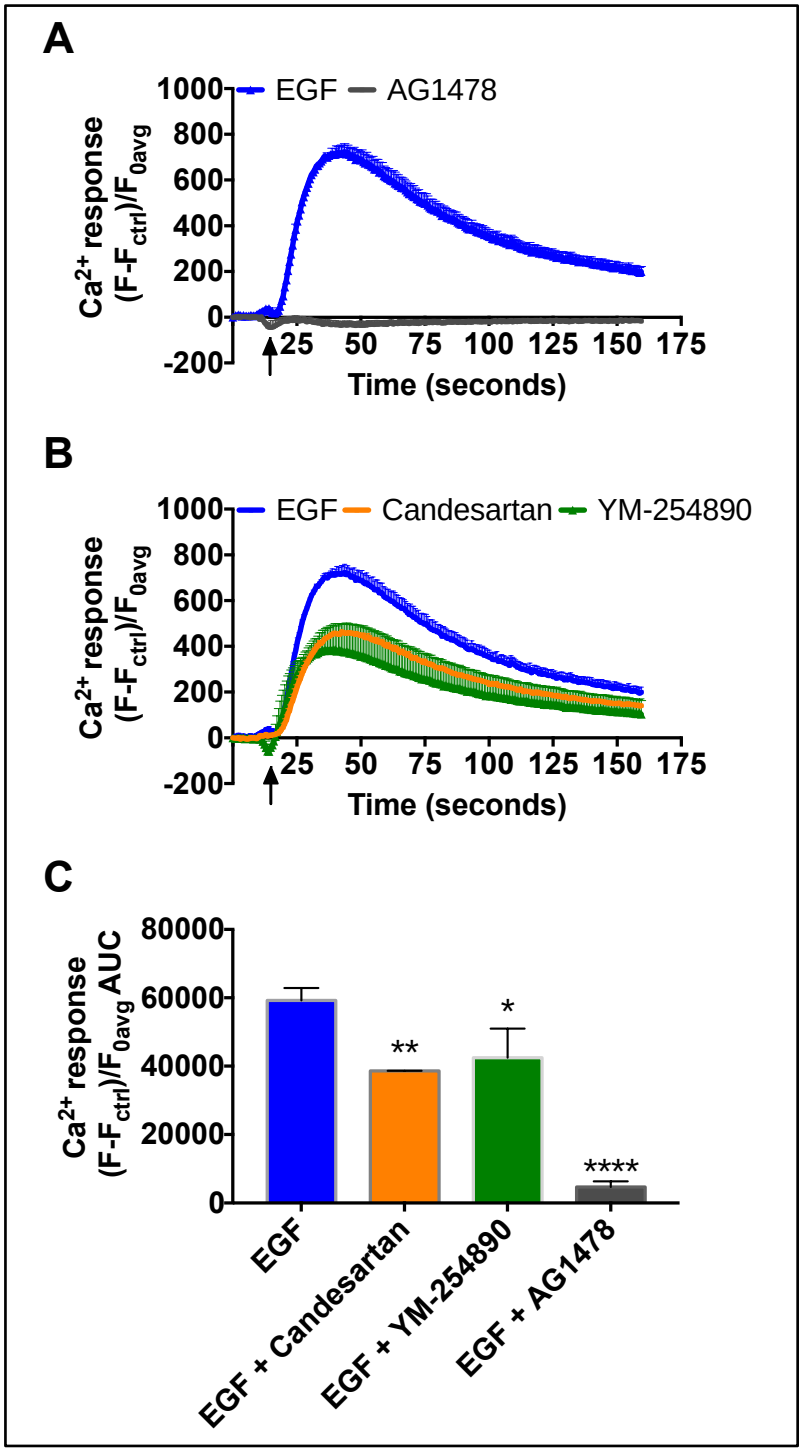


Figure 5.01: The effects of AT_1R and $G_{q/11}$ inhibition on EGFR-mediated Ca^{2+} response. **(A-C)** HEK293 cells stably expressing AT_1R were co-transfected with EGFR and GCaMP and treated with 1μM EGF or vehicle. EGFR-dependent Ca^{2+} response (corrected for vehicle response) were treated with **(A)** 500nM AG1478 (EGFR inhibitor), **(B)** 1μM YM-254890 ($G_{q/11}$ inhibitor) or 1μM Candesartan (AT_1R inverse agonist) for 30 minutes prior to stimulation. **(C)** Ligand-induced Ca^{2+} responses were quantified using area under the curve (AUC). Data represent mean ± SEM of 3 independent experiments. Statistical analysis by a one-way ANOVA with a Dunnett's post-test for multiple comparisons; ** $p<0.01$, * $p<0.05$, vs. EGF treated cells.

5.3.2 EGFR activation initiates β-arrestin recruitment to the AT_1R

Having observed that EGF-stimulation can potentially couple to $AT_1R/G_{q/11}$ activation, we next sought to demonstrate this activation directly using a BRET-based arrestin recruitment assay. This assay utilises an Rluc8-tagged AT_1R and a Venus-tagged β-arrestin2, whereby stimulation of AT_1R results in the recruitment of arrestin to the activated, phosphorylated AT_1R (Pfleger, Dromey, Dalrymple et al., 2006) (Figure 5.02A). In HEK293 cells, AngII-stimulation produced a robust and sustained ligand-dependent BRET signal, indicative of β-arrestin2 translocation and binding to the AT_1R (Figure 5.02B). Consistent with our hypothesis that EGF-stimulation leads to the activation of the AT_1R, we observed an EGF-mediated recruitment of β-arrestin2 to the AT_1R. This EGF-mediated activation of the AT_1R was slower and lower in magnitude compare to AngII (Figure 5.02B). The capacity of EGF to activate the AT_1R was also observed in VSMC (Figure 5.02C).

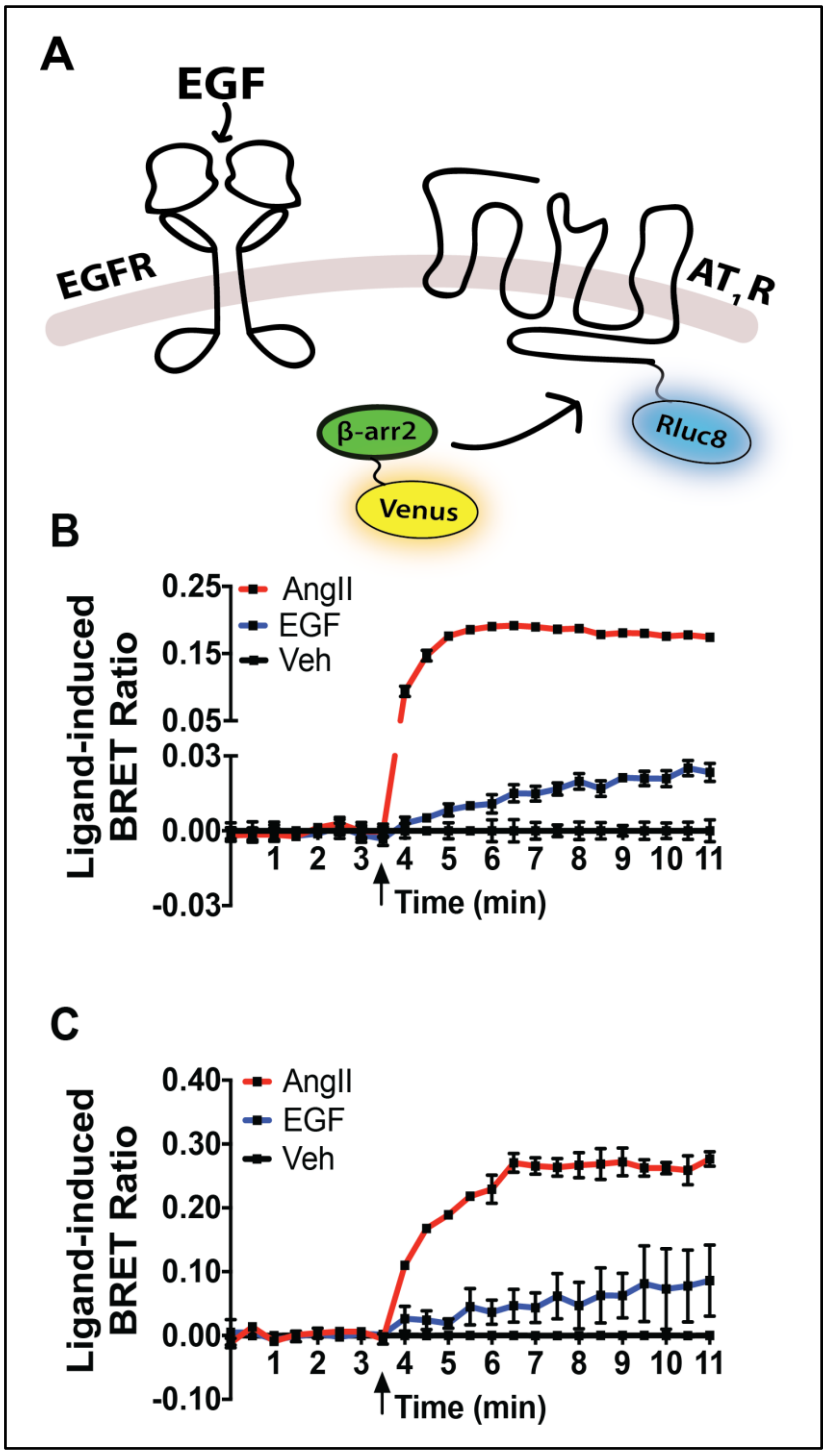


Figure 5.02: The development of a BRET-based assay to monitor EGFR-mediated AT_1R 'reverse transactivation'

(A) Schematic of EGFR-AT_1R BRET-based transactivation. AT_1R fused to a BRET donor (Rluc8), is co-transfected with β-arrestin2 adaptor protein tagged with a BRET acceptor (Venus) and the EGFR. Stimulation of the EGFR promotes activation of the AT_1R and recruitment of β-arrestin2. **(B)** HEK293 cells and **(C)** primary VSMC expressing EGFR, AT_1R-Rluc8, and β-arrestin2-Venus were treated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrow. Data represent mean ± SEM of 3 independent experiments.

5.3.3 Mechanisms underlying reverse transactivation

I next used pharmacological inhibitors to examine the molecular mechanisms underlying EGFR-stimulated AT_1R activation. EGF-mediated recruitment of arrestin to the AT_1R, was completely inhibited by the EGFR kinase inhibitor, AG1478. This activation of the AT_1R was also completely inhibited by pre-treatment with candesartan and YM-254890, indicating that arrestin recruitment was dependent on the active state of the receptor and $G_{q/11}$ (Figure 5.03A). We next adapted the BRET experiments to incorporate mutant versions of the AT_1R. Similarly, a truncated mutant of the AT_1R, [TK325]AT_1R, lacking 34 carboxyl terminal amino acids and deficient in ligand-mediated phosphorylation and recruitment of β-arrestins, was unable to recruit β-arrestin following EGF stimulation (Figure 5.04B). In contrast to previous results using YM-254890, a mutated AT_1R with a single point mutation, [Y215F]AT_1R, which can no longer couple to $G_{q/11}$, showed wild type levels β-arrestin2 recruitment (Figure 5.04B) in response to EGF-stimulation.

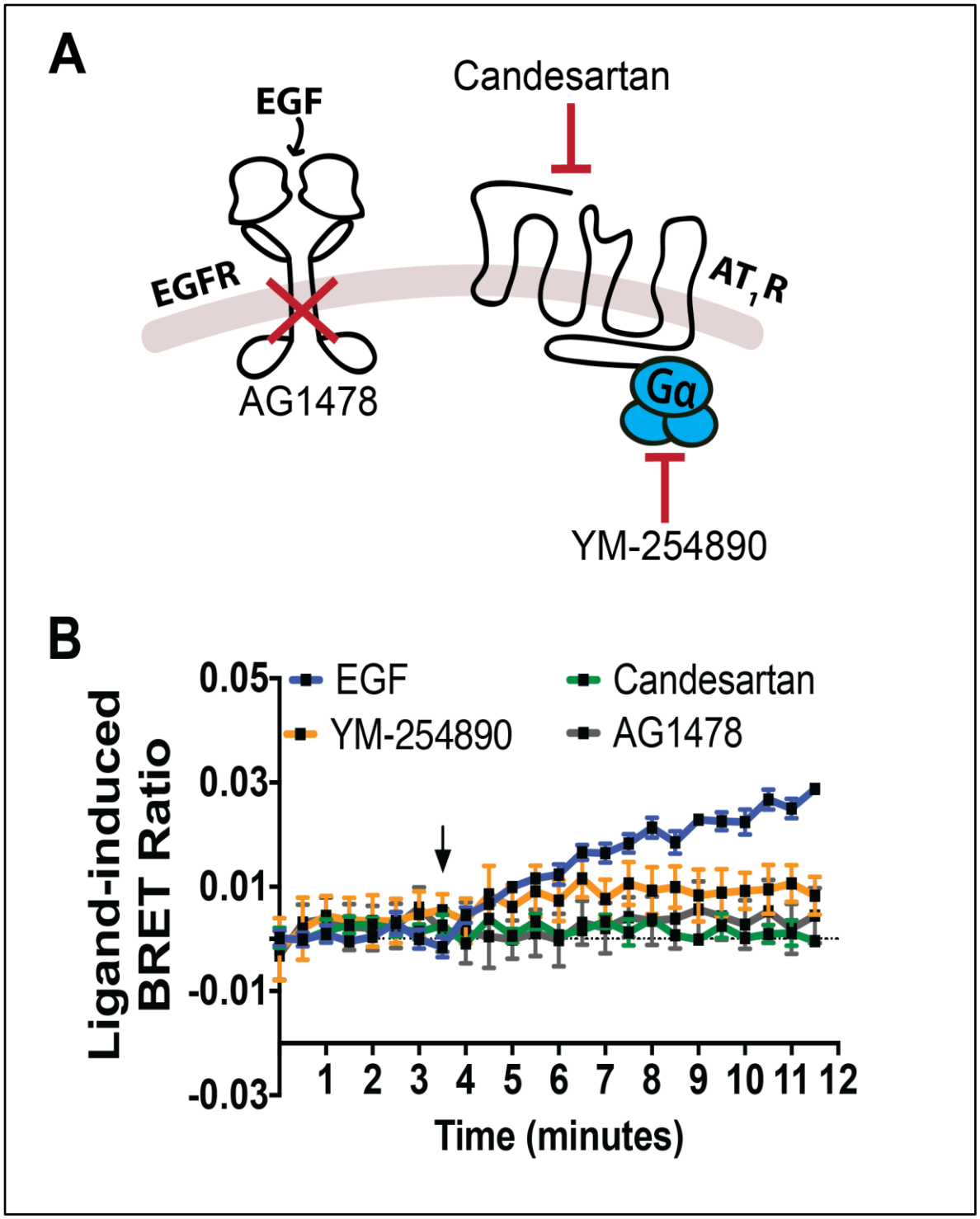


Figure 5.03: The use of pharmacological inhibitors in EGF-mediated β-arrestin2 recruitment to the AT_1R.
(A) Schematic of various pharmacological inhibitors and their bio-chemical targets. AG1478 inhibits the EGFR tyrosine kinase domain, candesartan is an inverse agonist of the AT_1R and YM-254890 blocks $G_{q/11}$ signalling. **(B)** HEK293 cells expressing EGFR, AT_1R-Rluc8, and β-arrestin2-Venus were treated with 500nM AG1478, 1μM YM-254890 and 1μM Candesartan for 30 minutes prior to stimulation with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3 independent experiments.

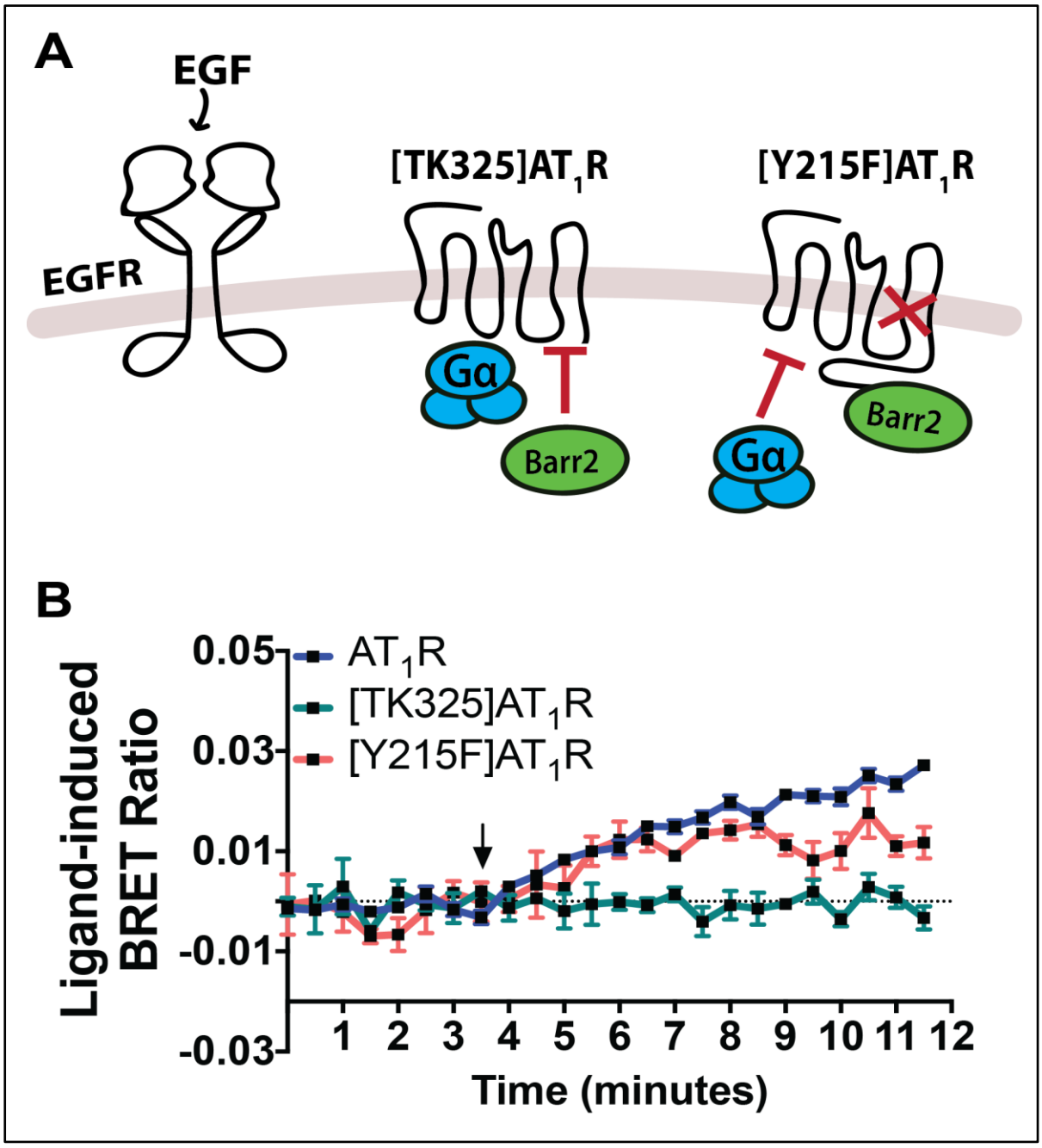


Figure 5.04: Dependence on β-arrestin and $G_{q/11}$ in EGF-mediated β-arrestin2 recruitment to the AT_1R.
(A) Schematic of wild type AT_1R and mutant AT_1R s - [TK325]AT_1R (truncated AT_1R deficient in recruiting β-arrestins) and [Y215F]AT_1R (inability to couple to $G_{q/11}$ signalling pathways). **(B)** HEK293 cells expressing AT_1R-Rluc8 and β-arrestin2-Venus, alongside wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3 independent experiments.

5.3.4 AT_1R Biosensors to monitor receptor conformational changes

To determine whether EGF-stimulation can mediate AT_1R conformational changes similar to AngII, we used AT_1R-based biosensors (Figure 5.05). The AT_1R biosensors report on ligand-induced conformational changes and have been reported to discriminate between biased and canonical ligands (Devost, Sleno, Pétrin et al., 2017). The biosensor harnesses BRET technology with a small fluorescent FlAsH molecule inserted at different positions (intracellular loop 2, ICL2; intracellular loop 3, ICL3; and the intracellular C-tail loop, C-tail) throughout the AT_1R and a donor Rluc8 located at the distal end of the carboxyl-tail (Figure 5.05A) (Devost, Sleno, Pétrin et al., 2017).

As shown in Figure 5.05B, there was a minimal response of the ICL2 AT_1R biosensor to AngII, as reported previously (Devost, Sleno, Pétrin et al., 2017), and to EGF . Relative to vehicle control, AngII-stimulation of the ICL3 AT_1R biosensor lead to a robust decrease in BRET ratio, consistent with an agonist response as previously described (Figure 5.05C). Interestingly, stimulation with EGF caused a decrease ICL3 BRET response relative to vehicle, which was significantly less than that promoted by AngII. Finally, using the C-tail AT_1R biosensor, AngII (but not EGF) led to a significant decrease in the BRET ratio (Figure 5.05D). Overall, these data support the idea that both AngII and EGF can both promote conformational changes, but these appear to be distinct.

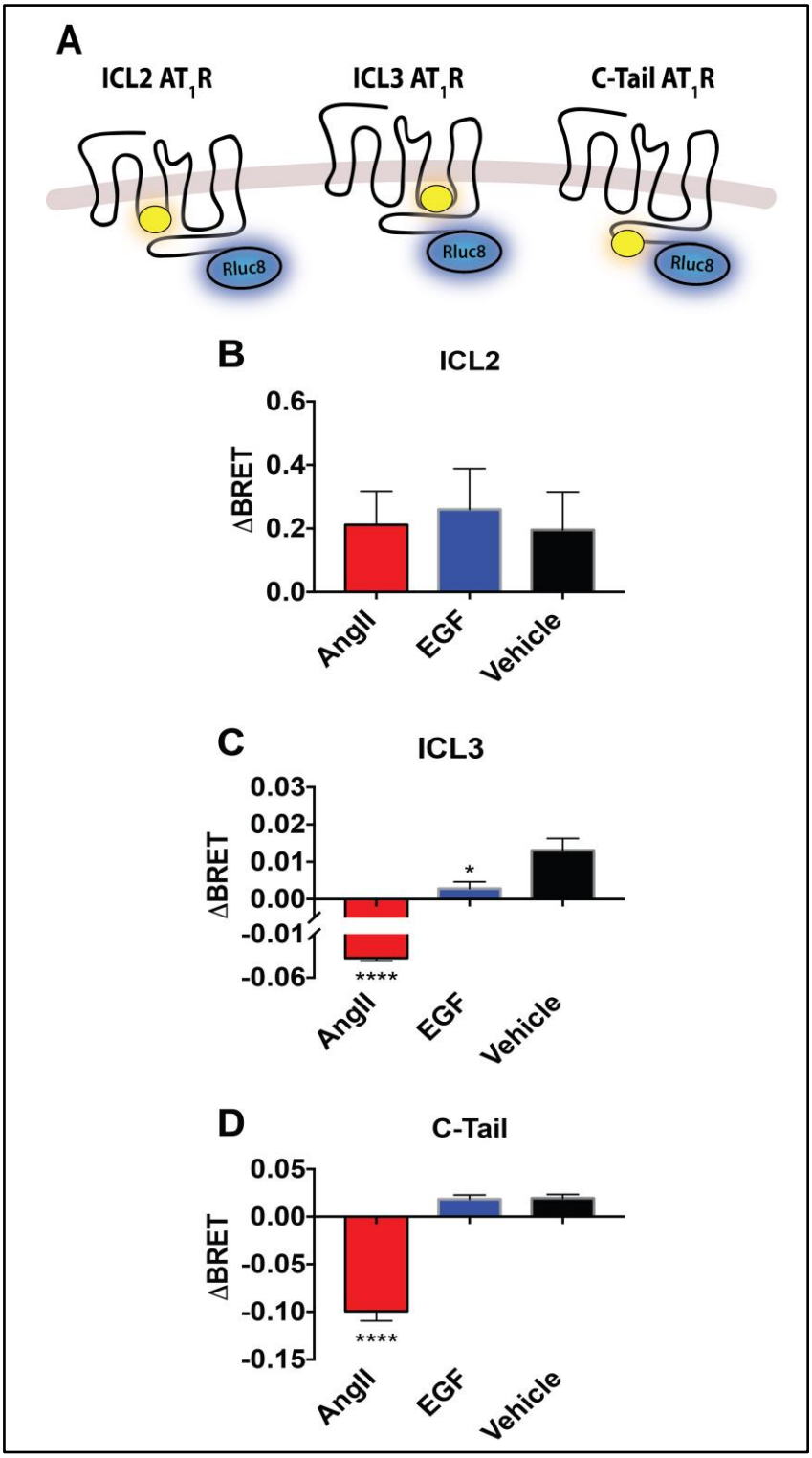


Figure 5.05: AT_1R FlAsH Biosensors to monitor AngII- and EGF-mediated receptor conformational changes.

(A) The small FlAsH sequence was incorporated to the 2nd and 3rd intracellular loop as well as the carboxyl terminal domain of the AT_1R. **(B)** ICL2 **(C)** ICL3 and **(D)** C-tail were transiently transfected into HEK293 cells and treated with 10μM AngII, 1μM EGF or vehicle. FlAsH-labelled and agonist-induced BRET was calculated as described in Chapter 2.6.4 Conformational Biosensor Assay. Data represent mean ± SEM of 4-5 independent experiments.

5.3.5 AT_1R trafficking and distribution

As a measure of AT_1R activation, we next examined the localisation of the AT_1R relative to a panel of cellular markers (K-ras for the plasma membrane; Rab4 for early endosome) using a BRET-based assays previously described (Figure 5.06) (Tiulpakov, White, Abhayawardana et al., 2016; White, Vanyai, See et al., 2017). Stimulation with AngII lead to a rapid internalisation of the receptor from the plasma membrane (Figure 5.06B) to intracellular vesicles (Figure 5.06C). In contrast, EGF-stimulation resulted in a slight increase in BRET signal with the plasma membrane marker K-ras, indicating that EGF activation of the AT_1R leads to retention/accumulation of the receptor at the plasma membrane (Figure 5.06B). In contrast to AngII treatment, following EGF-stimulation the AT_1R did not traffick to other subcellular markers (Figure 5.07).

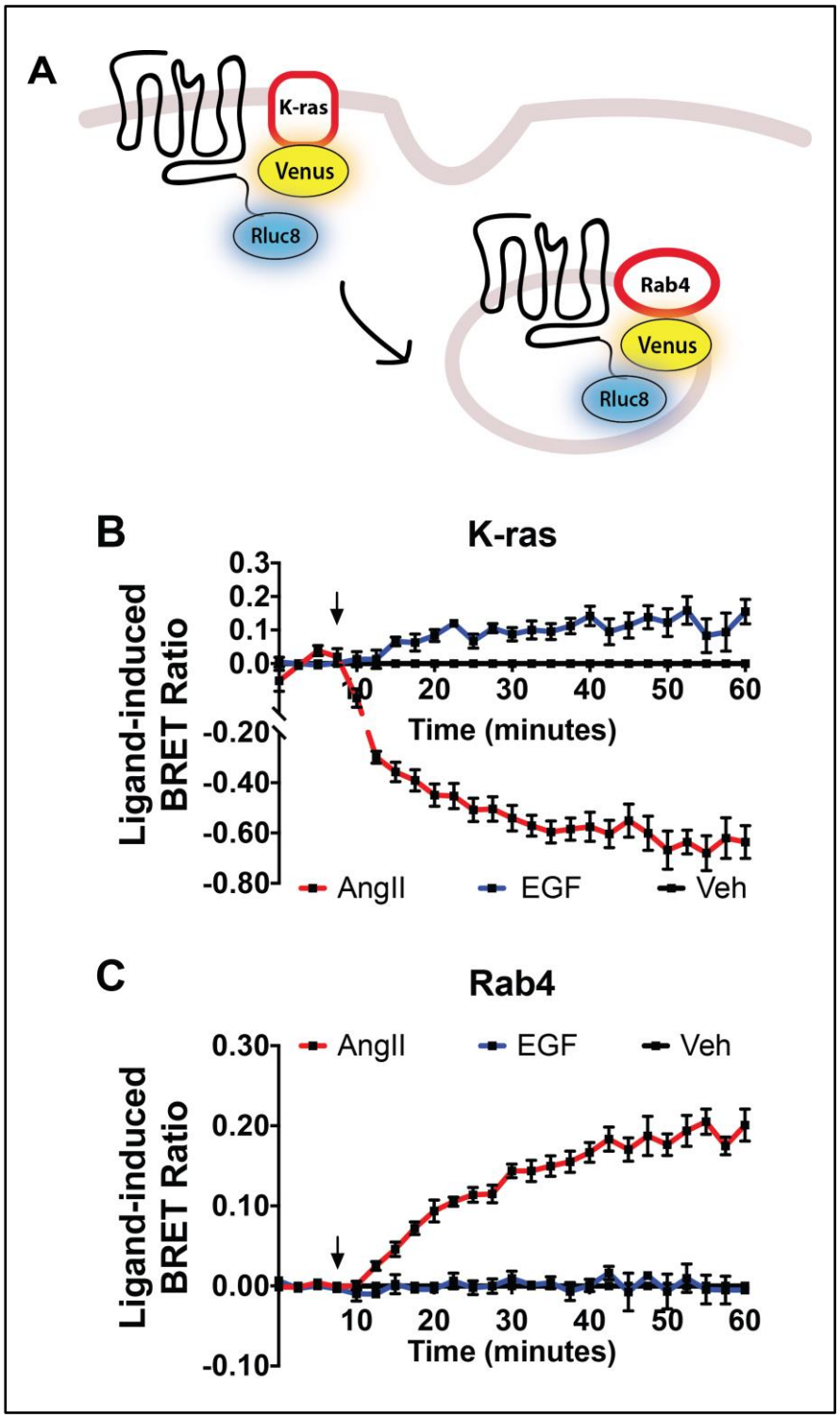


Figure 5.06: The use of membrane and subcellular markers to monitor AT_1R localisation and trafficking

(A) A simplified schematic representation of the plasma membrane marker, K-ras, and the early endosome marker, Rab4. Ligand-induced trafficking was monitored using Rluc8-tagged AT_1R by measuring proximity with Venus-tagged K-ras or Rab4. **(B)** HEK293 cells were transfected with AT_1R-Ruc8 and K-ras-Venus or **(C)** Rab4-Venus and treated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 3 independent experiments.

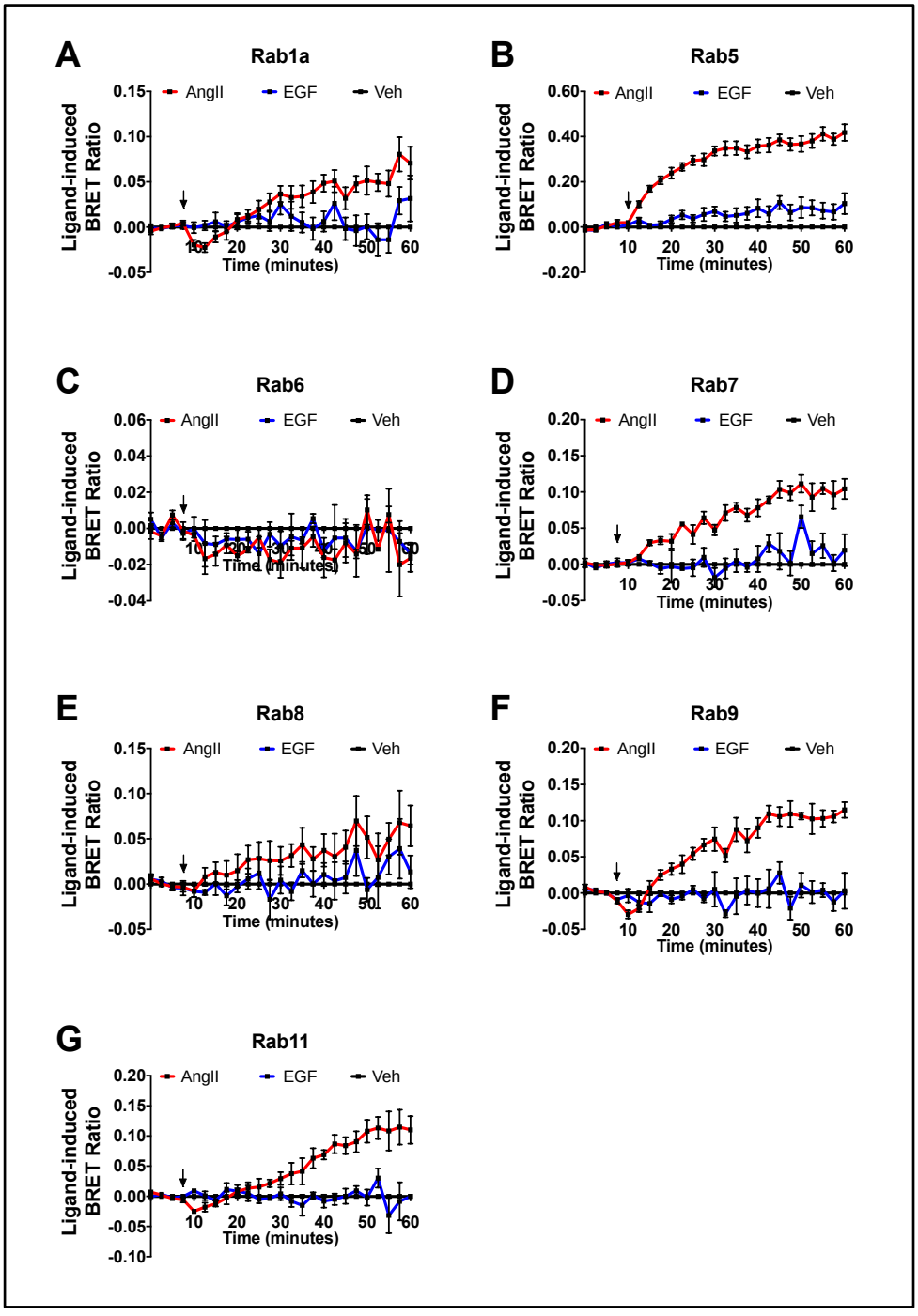


Figure 5.07: The use of subcellular markers to monitor AT_1R localisation and trafficking

HEK293 cells were transfected with AT_1R-Rluc8 and one of the subcellular markers **(A)** Rab1a, endoplasmic reticulum trafficking to the *cis*-Golgi **(B)** Rab5, early endosome, **(C)** Rab6, Golgi and trans-Golgi network, **(D)** Rab7, late endosome/lysosome, **(E)** Rab8, trans-Golgi network to the plasma membrane, **(F)** Rab9, late endosome trafficking to the trans-Golgi network, **(G)** Rab11, recycling endosomes, and treated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 5-6 independent experiments.

5.4 Discussion

The work described in this chapter work came from a serendipitous observation that AT_1R and $G_{q/11}$ inhibitors reduced an EGF-induced PLC-γ mediated Ca^{2+} signal. Our results indicate that EGF-stimulation can activate the AT_1R as measured by β-arrestin2 recruitment to the activated AT_1R. The capacity of EGF to recruit arrestin to the AT_1R is apparently dependent on the EGFR kinase domain as well as AT_1R active, $G_{q/11}$ coupled state, and AT_1R C-tail phosphorylation. Using AT_1R-based biosensors, we provide additional, direct evidence that EGF-activation promotes an AT_1R conformational change that may be distinct from the AngII-bound AT_1R. Functionally this may be important, because it predicts that treatment with non-peptide AT_1R antagonists (such as candesartan) may prevent a significant proportion of EGF-mediated signalling. This may have fundamental implications in the understanding of GPCR and RTK signalling.

To confirm AT_1R reverse transactivation, we used a BRET-based assay that quantitatively measures β-arrestin2 recruitment to the AT_1R. As an internal control, arrestin recruitment was readily demonstrable upon AT_1R activation, indicative of arrestin binding to the activated AT_1R and its subsequent desensitisation and internalisation. We initially tested whether EGFR stimulation could also promote β-arrestin2 translocation and interaction with AT_1R. Although EGF-mediated arrestin translocation was also apparent, it was consistently lower in magnitude and delayed, suggestive that arrestin binds at a much slower rate. Furthermore, we show that AG1478 completely inhibited EGF-induced arrestin, indicating that the EGFR kinase domain is absolutely required for EGF-induced arrestin recruitment to the AT_1R. Interestingly, cells pre-treated with YM-254890 and candesartan completely attenuated arrestin recruitment, indicating that EGF-mediated transactivation of the AT_1R is dependent upon the co-activation of the AT_1R and its capacity to couple to $G_{q/11}$.

To extend our observations that AT_1R transactivation is dependent on receptor phosphorylation, we further adapted the arrestin-BRET-based assay to include mutant versions of the AT_1R. A carboxyl-terminal truncated AT_1R that lacks receptor phosphorylation sites, [TK325]AT_1R, was incapable of recruiting β-arrestin2 following EGF-treatment. This data confirms that EGF-induced β-arrestin2 binding does not reflect

bystander (non-specific) BRET, as AT_1R-β-arrestin2 association was attenuated when the truncated form of the AT_1R was used. Furthermore, the use of a single point AT_1R mutant, [Y215F]AT_1R, that can no longer couple to $G_{q/11}$ mediated pathways, revealed β-arrestin2 recruitment approximately equivalent to the wild type AT_1R. This suggests that the EGFR can still activate [Y215F]AT_1R, as measured by arrestin recruitment.

At the time of making this observation, I became aware of a study (Tóth, Prokop, Gyombolai et al., 2018) into AT_1R heterologous phosphorylation and arrestin recruitment, where a similar 'reverse transactivation' was observed. Although they didn't directly focus on this observation, they did show the ability of EGF to activate arrestin trafficking to the AT_1R. They provide evidence that activation of PKC (whether it is by the EGFR or other GPCRs) recruits β-arrestin2 to the AT_1R even in the absence of AT_1R ligand. Contrary to our data, they suggest a mechanism whereby β-arrestin2 recruitment does not require the activate state of the AT_1R. Given that the significant proportion of the EGF signalling is blocked by the inverse agonist candesartan, we would argue the possibility exists that the EGFR can directly activate the AT_1R and its downstream $G_{q/11}$-mediated events.

Although bidirectional cross-communication between RTKs and GPCRs have been previously reported, the evaluation of reverse transactivation is not an exhaustively studied phenomenon. Various studies have suggested that RTK-induced GPCR transactivation is mediated exclusively through intracellular signalling, while other have proposed that growth factor receptors trigger the extracellular release of GPCR ligands. Both transcriptional upregulation and enzymatic activation can contribute to the synthesis and secretion of GPCRs cognate ligand. It is important to note that transcriptional dependent mechanism has only been noted for the chemokine receptor, CCR5, whereby IGF1 stimulation upregulates transcription and synthesis of RANTES, CCR5 natural ligand (Mira, Lacalle, González et al., 2001). In the case of platelet-derived growth factor (PDGF) and IGF1, RTK activation mediates the production of a GPCR ligand that binds to and activates the GPCR in an autocrine and/or paracrine manner, however this has only been exhibited for selective GPCRs (El-Shewy, Johnson, Lee et al., 2006; Hobson, Rosenfeldt, Barak et al., 2001).

Alternatively, others have suggested that GPCR transactivation involves the formation of GPCR-RTK complexes and the phosphorylation of transactivated GPCRs (Akekawatchai, Holland, Kochetkova et al., 2005). This complex formation provides a platform by which RTK and GPCRs signals can integrate and activate downstream MAPK signalling pathways more efficiently by way of a mechanism independent of ligand production. It has been proposed that the formation of GPCR-RTK signalling platforms not only facilitate the cross-communication between both receptor systems, but RTK form constitutive complexes with the GPCR partner. Interestingly, association of Sphingosine-1-phosphate receptor (SIP1) with PDGFR facilitates tyrosine phosphorylation of $G\alpha_i$ by PDGFR kinase activity, a process that prevents $G\alpha$ re-association with $G\beta\gamma$ subunits, prolonging the duration of G protein activation. Scaffolding proteins are thought to be a major driver of GPCR-RTK complexes (Ango, Prézeau, Muller et al., 2001), however as already mentioned in Chapter 4.0, how these functional GPCR-RTK complexes occur is still an open question.

One major question that remains unresolved is whether EGF-treatment promotes distinct patterns of AT_1R conformational changes compared to the AT_1R canonical ligand, AngII. We interrogated the conformational changes of the receptor using a conformational biosensor as reported previously (Devost, Sleno, Pétrin et al., 2017). While we were able to reproduce the AngII-mediated conformational changes in these biosensors, similar conformational changes were not observed in response to EGF. There are two possible explanations. Firstly, EGF-stimulated AT_1R activation is modest, and therefore, the conformational changes may reflect this. Indeed, the changes relative to vehicle ICL3 are of the same direction of AngII, but weaker. Similar logic cannot be applied to the C-tail sensor, which shows robust changes to AngII, but not EGF. Secondly, an alternative possibility is that EGF-stimulation is putting the AT_1R in a different conformation and this conformation can provide some signalling and lead to phosphorylation of the receptor, arrestin trafficking, but importantly does not sustain/initiate internalisation of the AT_1R. On balance, these data can be interpreted to suggest that there is a different conformational state mediated by EGF-stimulation relative to AngII-stimulation.

Although there is a clear link between EGFR and cancer, there remains significant controversy as to the potential role of the RAS (AngII/AT_1R) in cancer (George, Thomas &

Hannan, 2010; Herr, Rodewald, Fraser et al., 2008; Miyajima, Kosaka, Kikuchi et al., 2015; Xu, Fan, Wu et al., 2017). Some may argue that the capacity of the AT_1R to transactivate the EGFR may play some role in such a possibility (Greco, Muscella, Elia et al., 2003). Many studies have been able to show AngII-mediated growth and proliferation in variety of models; the epidemiological data of large cohort population studies of people treated with ACE inhibitors and ARB are ambiguous (Chae, Valsecchi, Kim et al., 2011; Christian, Lapane, Hume et al., 2008; Fryzek, Poulsen, Lipworth et al., 2006; Li, Malone, Weiss et al., 2003; Wang, Liu, Chao et al., 2013). One of the avenues opened by the current study is the possibility that some of the actions of the EGFR, be it in normal physiology or disease, may necessitate the concomitant activation of other receptors e.g. GPCRs, including the AT_1R. As far as we know, this is not been widely studied but my data provide the platform for considering this possibility.

5.5 Summary

The results of this study demonstrate that by blocking AT_1R signalling (whether it be direct inhibition of the AT_1R or downstream $G_{q/11}$ signalling), in part, also blocks a significant proportion of EGF-mediated signalling. Such an observation could have enormous implications clinically. While therapeutics that target EGFR activity are mainstream treatments for a variety of cancers, resistance to cancer treatments limit the efficacy of these reagents and remains the major challenge for targeted cancer therapy. This study gives rise to the notion that EGFR may necessitate the concomitant activation of the AT_1R and may provide an alternative avenue by which cancer cells can signal and be modulated.

Chapter Six

The contribution of BMX, CHKA and TRIO in AT_1R-EGFR transactivation.

6.0 The contribution of BMX, CHKA and TRIO to AT_1R-EGFR transactivation

6.1 Introduction

The exact mechanism of EGFR transactivation remains contentious. In addition to the TMPS paradigm described previously, a number of second messengers have been reported to facilitate AT_1R-EGFR transactivation, including PKC, Src, Ca^{2+}, and ROS. Many research groups, including our own, have focused on defining the mechanism of GPCR-EGFR transactivation in various cells/tissues, but despite a tremendous amount of hard work and a lot of publications, the field has stalled in a mechanistic sense. This prompted the Thomas laboratory, in collaboration with Dr Ross Hannan (George, Purdue, Gould et al., 2013), to instead employ a functional genomics-approach to identify novel genes modulating AngII-mediated EGFR transactivation. Based on the assumption that kinases are likely involved in mediating AT_1R-EGFR transactivation, the developed RNAi screen focused on identifying kinases involved in EGFR-mediated ERK1/2 activation following AngII stimulation. This screen identified known genes previously involved in EGFR function (EGFR, HER2, Akt, MAPK1) as well as identifying other genes that have not been previously associated with EGFR transactivation. The three most interesting were the choline kinase alpha (CHKA), the triple function domain protein (TRIO) and the bone marrow kinase X-linked (BMX) kinase.

BMX (also termed epithelial tyrosine kinase, ETK) is a non-receptor tyrosine kinase that belongs to the Tec family kinases. Although BMX has not been directly implicated in EGFR transactivation, it has been recently suggested to play a role in ischemic preconditioning, and cardiac functional phenotype, particularly pressure overload (Mitchell-Jordan, Holopainen, Ren et al., 2008; Zhang, Franklin, Li et al., 2010). BMX has a typical array of regulatory domains and a highly conserved carboxyterminal kinase domain (Figure 6.01) (Qiu, Wang, Liu et al., 2014; Qiu, Robinson, Pretlow et al., 1998). The amino-terminus contains a pleckstrin homology (PH) domain that mediates membrane localisation (Chen, Jiang, Gewinner et al., 2013). The PH domain is followed by an array of zinc-binding Bruton's tyrosine kinase (BTK) homology (BH) domains as well as a SH3 and SH2 domain. These domains are thought to mediate inter- and intra-molecular protein interactions (Chen, Jiang, Gewinner et al., 2013; Joseph & Andreotti, 2009; Pursglove, Mulhern, Mackay et al., 2002;

Qiu, Wang, Liu et al., 2014).

CHKA is responsible for the generation of membrane phospholipids (Lacal, 2015) and has been demonstrated to play an important role in cell transformation and tumorgenesis (Hernando, Sarmentero-Estrada, Koppie et al., 2009). CHKA is a cytosolic enzyme that catalyses the phosphorylation of free intracellular choline to phosphocholine, the first step of the Kennedy pathway. The Kennedy pathway is the major contributor to the biosynthesis of phosphatidylcholine (PtdCho) in mammalian cells (Gruber, Too, Wong et al., 2012). Together, PtdCho and phosphoethanolamine (the other major product from the Kennedy pathway) is the major constituent of cell membranes, contributing to approximately 50% of the total phospholipid species in eukaryotic membranes (Gruber, Too, Wong et al., 2012).

At least three isoforms of choline kinase exist in mammalian cells and are encoded by two genes: CHKα (CHKA) and CHKβ (CHKB), which display 60% similarity and are ubiquitously expressed in different tissues (Gallego-Ortega, Ramirez de Molina, Angeles Ramos et al., 2009). CHKA has been largely implicated in the development of carcinogenic processes and is over expressed in a variety of human cancers and late stage malignant tumours (Gallego-Ortega, Gomez del Pulgar, Valdes-Mora et al., 2011; Lin, Hu, Gu et al., 2017), having recently been suggested as an appropriate marker for poor clinical outcome and tumour progression in breast carcinomas (de Molina, Gutierrez, Ramos et al., 2002; Miyake & Parsons, 2012), bladder carcinomas (Hernando, Sarmentero-Estrada, Koppie et al., 2009), lung cancer (Glunde & Bhujwalla, 2007; Ramirez de Molina, Sormentero-Estrada, Beldo-Iniesta et al., 2007) and prostate cancer (Challapalli, Trousil, Hazell et al., 2015). A consistent metabolic hallmark is the overexpression of both CHKA and altered choline phospholipid metabolism, leading to an increase in total choline containing metabolites, as well as an increased pool of phosphocholine available for breakdown and synthesis of PtdCho (Glunde, Bhujwalla & Ronen, 2011). The synthesis of PtdCho is vital, and plays an essential role in the structural maintenance of the plasma membrane as well as a key precursor for the generation of various lipid second messengers (Falcon, Hudson, Huang et al., 2013) and the propagation of mitogenic signalling events (Rodriguez-Gonzalez, Ramirez de Molina, Benitez-Rajal et al., 2003; Yalcin, Clem, Makoni et al., 2010). Phospholipase D (PLD) hydrolyses PtdCho, forming the negatively charged phospholipid, phosphatidic acid (PA) (Jenkins & Frohman, 2005). Generation of PA is known to interact with positively

charged effector proteins, including the well-characterised lipid interacting domain, PH-domain, of SOS (Zhao, Du, Skowronek et al., 2007). This initiates the translocation of various signalling proteins and mediates downstream mitogenic signalling.

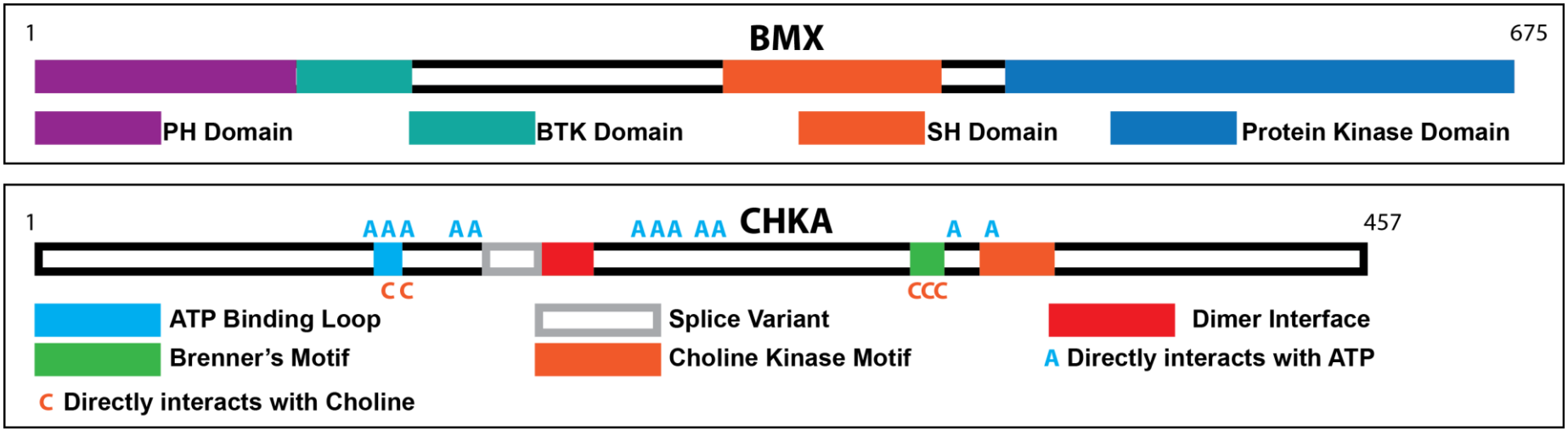


Figure 6.01: Schematic representation of BMX and CHKA structure and functional domains
The top figure shows that BMX N-terminus contains the PH domain is immediately followed by the conserved Btk motif. The C-terminus contains the kinase domain preceding the SH3 and SH2 binding motifs. Below, CHKA consists of 457 amino acids and is classified an 'atypical' kinase – a protein with confirmed protein kinase activity however has little sequence similarity to any known eukaryotic protein kinases. The N-terminal domain is composed of a single α-helix sheet and five-stranded antiparallel β-sheets, which is thought to stabilise the protein as a dimer (Dimer interface). Prior to this is an 18 amino acid insertion that is evident in CHKA2 and not CHKA1, its function is not clear. The C-terminal domain is primarily helical and contains many of the conserved regions - the Brenner's motif and the choline kinase motif. These regions are highly conserved among other atypical kinases, namely choline kinases, eukaryotic protein kinases and aminoglycoside phosphotransferases kinases.

Lastly, TRIO is a large multifunctional protein (3097 amino acid protein) that displays 3 enzymatic domains - a serine/threonine kinase domain, as well as two functional GEF domains (specific for GDP/GTP exchange on Rac1 and RhoA) (Bellanger, Lazaro, Diriong et al., 1998; Medley, Serra-Pagès, Iannotti et al., 2000). At present, no publications link AngII activation to TRIO activity, however AngII does engage other RhoA GEFs, such as Arhgef1 (Carbone, Brégeon, Devos et al., 2015; Guilluy, Bregeon, Toumaniantz et al., 2010). Intriguingly, a forward genetic screen revealed that the TRIO homologue in *C.Elegans*, UNC-73, is a major mediator of $G_{q/11}$ signalling (Williams, Lutz, Charlie et al., 2007). Furthermore, $G_{q/11}$ can activate the C-terminal Rho-specific DH-PH domain of TRIO (Rojas, Yohe, Gershburg et al., 2007). Most relevant to my studies is a recent genome-wide RNAi screen performed in *Drosophila*, which found that TRIO activation is required for G_q mediated mitogenic signalling, as well as sustained cellular growth (Vaque, Dorsam, Feng et al., 2013).

Given the accumulating literature linking GPCR-EGFR transactivation to tissue remodelling and disease state, a broader mechanistic picture of the underlying biology is required. Having already optimised a sophisticated BRET-based approach that interrogates AT_1R-EGFR transactivation, it is of great interest to confirm the involvement of these three novel proteins in the 'crosstalk' between the AT_1R and the EGFR.

6.2 Materials & Methods

Methods used throughout this chapter are detailed at length in Chapter 2.0, this includes cell culture (Section 2.4), ligand-induced BRET assays (Section 2.6.1) and confocal imaging (Section 2.6.2).

6.2.1 Materials

HEK293 cells were obtained from American Type Culture Collection. HEK293FT were obtained by Thermo Fisher Scientific. AngII was obtained from Auspep or Sigma Aldrich and EGF from R&D Systems or Peprotech. [Methyl-^{14}C]-Choline Chloride was supplied by Perkin Elmer. Thin layer chromatography (TLC) plates were supplied by Sigma Aldrich.

6.2.2 DNA Constructs

CHKA was generated by GeneArt (Thermo Fisher Scientific, Regensburg, Germany). pDONR223-BMX cDNA was a gift from William Hahn & David Root (Addgene plasmid # 23936). TRIO-GFP was kindly provided by Dr Anne DeBant (Cell Biology Research Institute of Montpellier, France). c-Src was kindly provided by Sarah Parsons (University of Virginia, VA, USA). mCherry-CHKA was kindly provided by Dr Aria Baniahmad (University of Jena, Germany). Venus-BMX, CHKA-Venus and Venus-CHKA were generated by inserting BMX and CHKA into pcDNA3-Venus, kindly provided by Dr Kevin Pfleger. Rluc8-BMX was generated by inserting BMX from Venus-BMX into pcDNA3-Rluc8, kindly provided by Dr Kevin Pfleger. CHKA with no additional tags was generated by inserting CHKA into pcDNA3. Preparation of the following cDNA constructs has been previously described: AT_1R, [TK325]AT_1R and [Y215F]AT_1R (Smith, Chan, Qian et al., 2011), EGFR-Rluc8 and Grb2-Venus (Ayoub, See, Seeber et al., 2013), K-ras-Venus and all subcellular compartment markers (Rabs-Venus) (Tiulpakov, White, Abhayawardana et al., 2016), β-arrestin2-GFP (Dinh, Qian, Seeber et al., 2004). AT_1R-Rluc8 was generated by inserting AT_1R from AT_1R-Venus into pcDNA3-Rluc8, prepared previously from cDNA kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, California, USA.

6.3 Results

6.3.1 BMXs involvement in AT_1R-EGFR transactivation

No previous data has linked BMX to EGFR transactivation, except the RNAi screen performed prior to this work (George, Purdue, Gould et al., 2013). We hypothesis that BMX interacts with both the AT_1R and EGFR, as has been reported for the PAR1 and other members of the RTK family (Cohen, Maoz, Turm et al., 2010). We directly examined BMX potential interactions using a BRET-based approach. A suitable vector containing an in frame Venus molecule was genetically fused to the N-terminus of BMX was generated (Venus-BMX). Firstly, to investigate the role of BMX in AT_1R-EGFR transactivation, we monitored the recruitment of Venus-BMX with EGFR-Rluc8 (Figure 6.02A). As shown in Figure 6.02B, following EGF-stimulation, a ligand-dependent BRET signal was observed in cells co-transfected with Venus-BMX and EGFR-Rluc8, indicative of BMX translocation and interaction with the EGFR. This association was further enhanced with the addition of AT_1R DNA (Figure 6.02B).

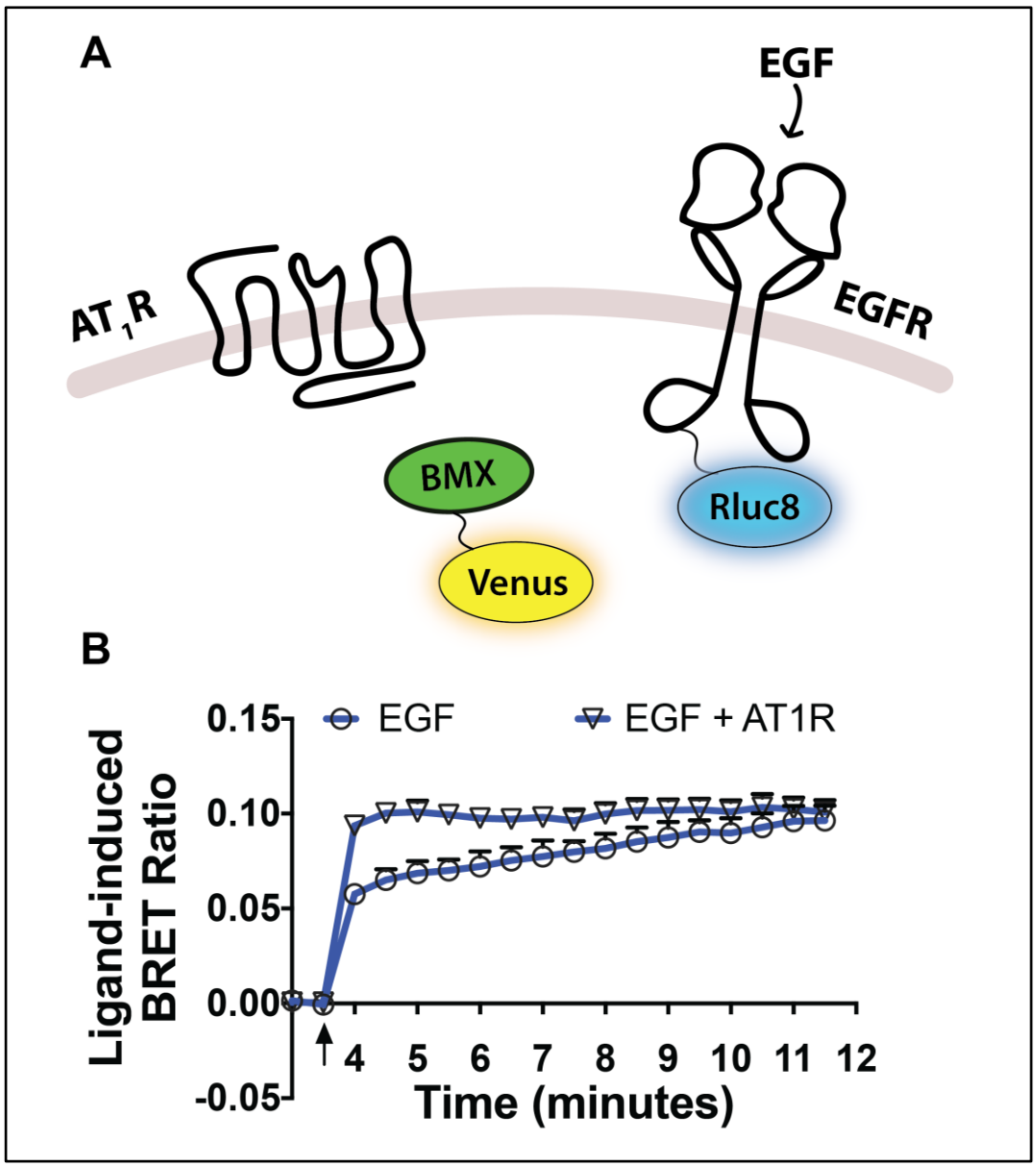


Figure 6.02: Kinetic analysis of EGF-mediated recruitment of BMX to the EGFR.
(A) Schematic illustration of BMX-EGFR BRET-based assay. EGFR fused to a BRET donor (Rluc8), is co-transfected with BMX tagged with a BRET acceptor (Venus) in the presence or absence of the AT_1R. **(B)** HEK293 cells expressing EGFR-Rluc8 and BMX-Venus in the presence or absence of AT_1R were treated with 1μM EGF or vehicle (indicated by arrow). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

We next sought to interrogate if this enhanced association between EGFR-BMX in the presence of additional AT_1R, is dependent upon $G_{q/11}$ coupling. In order to understand the molecular mechanisms underlying BMX-EGFR association, we used a single point mutant of the AT_1R, [Y215F]AT_1R, previously reported to retain high affinity for AngII, but an inability to couple to $G_{q/11}$ mediated signalling (Hunyady, Bor, Balla et al., 1995). Stimulation of the EGFR, in the presence of [Y215F]AT_1R, promoted BMX recruitment to the EGFR which was less than that promoted by the wild type AT_1R (Figure 6.03B). Similarly, we repeated the same experiment with a truncated mutant of the AT_1R, [TK325]AT_1R, lacking 34 carboxyl terminal amino acids and deficient in ligand-mediated phosphorylation and recruitment of β-arrestins (Qian, Pipolo & Thomas, 2001; Thomas, Motel, Kule et al., 1998). Likewise, [TK325]AT_1R also showed a decreased BMX recruitment compared to the wild type AT_1R (Figure 6.03B). It is important to note, although there is a change in kinetics, there is no decrease in the maximal EGFR-BMX recruitment, suggesting that AT_1R may influence EGFR-BMX kinetic profile but not the overall association.

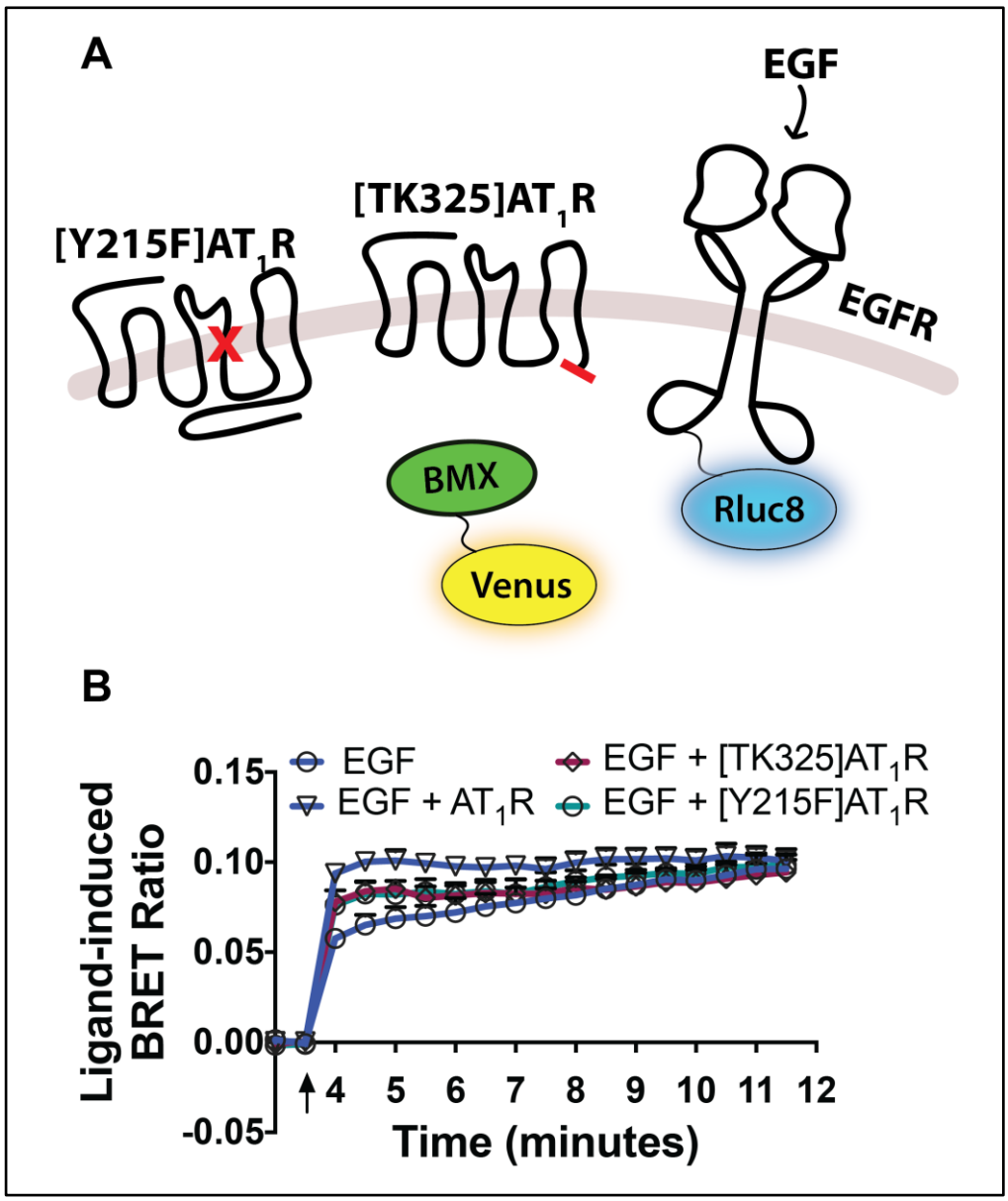


Figure 6.03: Kinetic analysis of EGF-mediated recruitment of BMX to the EGFR in the presence of mutant AT_1R.

(A) Schematic illustration of BMX-EGFR BRET-based assay. EGFR fused to a BRET donor (Rluc8), is co-transfected with BMX tagged with a BRET acceptor (Venus) in the presence of wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R. **(B)** HEK293 cells expressing EGFR-Rluc8 and BMX-Venus, alongside wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 1μM EGF or vehicle (indicated by arrow). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

Lastly, we examined whether AngII-stimulation could also recruit BMX to the EGFR as seen following EGF-stimulation (Figure 6.04A). Contrary to EGF-treatment, AngII-stimulation promoted a decrease in BRET ratio, suggesting AngII-stimulation promotes dissociation between EGFR and BMX (Figure 6.04B). This AngII-mediated BMX-EGFR dissociation was shown to be independent of $G_{q/11}$- and arrestin-coupling.

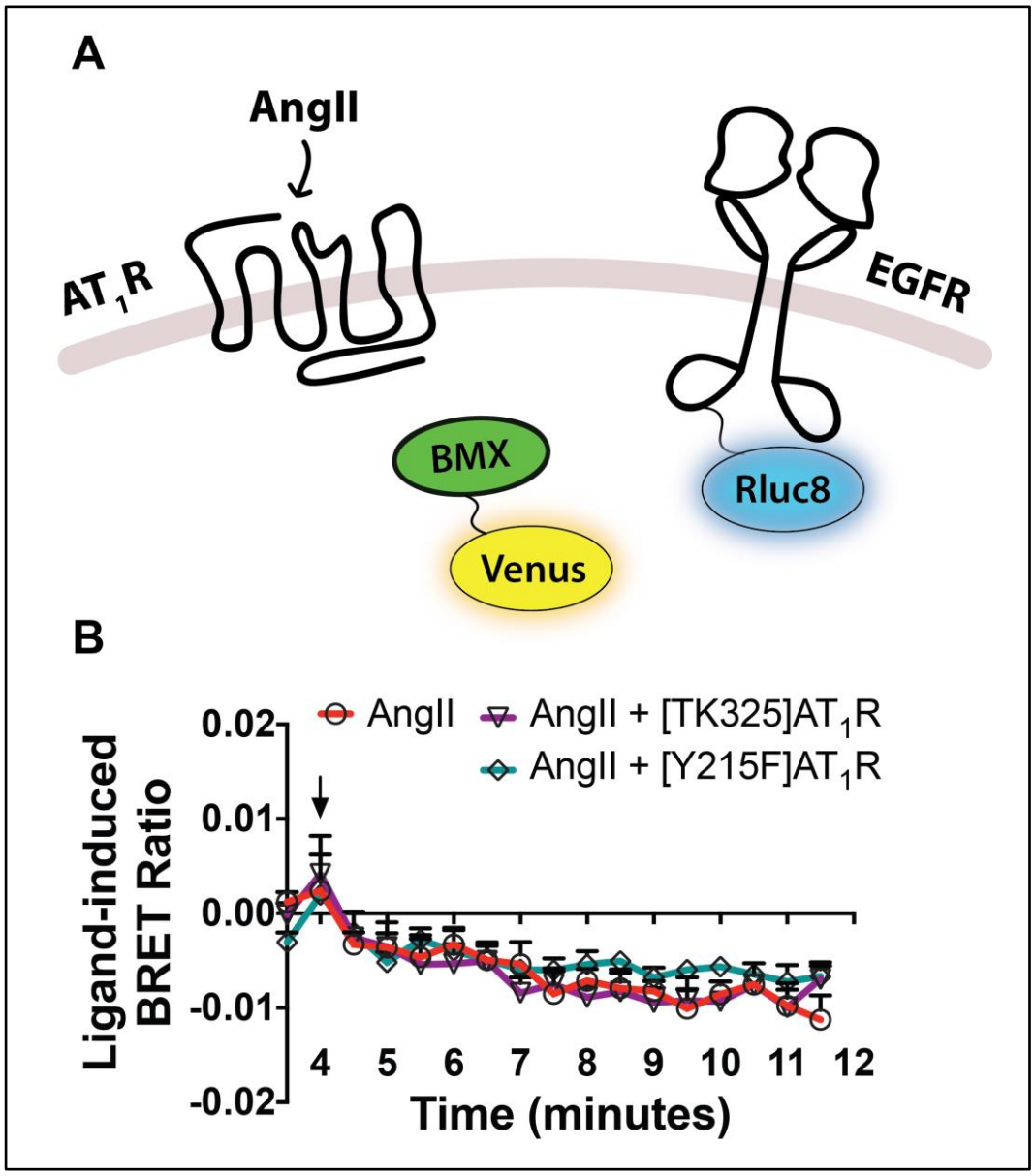


Figure 6.04: Kinetic analysis of AngII-driven recruitment of BMX to the EGFR.
(A) Schematic illustration of BMX-EGFR BRET-based assay. EGFR fused to a BRET donor (Rluc8), is co-transfected with BMX tagged with a BRET acceptor (Venus). **(B)** HEK293 cells expressing EGFR-Rluc8 and BMX-Venus, alongside wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII or vehicle (indicated by arrow). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

As shown in Figure 6.05A, we next investigated BMX association with the AT_1R, by co-transfecting cells with Venus-BMX and AT_1R-Rluc8. Similarly, AngII-treatment resulted in a decreased BRET response, indicative of BMX dissociation from the AT_1R, or a complex containing the AT_1R. In response to EGF-stimulation, we observed a partial increase in BRET ratio between AT_1R and BMX (Figure 6.05B). Together this data suggests that EGF-stimulation promotes BMX association with the EGFR, meanwhile AngII-treatment promotes BMX dissociation with both the AT_1R and EGFR.

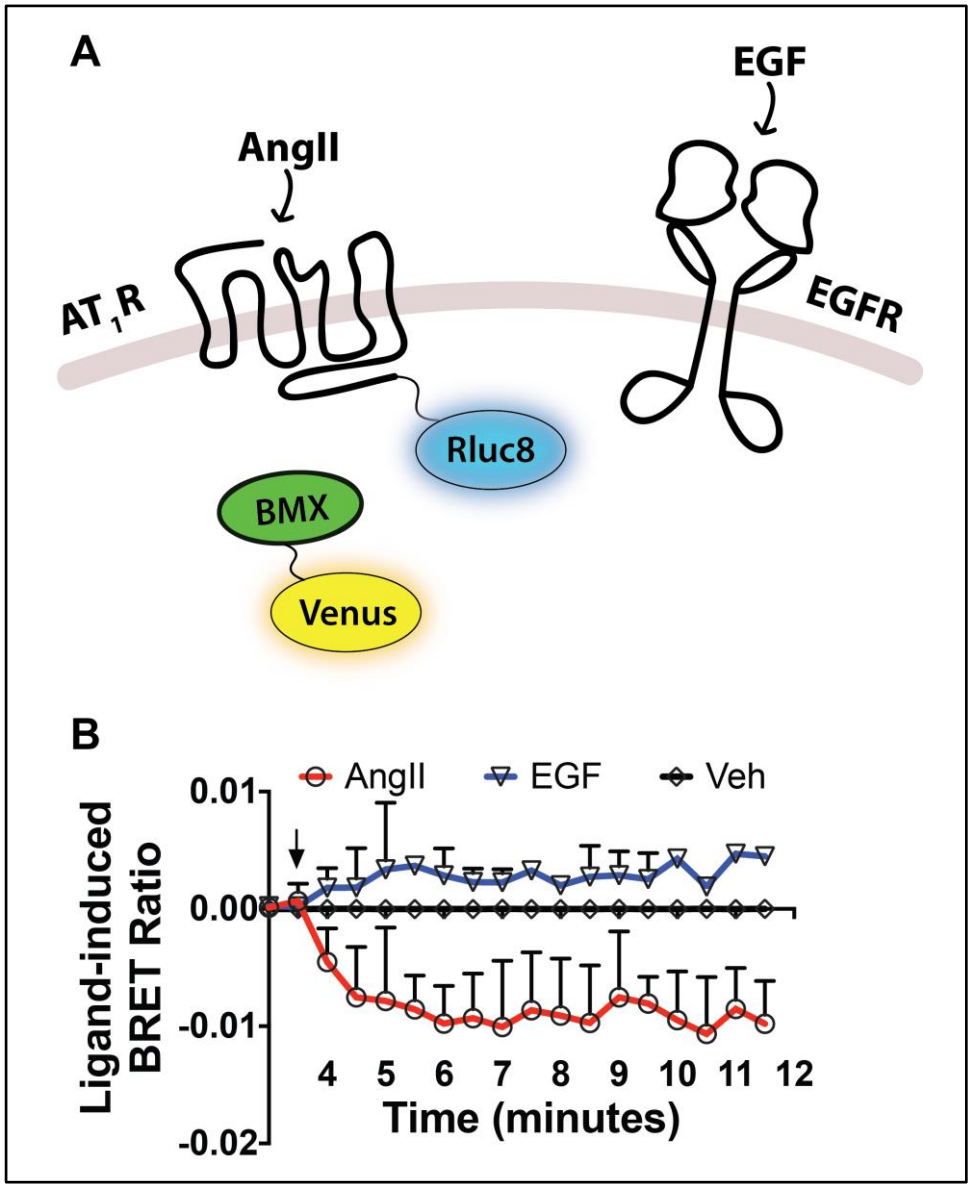


Figure 6.05: Kinetic analysis of ligand-driven recruitment of BMX to the AT_1R
(A) Schematic illustration of BMX-AT_1R BRET-based assay. AT_1R fused to a BRET donor (Rluc8), is co-transfected with BMX tagged with a BRET acceptor (Venus). **(B)** HEK293 cells were co-transfected with Venus-BMX and AT_1R-Rluc8 and stimulated with 10μM AngII, 1μM EGF or vehicle (indicated by arrow). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

To examine the distribution and trafficking of BMX, we used a novel BRET localisation assay approach. The use of validated Venus-tagged subcellular localisation markers enable us to monitor ligand-induced BMX trafficking in real-time and in live cells (as shown in Figure 6.06). As predicted, AngII-stimulation led to a decreased BRET response between BMX and K-ras, a marker localised to the plasma membrane, indicative of BMX internalising away from the membrane. Meanwhile EGF-treatment resulted in BMX trafficking to the plasma membrane (Figure 6.07). Interestingly, relative to vehicle treated cells, AngII- or EGF-treated cells did not observe any significant BRET responses between BMX and the remaining subcellular compartments (Figure 6.07).

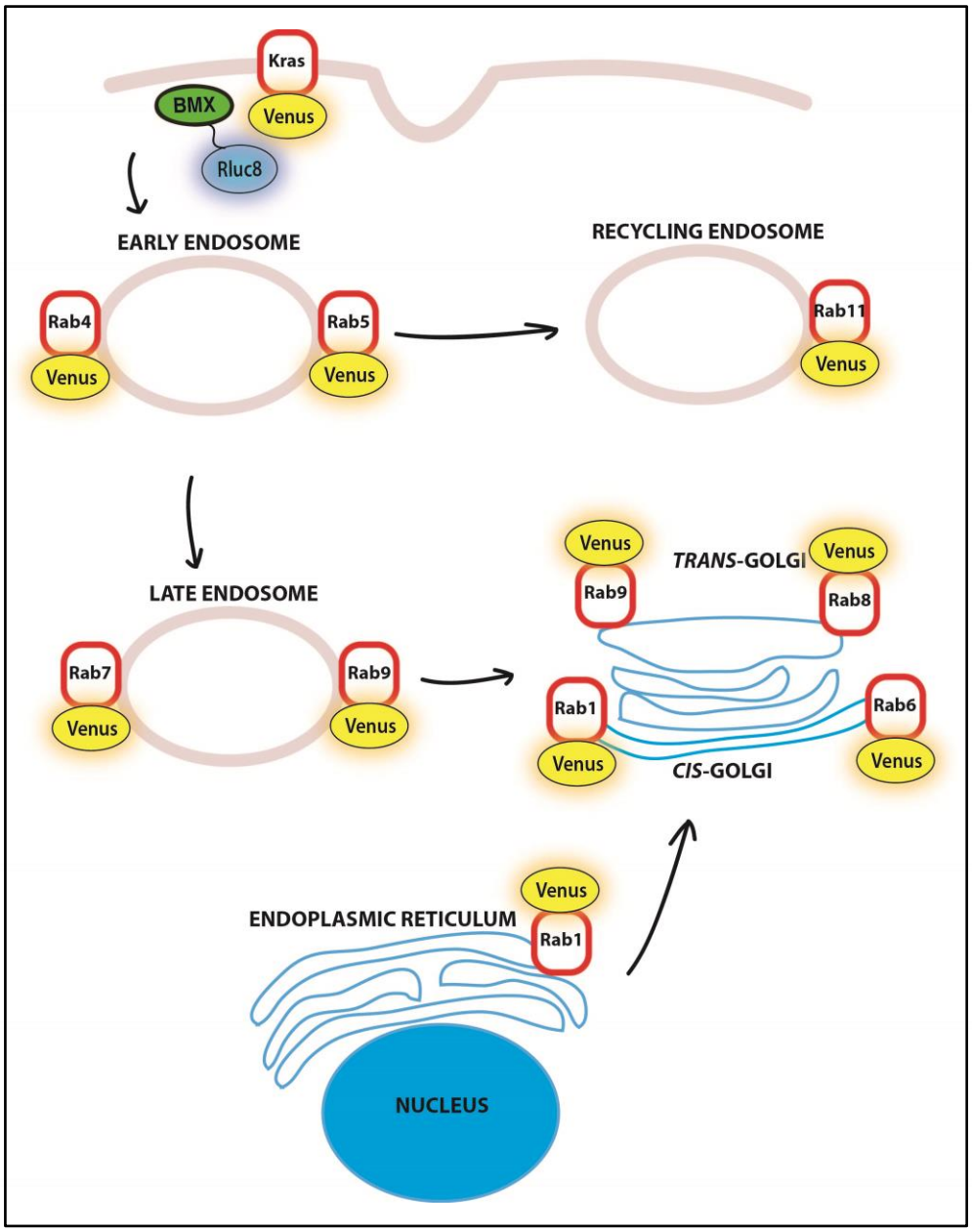


Figure 6.06: A simplified schematic representation of subcellular marker localisation and protein trafficking.
Ligand-induced trafficking was monitored using an Rluc8-tagged protein of interest (BMX-Rluc8) and measuring its proximity to Venus-tagged cellular markers. Extended BRET (eBRET) measures BMX interaction with plasma membrane marker, Venus-K-ras, or the subcellular compartment marker Rabs: early endosome (Venus-Rab5), early endosome recycling (Venus-Rab4), recycling endosome (Venus-Rab11), late endosome/lysosomes (Venus-Rab7), late endosome trafficking to the *trans*-Golgi network (Venus-Rab9), endoplasmic reticulum trafficking to the *cis*-Golgi (Venus-Rab1), Golgi apparatus and *trans*-Golgi network (Venus-Rab6), *trans*-Golgi network to plasma membrane (Venus-Rab8).

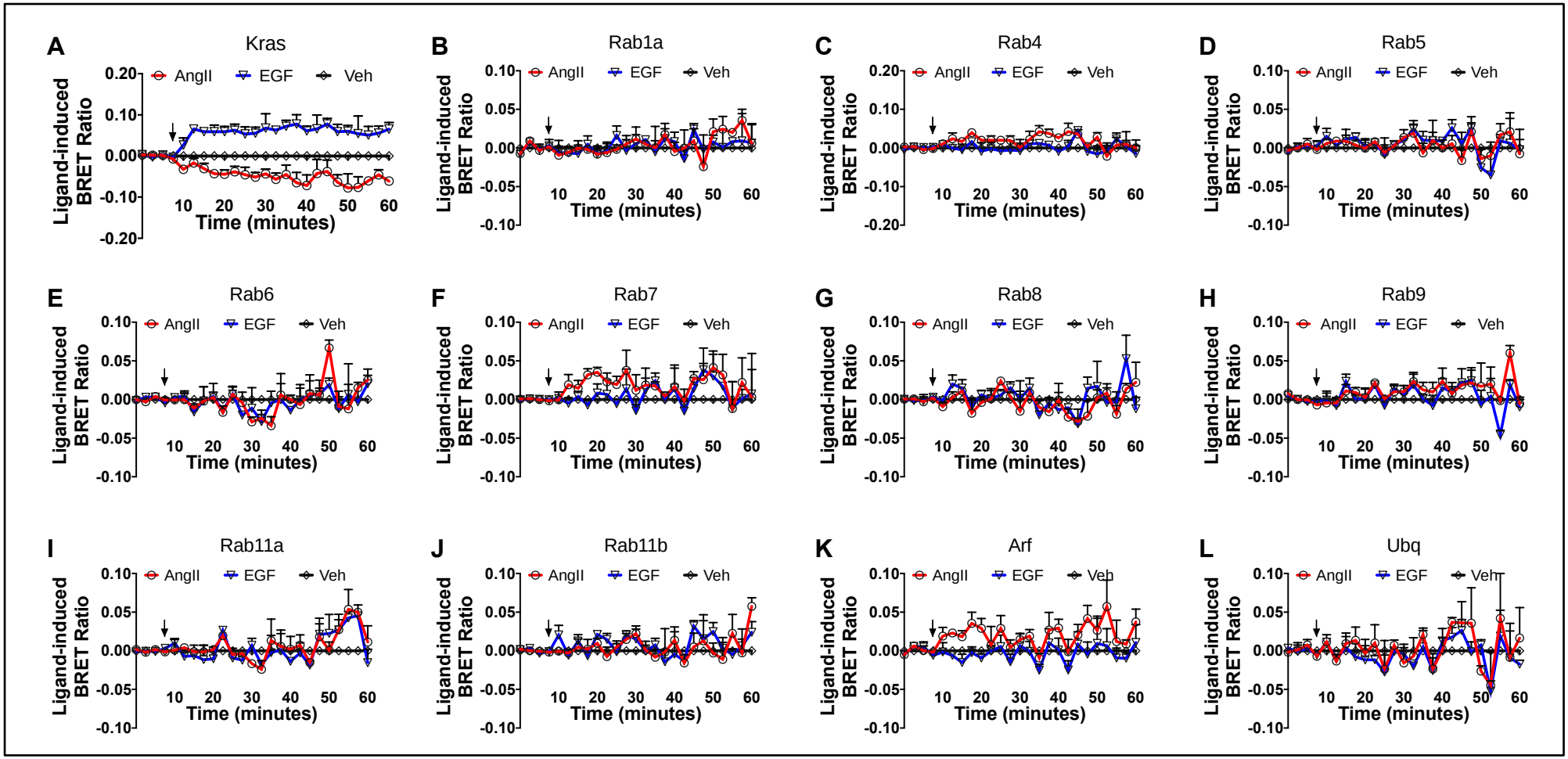


Figure 6.07: Kinetic profiling of the trafficking properties of BMX

(A-L) HEK293 cells were transiently transfected with Rluc8-BMX and Venus-tagged cellular markers (as per the title of each graph). Cells were stimulated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by the arrow. BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represents mean ± SEM of 4 independent experiments with triplicates within.

6.3.2 CHKAs involvement in AT_1R-EGFR transactivation

To determine if AT_1R activation leads to CHKA relocation, I generated and transfected vectors containing an in-frame Venus molecule fused to the N- and C-terminus of CHKA: Venus-CHKA and CHKA-Venus, respectively. Correct localisation of the Venus-tagged CHKA constructs were initially confirmed using confocal microscopy (Figure 6.08). As a positive control, we observed rapid and sustained β-arrestin2-GFP translocation to the plasma membrane following AngII-stimulation (Figure 6.08A-C). We next examined whether AngII-stimulation also resulted in CHKA translocation. In contrast, HEK293 cells expressing Venus-CHKA (Figure 6.08D,E) and CHKA-Venus (Figure 6.08F-H) and treated with AngII and did not display CHKA trafficking at 1 minute or 10 minute post agonist treatment.

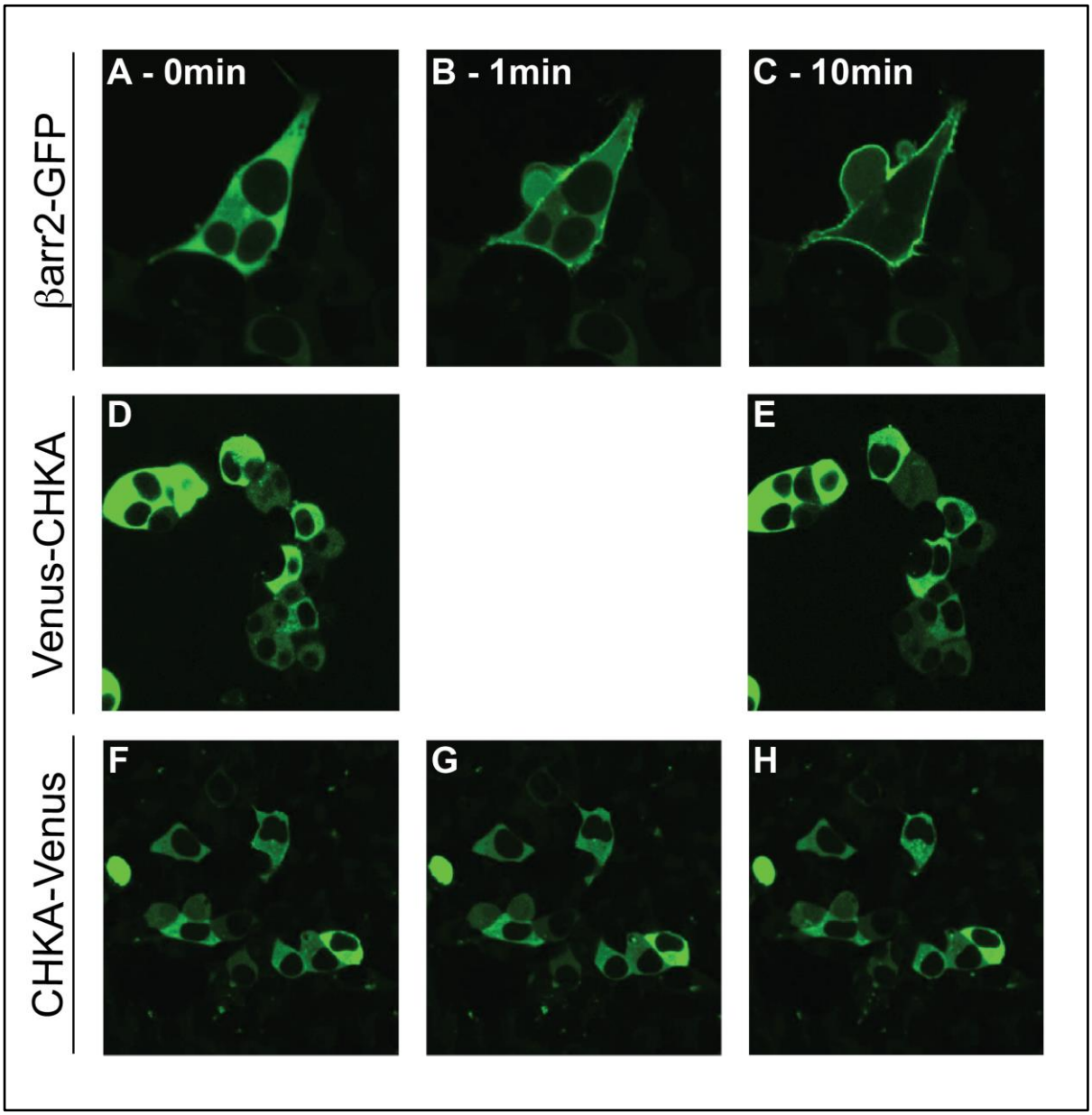


Figure 6.08: CHKA Trafficking in response to AngII-stimulation
(A-C) As a positive control, AT_1R was transiently co-expressed with β-arrestin2-GFP in HEK293 cells. **(D, E)** Venus-CHKA and **(F-H)** CHKA-Venus were transiently co-expressed with AT_1R in HEK293 cells. All cells were stimulated with 100nM AngII at 37°C. The distribution of β-arrestin2-GFP and Venus-tagged CHKA was visualised under confocal microscopy **(A)** prior to AngII-stimulation, **(B)** 1 minute post AngII-stimulation and **(C)** 10 minutes post AngII-stimulation.

Through the use of co-immunoprecipitation assays, CHKA has previously been shown to form a complex with the EGFR kinase domain in a Src-dependent manner. To determine whether CHKA physically associates with EGFR, we tested if EGF-stimulation could mediate CHKA interaction with the EGFR, comparing both CHKA-Venus & Venus-CHKA interaction with EGFR-Rluc8 in HEK293 and COS cells. Various combinations of EGFR-Rluc8 and tagged-CHKA were tested, however, no association between EGFR and CHKA was evident in HEK293 (Figure 6.09) and COS cells (Figure 6.10).

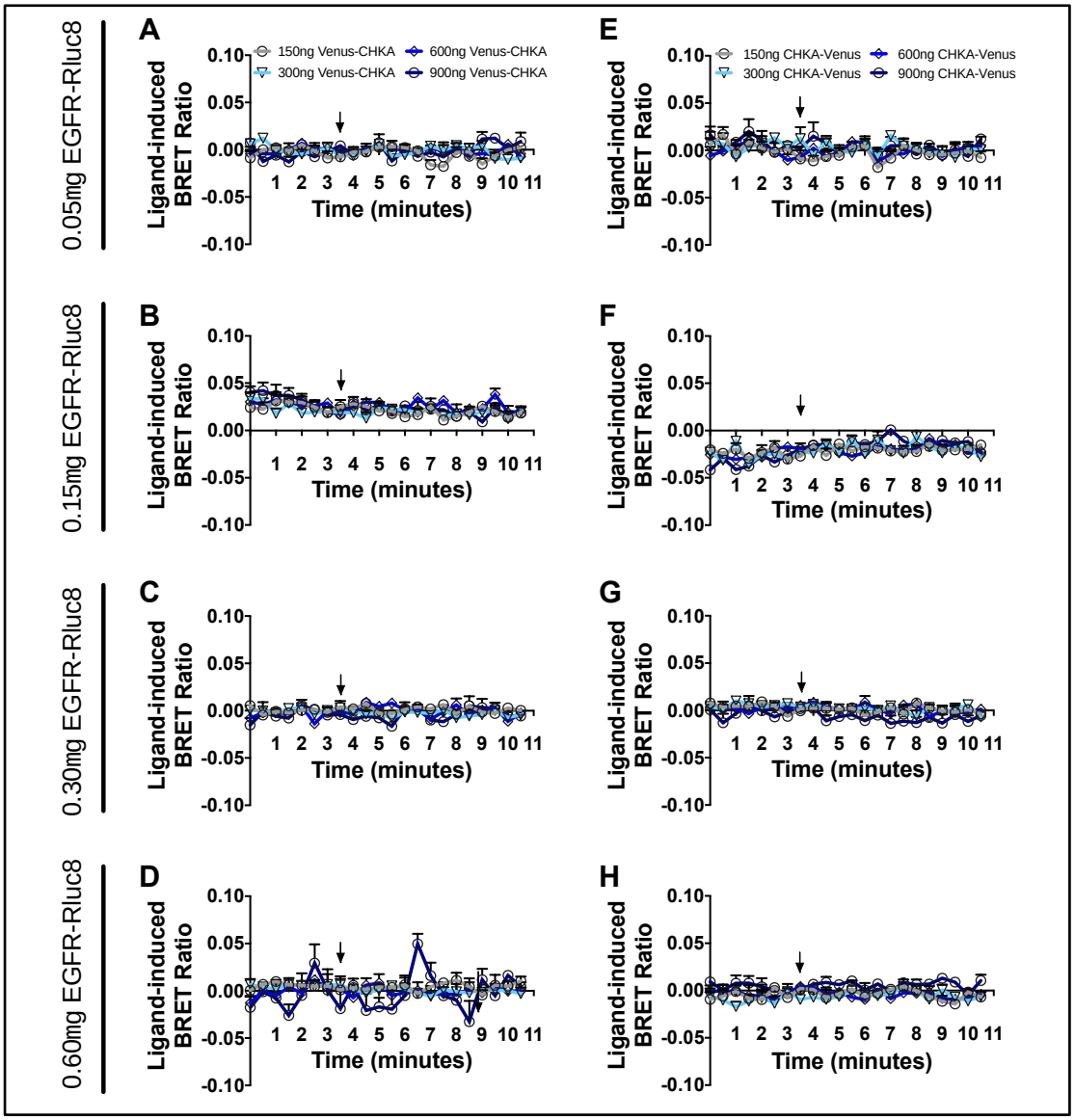


Figure 6.09: Kinetic analysis of EGF-mediated recruitment of CHKA to the EGFR in HEK293 cells

An increasing amount of EGFR-Rluc8, **(A, E)** 0.05µg, **(B, F)** 0.15µg, **(C, G)** 0.3µg and **(D, H)** 0.6µg were co-transfected with various amounts (0.15µg, 0.3µg, 0.6µg and 0.9µg) of **(A-D)** Venus-CHKA and **(E-H)** CHKA-Venus in HEK293 cells. Cells were treated with 1µM EGF or vehicle (indicated

by arrows). BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

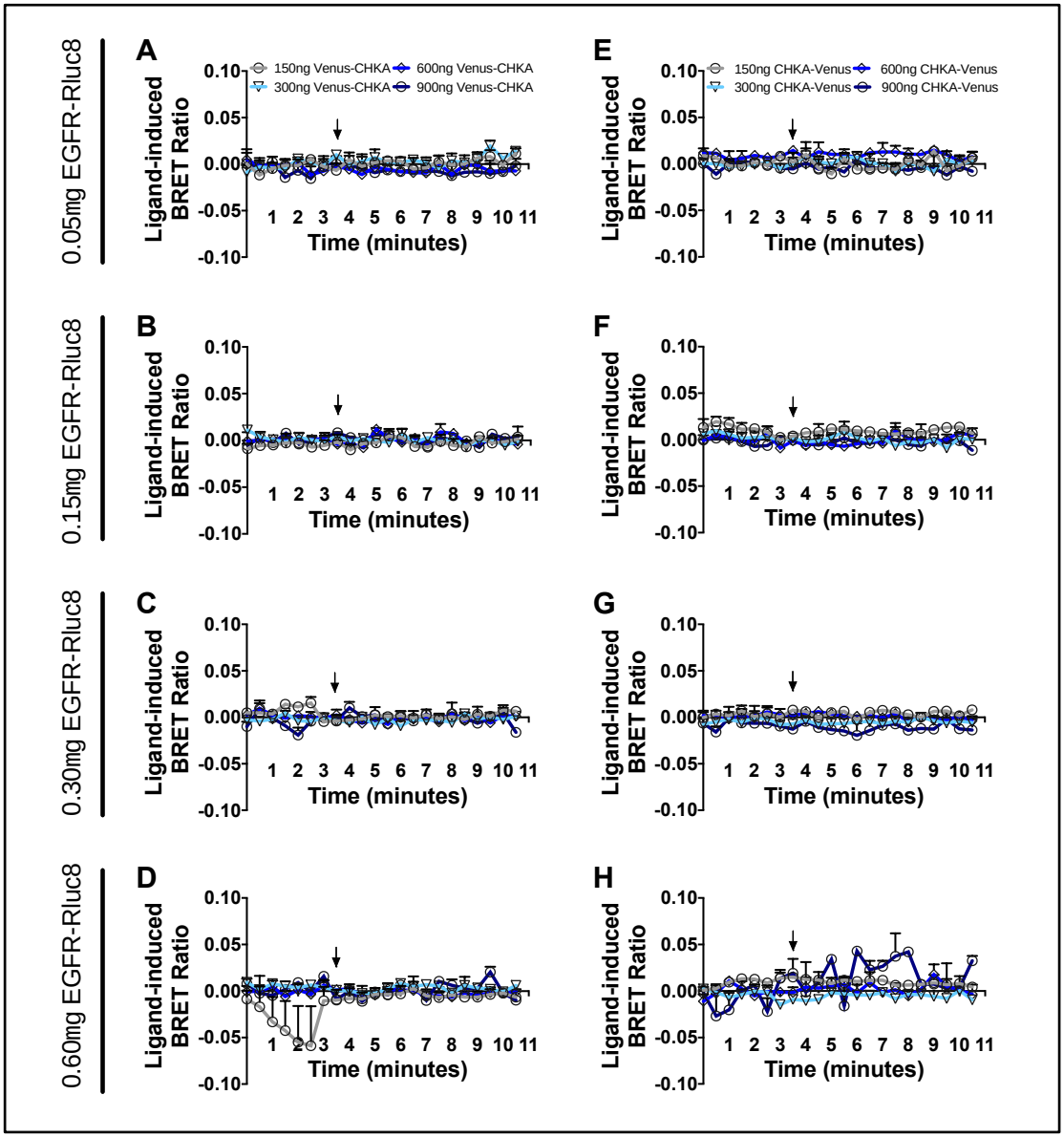


Figure 6.10: Kinetic analysis of EGF-mediated recruitment of CHKA to the EGFR in COS cells

An increasing amount of EGFR-Rluc8, **(A, E)** 0.05µg, **(B, F)** 0.15µg, **(C, G)** 0.3µg and **(D, H)** 0.6µg were co-transfected with various amounts (0.15µg, 0.3µg, 0.6µg and 0.9µg) of **(A-D)** Venus-CHKA and **(E-H)** CHKA-Venus in COS cells. Cells were treated with 1µM EGF or vehicle (indicated by arrows). BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 3 independent experiments with triplicate measurements.

Others have shown that either mutation or truncation of CHKA N-terminus has no significant impact on CHKA kinase activity; therefore all follow-up experiments were conducted with Venus-CHKA (Clem, Clem, Yalcin et al., 2011; Malito, Sekulic, Too et al., 2006). Miyake & Parsons proposed that EGFR forms a complex with the cytosolic kinase c-Src, which recruits CHKA to the cell membrane (Miyake & Parsons, 2012). I recapitulated the above experiments with the addition of c-Src in a hope that c-Src is crucial for mediating the interaction of CHKA with the EGFR; this also showed no association (Figure 6.11).

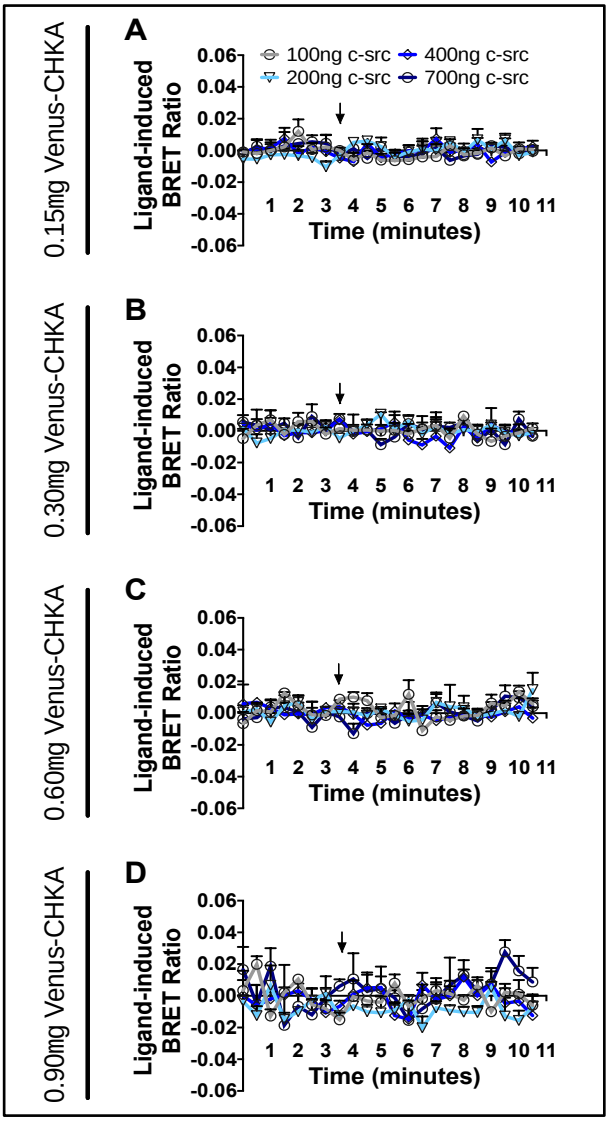


Figure 6.11: Kinetic analysis of EGF-mediated recruitment of CHKA to the EGFR with the addition of c-Src

Increasing amounts of Venus-CHKA and c-Src were co-transfected with EGFR-Rluc8 in HEK293 cells. HEK293 cells were transfected with 0.15µg of EGFR-Rluc8, alongside **(A)** 0.15µg, **(B)** 0.3µg, **(C)** 0.6µg and **(D)** 0.9µg of Venus-CHKA and increasing amounts of c-Src (as per the title of the top graph). Cells were treated with 1µM EGF or vehicle (indicated by arrows). BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 2 independent experiments with triplicate measurements.

CHKAs active enzyme consists of dimers or tetramers, and one confounder may be that the presence of a Venus-tag may interfere with activity. One final consideration was to transfect Venus-CHKA alongside CHKA with no additional tags (pcDNA3-CHKA). The addition of pcDNA3-CHKA was presumed to allow for CHKA-Venus to dimerise and form active dimers. This experiment also showed no increase in BRET ratio between Venus-CHKA and EGFR-Rluc8 following EGF-stimulation (Figure 6.12). As a final test, CHKA activity was confirmed directly using an *in vitro* CHKA activity assay.

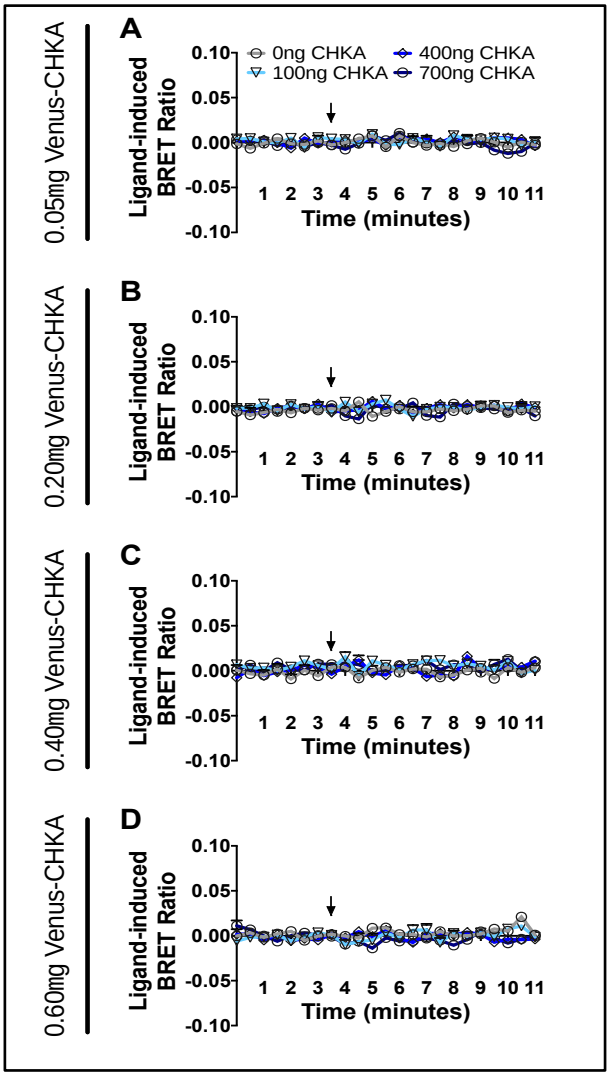


Figure 6.12: Kinetic analysis of fluorescently tagged CHKA and its interaction with EGFR with the addition of untagged-CHKA

EGFR-Rluc8 was transiently transfected into HEK293 cells alongside increasing amounts of Venus-CHKA and untagged-CHKA. HEK293 cells expressing 0.15µg EGFR-Rluc8, **(A)** 0.05µg Venus-CHKA, **(B)** 0.2µg Venus-CHKA, **(C)** 0.4µg Venus-CHKA and **(D)** 0.6µg Venus-CHKA alongside increasing amounts of untagged-CHKA (as per the title of the top graph). Cells were treated with 1µM EGF or vehicle (indicated by arrows). BRET measurements were taken using the CLARIOstar Microplate Reader (BMG LabTech). Data represent mean ± SEM of 2 independent experiments with triplicate measurements.

As shown in Figure 6.13, I tested whether the CHKA fusion constructs were enzymatically active. Current methods for studying CHKA enzymatic activity involve labelling cells with radiolabelled choline and separating choline and phosphocholine (P-Choline) metabolites using thin layer chromatography (Figure 6.13). As a positive control, cells were transfected with CHKA plasmid with no additional tags (pcDNA3-CHKA), and was able to produce P-Choline (Figure 6.13). I was unable to detect a P-Choline band for both Venus-tagged CHKA plasmids, suggestive that both CHKA-Venus and Venus-CHKA are kinase defective.

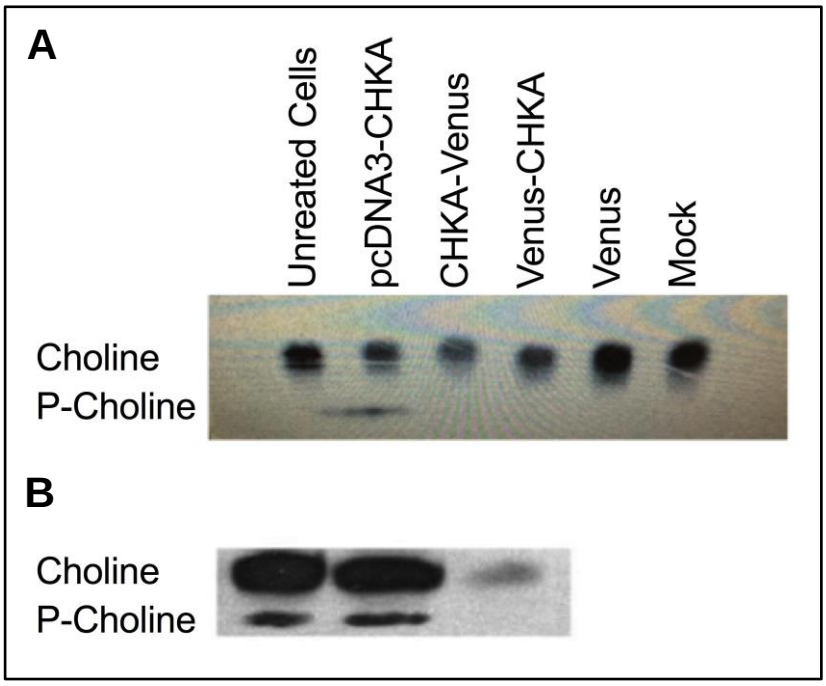


Figure 6.13: *In vitro* CHKA Activity Assay.
(A) HEK293 cell were transfected with 1µg of CHKA, CHKA/Venus, Venus/CHKA, empty Venus plasmid and mock plasmid. 24hours following transfection, cells were labelled with methyl choline-chloride carbon^{14} for 1hour. HEK293 cells were fixed, washed and resolved on thin layer chromatography plates. Choline & phosphocholine levels were imaged using a phosphorimager. A representative image of choline and phosphocholine levels showing CHKA plasmid with no additional tags was able to produce phosphocholine. **(B)** A representative image illustrating choline and phosphocholine bands.

During my canditure, Asim et al., 2016 generated the first fluorescently labelled CHKA construct, mCherry-CHKA (Asim, Massie, Orafidiya et al., 2016). The authors kindly donated this construct, and I recapitulated previously performed experiments using NanoBRET. Unfortunately, this also showed no EGFR-CHKA association following EGF-stimulation (Figure 6.14).

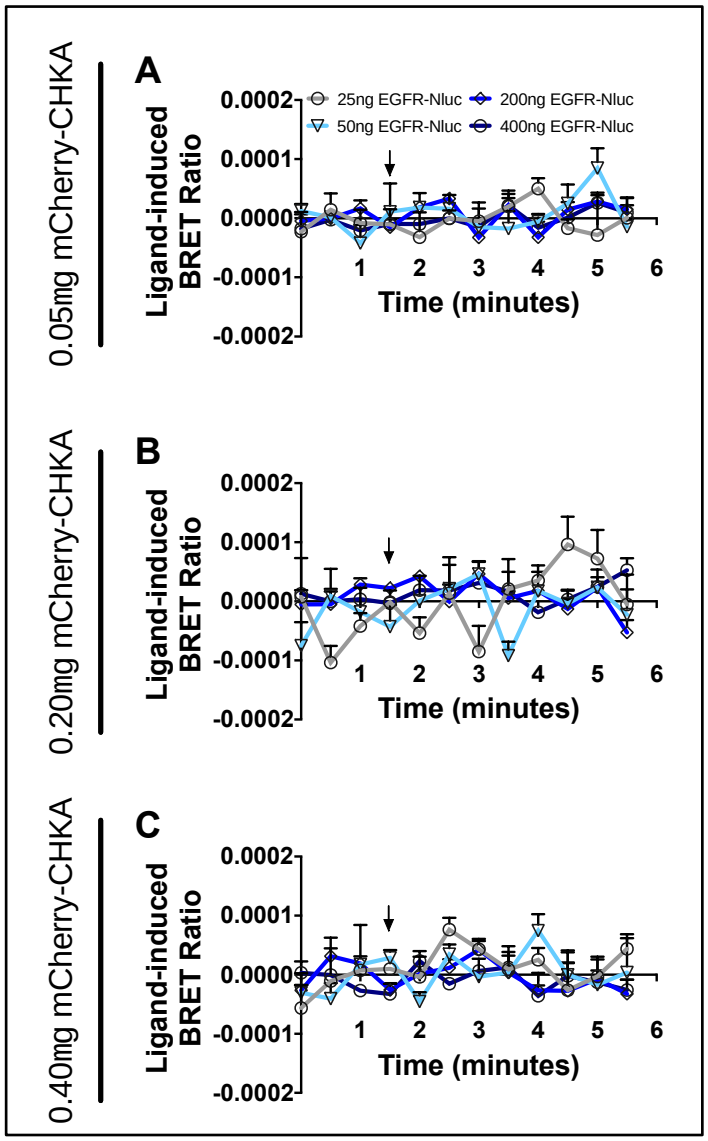


Figure 6.14: Kinetic analysis of CHKA interaction with EGFR using a Nano-BRET approach

Increasing amounts of EGFR-Nluc and mCherry-CHKA were co-transfected into HEK293 cells. **(A)** 0.05µg, **(B)** 0.2µg and **(C)** 0.4µg of mCherry-CHKA were transiently transfected with increasing amounts of EGFR-Nluc (as per the title of the top graph). Cells were treated with 1µM EGF or vehicle (indicated by arrows). Data represent mean ± SEM of 2 independent experiments with triplicate measurements.

To better understand CHKA's involvement in AT_1R-EGFR cross-talk, increasing amounts of CHKA were transfected alongside EGFR/Rluc8, Grb2/Venus & AT_1R. My hypothesis was that, if CHKA does play a role in transactivation, increasing the amount of the protein may be expected to enhance transactivation. The over-expression of CHKA increased the BRET ratio between EGFR/Rluc8 & Grb2/Venus following EGF-stimulation (Figure 6.15A and 6.15B) and AngII-stimulation (Figure 6.15C and 6.15D). Additionally, using endogenous levels of CHKA expressed in HEK293 cells, inhibition of CHKA with CK37 enhanced EGFR and Grb2 association following AngII-stimulation (Figure 6.15E and 6.15F).

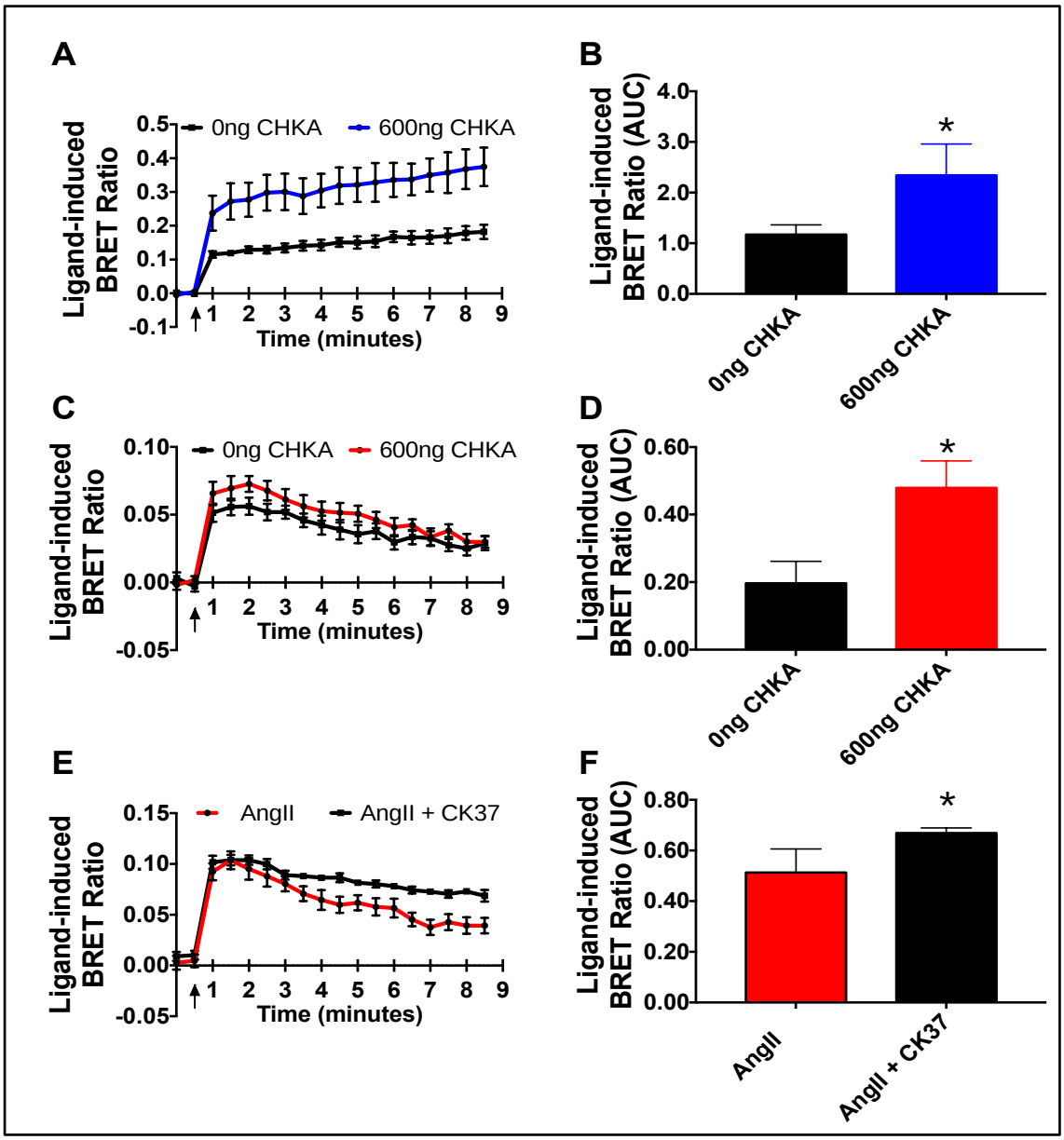


Figure 6.15: CHKAs involvement in EGF- and AngII-induced Grb2 recruitment to the EGFR

(A) HEK293 cells co-transfected with AT_1R, EGFR/Rluc8 and Grb2/Venus in the presence of overexpressed CHKA or endogenous levels of CHKA (co-transfected with equal parts of mock plasmid) and treated with **(A, B)** 1μM EGF, **(C, D)** 10μM AngII or vehicle. Quantification of ligand-induced BRET ratio (AUC) between EGFR-Rluc8 and Grb2-Venus with and without the addition of CHKA following **(B)** EGF-stimulation or **(D)** AngII-stimulation. (*$p<0.05$ vs. cells transfected with 0ng CHKA) **(E)** HEK293 cells co-transfected with AT_1R, EGFR/Rluc8 and Grb2/Venus were treated with CK37 (CHKA inhibitor) for 30 minutes prior to stimulation with AngII or vehicle. **(F)** Quantification of ligand-induced BRET ratio (AUC) between EGFR-Rluc8 and Grb2-Venus following AngII-treatment and in the presence CK37. *$p<0.05$ vs. AngII treated cells. Agonist stimulation is indicated by arrows. Data represents mean ± SEM of 3 independent experiments. Statistical analysis by a one-way ANOVA with a Dunnett's post-test for multiple comparisons;

6.4 Discussion

Using a cellular model of human AT_1R-EGFR transactivation, the Thomas laboratory has previously performed a functional siRNA screen of the human kinome in an attempt to interrogate AT_1R transactivation of the EGFR. The screen identified a suite of genes that have been previously established to regulate AT_1R-EGFR transactivation, in addition to others that have not been previously implicated (including BMX, CHKA and TRIO). In summary, individual knockdown of each molecule attenuated tyrosine phosphorylation of the EGFR following AngII-stimulation, and was shown to function downstream of the AT_1R. Although they have not been previously linked with AT_1R-EGFR cross-talk, previous literature has provided clues as to how they might be associated. Therefore, it was of great interest to further interrogate the molecular basis for the requirement for BMX, CHKA and TRIO in AT_1R-EGFR transactivation.

Although BMX has not been directly implicated in AT_1R-EGFR transactivation, BMX can be activated by a number of cell surface receptors, namely the IGF receptor and the vascular endothelial growth factor receptor (VEGFR) (Chen, Jiang, Gewinner et al., 2013; Zhang, Xu, Ekman et al., 2003). To confirm whether BMX directly binds with the EGFR, we used a BRET-based assay that quantitatively measures BMX recruitment to the EGFR. Following EGF-treatment, BMX translocation and direct association with the EGFR was readily demonstrable. This is consistent with previous studies, with co-immunoprecipitation studies showing that EGF-treatment results in BMX tyrosine phosphorylation and a marked increase in BMX-EGFR association (Jiang, Borgesi, McKnight et al., 2007). Complementary to our studies, others have shown that following EGF-treatment, BMX translocates to the membrane, where its kinase activity is activated by Src kinase (Chen, Huang, Kung et al., 2004). In fact, the activation of Btk family kinases by direct phosphorylation by Src family kinases has been well documented (Chen, Kim, Li et al., 2001; Rawlings, Scharenberg, Park et al., 1996; Tsai, Su, Fang et al., 2000). Signalling molecules that bear phosphotyrosine- and proline-rich domains disrupt the intramolecular interaction of the BMX protein, leading to unfolding of the kinase domain and exposing BMX potential phosphotyrosine sites (Qiu, Robinson, Pretlow et al., 1998; Semaan, Alsaleh, Gottenberg et al., 2008). Future experiments include the use of pharmacological inhibitors that target Src, EGFR tyrosine

domain and Ca^{2+} to better understand the molecular requirements that drive BMX-EGFR association. Additionally, it is still unknown the exact EGFR residues and/or receptor domains that recruit BMX following EGF-stimulation. Part of ongoing studies is to better define BMX mechanism through the use of a kinase defective BMX (K455M) in combination with mutant forms of the EGFR whereby key receptor tyrosine residues are individually mutated.

BMX-EGFR association was further enhanced in cells co-expressing AT_1R. We were able to show that this enhanced BMX-EGFR association, was partially dependent on G_q coupling as well as AT_1R phosphorylation and arrestin recruitment. Based on the above observations, we next tested BMX-EGFR association following AngII-treatment. Interestingly, we observed a decreased EGFR-BMX BRET response following AngII-treatment. A final consideration was to profile BMX distribution and trafficking through multiple subcellular compartments. Following EGF-treatment, we noted a robust recruitment of BMX to the plasma membrane, and as predicted, a dissociation following AngII-treatment. Interestingly, we did not detect ligand-induced changes in proximity for the remaining cellular markers.

Given that BMX-EGFR disassociated following AngII stimulation, it was compelling to test BMX association with AT_1R. Similarly to BMX disassociation with the membrane marker K-ras, a dramatic decrease in BMX-AT_1R association was observed following AngII-stimulation. BMX, via its PH domain, has been found to bind to the thrombin activated GPCR, PAR1, and this association drives Shc recruitment and oncogenic activity (Cohen, Maoz, Turm et al., 2010). Given that BMX is predominantly a cytosolic protein, this suggests that BMX would first have to translocate to the plasma membrane, maybe a small percentage is only required at the cellular membrane. Others have also reported that G proteins directly bind to BMX, and this interaction is important for BMX activation (Bence, Ma, Kozasa et al., 1997; Ma & Huang, 1998; Mao, Xie, Yuan et al., 1998; Tsukada, Simon, Witte et al., 1994). Our data suggests that the AT_1R and BMX exist as a preformed complex at the membrane. Part of ongoing studies is the use of BiFC (as outlined in Chapter 4.0), a fluorescence-based approach that enables direct visualisation of protein interactions in living cells. Tagging BMX and AT_1R with two complementary non-fluorescent YFP fragments will allow for YFP to reform its native three-dimensional structure and emit fluorescent

signal if BMX and AT_1R exist as a complex. We predict this association will occur at the plasma membrane and may involve AT_1R coupling to $G_{q/11}$.

It remains undetermined why BMX dissociates from the EGFR following AngII-treatment. Mechanistically, it would seem logical for BMX to associate with EGFR following AngII stimulation. The PH-domain of BMX has been shown to interact with various protein partners and it is thought to spatially regulate BMX interactions. It could be speculated that BMX can modulate both GPCR and RTK function by acting as a signalling scaffold that allows for the recruitment of other binding partners and effector proteins, or as a second messenger that modulates downstream signalling activities. As part of their signalling repertoire, AT_1R and EGFR regulates BMX activation and trafficking contrarily, however how this influences AT_1R-EGFR transactivation remains unknown.

A number of papers have highlighted BMX relevance and role in the cardiovascular system. BMX is highly expressed in the endothelium and endocardium of mouse embryo's large arteries (Rajantie, Ekman, Iljin et al., 2001). Genetic ablation of BMX does not result in any obvious developmental phenotype in mice, however has been implicated a variety of pathological states, including inflammatory angiogenesis, pressure overload-induced hypertrophic growth and arteriogensis (Holopainen, Rasanen, Anisimov et al., 2015; Mitchell-Jordan, Holopainen, Ren et al., 2008; Paavonen, Ekman, Wirzenius et al., 2004; Zhang, Xu, Ekman et al., 2003). During my canditure, Holopainen et al., 2015 showed that BMX inactivation, and/or mice deficient of BMX, attenuates the development of AngII-induced cardiac hypertrophy (Holopainen, Rasanen, Anisimov et al., 2015). All preliminary data has been conducted in HEK293 cells, and we are aware that interpolation to other cell types must be performed with caution. Having already demonstrated BRET-based assays in VSMC, we hope to recapitulate any major findings in primary cells.

Having focused on BMX, I next interrogated CHKAs potential role in EGFR transactivation. Due to recent findings, much effort is being invested into the molecular mechanisms by which CHKA and associated metabolites contribute to the cholinic phenotype in cancer state. The overexpression of CHKA and increased activity in cancer state renders it a very attractive therapeutic, with CHKA inhibitors showing antitumoral and antiproliferative activity (Al-Saffar, Troy, Ramirez de Molina et al., 2006; Granata, Nicoletti, Tinaglia et al., 2014; Lacal, 1999; Rodriguez-Gonzalez, de Molina, Fernandez et al., 2004). A variety of

novel cancer drugs have been generated showing promising results in pre-clinical trials (Lacal & Campos, 2015) with a CHKA inhibitor, TCD-717, approved to enter first stage clinical trials (de la Cueva, Ramirez de Molina, Alvarez-Ayerza et al., 2013). Although CHKA has not been directly implicated in the transactivation process, the Thomas Lab has proposed a mechanistic model that incorporates recent key findings from the literature (Figure 6.16). It has been shown that CHKA forms a complex with the EGFR kinase domain in a Src dependent manner (Miyake & Parsons, 2012). More recently, confocal-based immunofluorescence imaging has revealed evidence of membrane co-localisation between EGFR and CHKA (Lin, Hu, Gu et al., 2017), with CHKA-EGFR association shown to enhance following EGF-stimulation (Miyake & Parsons, 2012). It is proposed that CHKA subsequently converts choline to phosphocholine, contributing to a local increase of PtdCho immediately surrounding the complex. PLD is recruited to the EGFR and hydrolyses PtdCho, generating PA and choline. The negatively charged PA has been show to be a critical upstream regulator of Ras signalling through its interaction with the PH domain of Sos and initiating Ras-triggered mitogenic signalling pathway (Zhang & Du, 2009; Zhao, Du, Skowronek et al., 2007).

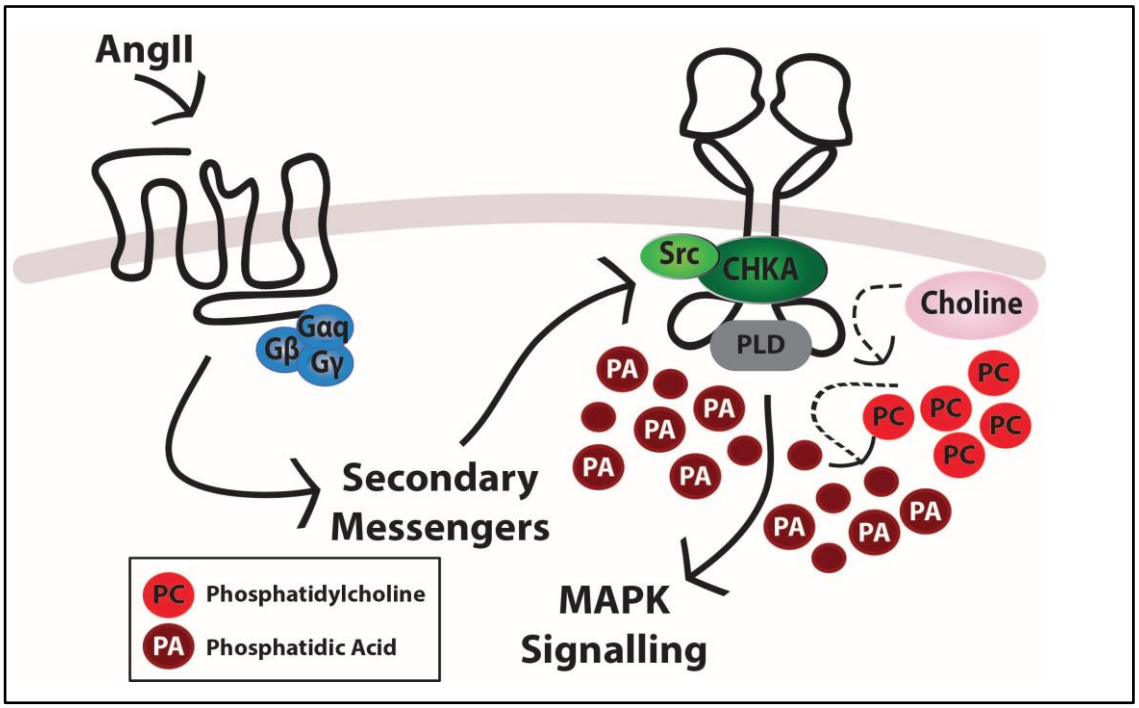


Figure 6.16: CHKAs proposed involvement in AT1R-EGFR transactivation.
Following AngII-stimulation, through the aid of second messengers, EGFR is activated and initiates the formation of the Src/EGFR/CHKA complex. CHKA subsequently produces a local phosphatidic acid pool surrounding the EGFR, triggering the mitogenic signalling pathway.

To validate the proposed model of CHKA action, a BRET-based assay was employed to determine if CHKA interacts with the EGFR, or alternatively, with the AT_1R. In accordance with previous studies, we initially aimed to determine whether stimulation of the EGFR could promote CHKA translocation and interaction with the EGFR in a ligand-dependent manner. In our hands, we were unable to observe an increased association between CHKA and EGFR in both HEK293 and COS cells. However, a major caveat of BRET is that it cannot readily distinguish between conformational rearrangements within a protein complex or proteins association/dissociation. The use of a BRET-based approach may not be able to capture a pre-formed EGFR-CHKA complex, or able to report an increased association/rearrangement between EGFR and CHKA following EGF-stimulation.

Furthermore, it cannot be decisively said whether the addition of the Venus tag rendered CHKA inactive. A limitation of BRET is the addition of the Venus-tag can interfere with proteins functionality. CHKAs active enzyme consists of dimers (homo- or hetero-dimers) or as tetramers. Both the N-terminal region, as well as the N-terminus proximity to the C-terminal region, is necessary for the formation of homo- or hetero-dimer complexes (Aoyama, Liao & Ishidate, 2004; Malito, Sekulic, Too et al., 2006). Due to the steric involvement of sequences close to the C- and N-terminal sites in formation of catalytic cavities, the addition of the Venus tag may prevent accurate protein folding and/or the formation of active CHKA. To date, two separate groups have published a fluorescently tagged CHKA, one of which I tested. Unfortunately, both studies did not report if the addition of a fluorescent tag interfered with CHKA kinase activity (Lin, Hu, Gu et al., 2017; Miyake & Parsons, 2012). Although the use of BRET[1] appears to be an insufficient approach to monitor CHKA's potential interactions, the development of techniques that use much smaller tags, namely nanoBRET and FlAsH, may overcome this issue and should be considered for future experiments. Although other groups have reported the existence of CHKA-EGFR complexes (Lin, Hu, Gu et al., 2017; Miyake & Parsons, 2012), it must be considered that transactivation may not involve CHKA's direct association with EGFR.

We were able to demonstrate that the addition of CHKA does enhance both AngII- and EGF-mediated Grb2 recruitment to the EGFR. Although this 'over-expression experiment' does not give any further insight into CHKA's involvement mechanistically, it is reassuring that CHKA does influence AngII-mediated transactivation. Interestingly, we report that pre-

treatment with a small molecule inhibitor of CHKA, CK37 produced a sustained Grb2 association with the EGFR following AngII-stimulation. Interestingly, siRNA knockdown of CHKA blunted ERK1/2 phosphorylation following AngII-treatment, an observation that needs reconciling.

CHKA catalytic properties are critical for cell growth and survival, with a variety of studies showing that a depletion of CHKA protein (i.e., siRNA and shRNA techniques) reduce intracellular phosphocholine levels and a decrease in cell viability (Bañez-Coronel, de Molina, Rodríguez-González et al., 2008; Clem, Clem, Yalcin et al., 2011; Mori, Glunde, Takagi et al., 2007). However, CHKA has also been shown to associate with other oncogenic proteins and can act as a molecular chaperone, raising the possibility that CHKAs protein itself, rather than it catalytic function, may be crucial for cell survival and growth (Asim, Massie, Orafidiya et al., 2016; Rauch, Volinsky, Romano et al., 2011). In an attempt to differentiate CHKAs catalytic and non-catalytic role in cancer cell survival, Falcon and colleagues compared siRNA-targeted deletion of CHKA to selective inhibition of CHKA (Falcon, Hudson, Huang et al., 2013). They observed marked difference in cell phenotype using siRNA-induced knockdown of CHKA compared to selective inhibition of CHKA catalytic activity. This non-catalytic 'scaffolding-role' of CHKA may explain this discrepancy in CHKAs relative role in EGFR transactivation, and warrants further study.

6.5 Summary

Given the accumulating literature linking GPCR-EGFR transactivation to tissue remodelling and disease state, a broader mechanistic picture of the underlying biology is required. The development of an unbiased functional siRNA screen identified three novel proteins that had not been previously implicated in EGFR transactivation. The next generation of RAS/AT_1R /AngII modifying agents will provide increased fidelity by targeting unique and specific molecules and pathways (in contrast to merely blocking the receptor *in toto*). Interrogating the mechanism by which these novel proteins are involved will provide new insights into this process and may represent a superior target for future drug development.

Chapter Seven

General Discussion.

7.0 General Discussion

The capacity of GPCRs to "transactivate" growth factor receptors has been an exemplar of receptor crosstalk since the initial observation by Axel Ullrich over 20 years ago (Daub, Weiss, Wallasch et al., 1996)**.** This paradigm of crosstalk has been confirmed for a range of GPCRs, including the AT_1R, where transactivation of the EGFR has been associated with cellular growth, remodelling, and apoptosis in the heart, kidney, and blood vessels as well as cancerous cell growth (Chan, Jenkins, Pipolo et al., 2006; Chen, Chen, Neilson et al., 2006; Greco, Muscella, Elia et al., 2003; Itabashi, Maesawa, Oikawa et al., 2008; Lin & Freeman, 2003; Ohtsu, Higuchi, Shirai et al., 2008; Thomas, 2001)**.** Various models have been evoked to explain GPCR-EGFR transactivation – Ullrich and colleagues proposed the TMPS model, whereby activated GPCRs stimulate MMPs and ADAMs to cleave inactive membrane-bound EGF ligands to stimulate the EGFR. While there has been general acceptance of the TMPS model, the exact MMP/ADAM/EGF ligand/EGFR engaged by a given GPCR in a specific tissue/cell, in a particular context, has been challenging to ascertain unambiguously. Moreover, alternative mechanism for EGFR transactivation exists, which may involve non-receptor tyrosine kinases (Pyk2 and Src, as well as BMX, CHKA and TRIO), additional second messengers (Ca^{2+}, PKC, ROS) and arrestins. Many research groups, including our own, have focused on defining the mechanism of GPCR-EGFR transactivation in various cells/tissues, but despite a tremendous amount of work and publications, it would be fair to conclude that the field has stalled in a mechanistic sense.

To address this knowledge gap, we rationalised that there was a need to develop new approaches/technologies that could better define the molecular, temporal and spatial aspects of AT_1R-EGFR transactivation, while also interrogating the relative contribution of gene(s) modulating this process. One major factor was that endpoint readouts of EGFR transactivation signalling are often downstream of highly amplified and integrated signal transduction pathways (i.e. ERK1/2). Although the activation of ERK1/2 appears to be a critical component in eliciting mitogenic responses, a single readout of ERK1/2 almost certainly does not always reflect only EGFR transactivation. We reasoned that a more robust and direct readout would be the activation and autophosphorylation of the EGFR, the convergence point for many growth regulatory signals, including that of transactivation. A

number of groups have measured EGFR phosphorylation (particularly phosphotyrosine residues Tyr1068 and Tyr1173) as readouts of EGFR transactivation (Ohtsu, Higuchi, Shirai et al., 2008; Shah, Farshori & Catt, 2004; Shah, Farshori, Jambusaria et al., 2003), however this end-point assay can be laborious, insensitive and challenging.

In recent years, a number of new technologies have been developed that overcome the inadequacies associated with current readouts of EGFR transactivation. Advancements in biophysical methods (these include BRET and FRET techniques) have allowed for live cell proximity detection and have been instrumental in determining the functional organisation and compartmentalisation of receptors and proteins of interest (Johnstone & Pfleger, 2012; Kocan, See, Seeber et al., 2008; White, Vanyai, See et al., 2017). My current study is the first to explore the application of BRET technology, specifically the recruitment of Grb2 to the EGFR, to quantitatively monitor, in living cells and real-time, the proximal activation of EGFR. Grb2 binds directly to phosphotyrosine residues (Tyr1068 and Tyr1086) or forms a multiprotein complex at phosphotyrosine residues Tyr1045, Tyr1148 and Tyr1173 (Capuani, Conte, Argenzio et al., 2015; Schlessinger, 2000; Wu, Wee, Jiang et al., 2012) (Figure 7.01). Given that Grb2 recruitment links directly EGFR tyrosine kinase to the downstream activation of the Ras/Raf/ERK1/2 cascade, it seemed sensible to monitor EGFR-Grb2 recruitment as the most primal readout of EGFR transactivation. I was able to demonstrate that both EGF- and AngII-stimulation resulted in the recruitment of Grb2 to the EGFR and this could be recapitulated in a variety of immortalised and primary cell lines, validating the versatility of this BRET-based approach.

The BRET assay was then used to screen a panel of 19 GPCRs and their capacity to recruit Grb2 to the EGFR. Of the 19 GPCRs tested, the $V_{1b}R$ and AT_1R showed consistent EGFR transactivation. Initially, this observation was concerning, as a number of these 'non-transactivating' GPCRs have been shown to mediate AG1478-preventable ERK1/2 phosphorylation. As highlighted in the discussion of Chapter 4.0, these 'non-transactivating' GPCRs were tested to be functional. As part of their biology, these 'non-transactivating' GPCR may not be recruiting Grb2 as part of their activation process, instead activating alternative effector molecules (as shown in Figure 7.01).

As illustrated in Figure 7.01, upon EGFR activation, the EGFR can bind directly to a number of adaptor molecules and effector proteins, regulating the activation of specific downstream pathway. These 'non-transactivating' GPCRs may not engage EGFR-Grb2 association and instead promote EGFR activation with alternative effector molecules. Understanding the details of this biased signalling requires a multifaceted approach that can simultaneously monitor the direct binding of a wide range of effector proteins to the EGFR. In a recent study, Schiffer and colleagues applied BRET technology to study the interaction of the EGFR with a number of effector proteins, namely Grb2, Shc42 (known activators of the MAPK proliferation pathway), p85 (activates PI3k-Akt survival pathway), PLCγ1 (activates PKC/Ca^{2+} signalling pathway) and STAT5a (activator of transcription pathways) (Tan, Wang, Littler et al., 2007). Such an approach not only measures the most proximal events in receptor activation, but also has the potential to compare a wide range of GPCRs and their effects on multiple EGFR effector-specific signalling pathways (Siddiqui, Cong, Daimon et al., 2013; Tan, Wang, Littler et al., 2007).

Tan et al., 2007 also applied this technology to screen nine additional RTK members, including HER4, highlighting that this technology can be further adapted for additional RTKs. Similarly, I compared the recruitment of Grb2 to additional members of the EGFR family of growth factor receptors. Our data demonstrates that the AT_1R is able to transactivate HER2 (the preferred dimerisation partner for other EGFR family members), and raises the possibility that the other receptor homologs (HER2, HER3 and HER4), and or combinations of dimers between these receptors, are selectively used by GPCRs. As already mentioned in Chapter 4.0, the classical view that EGFR monomers form activated dimers following EGF ligand binding has been replaced by more sophisticated models suggesting that dimerisation can occur in the absence of ligand (Fortian & Sorkin, 2014; Kozer, Barua, Henderson et al., 2014; Kozer, Barua, Orchard et al., 2013; Pinilla-Macua, Watkins & Sorkin, 2016). The use of a BRET-based assays that can report on effector-specific RTK activity offers the opportunity to not only catalogue GPCR multifaceted processes but also provides information to examine the relative contribution of HER receptor homologs in the transactivation process.

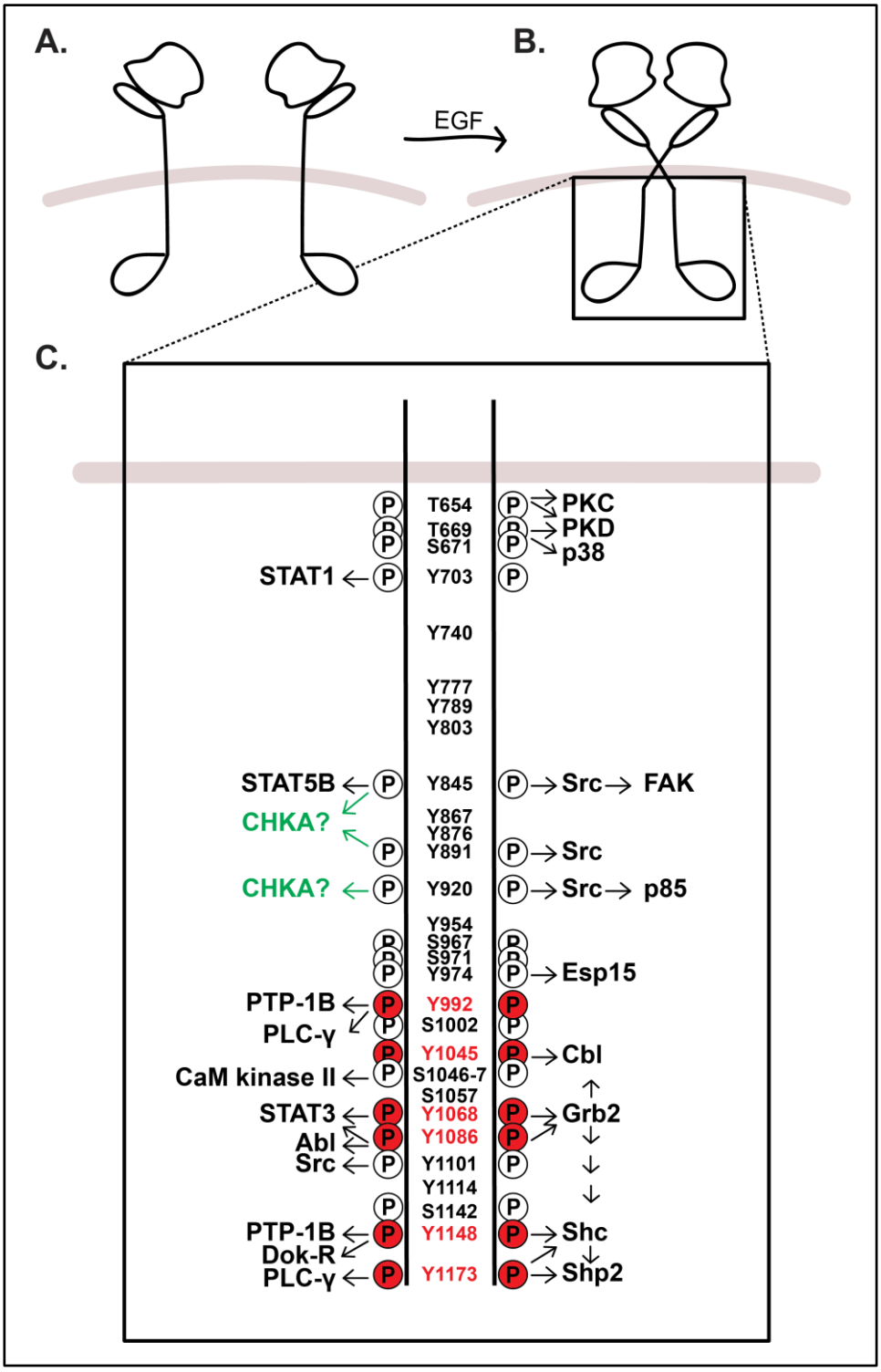


Figure 7.01: The recruitment of adaptor molecules to major EGFR tyrosine phosphorylation sites.

(A) The EGFR exists as monomers at the plasma membrane. Ligand binding to the extracellular region results in **(B)** ligand-induced receptor dimerisation **(C)** and the phosphorylation of distinct tyrosine as well as serine/threonine resides, acting as docking sites for a number of effector proteins. T: threonine residue, S: serine residue, white coloured P: phosphorylation sites, red coloured P: phosphorylation sites that bind Grb2. Figure adapted from Huang and Chang (Huang & Chang, 2011).

With these unique tools on offer, it is important to identify novel candidates that can precisely discriminate between individual signalling pathways. Of course, this reasoning lead to the development of the functional siRNA screen that identified three novel genes: BMX, CHKA and TRIO (George, Purdue, Gould et al., 2013). Accordingly, the first major undertaking of my project was to investigate the potential involvement of these novel proteins in controlling AngII-mediated EGFR transactivation. Given the anti-tumour effects of CHKA inhibitors (Challapalli, Trousil, Hazell et al., 2015; Granata, Nicoletti, Tinaglia et al., 2014; Lacal & Campos, 2015), and its reported association with the EGFR (Lin, Hu, Gu et al., 2017; Miyake & Parsons, 2012), my first priority was better understating CHKAs involvement in EGFR transactivation. Disappointingly, preliminary studies indicated that the addition of the BRET tags renders CHKA inactive and I was unable to use BRET to detect an association with the EGFR.

BMX, however, was shown to interact with the EGFR following EGF-stimulation, with this association further enhanced in cells co-expressing AT_1R. This association was partially dependent upon G_q coupling as well as AT_1R phosphorylation and arrestin recruitment. Interestingly, upon AngII-treatment, EGFR-BMX de-associated, with a similar observation observed when testing AT_1R-BMX interaction, It will be imperative to examine the contribution of BMX in additional relevant models of AT_1R-EGFR transactivation, predominantly in cardiac and vasculature systems. Although further experiments are required to better understand BMX function, it certainly supports the idea that BMX is a genuine mediator of transactivation (George, Purdue, Gould et al., 2013).

Despite the technical limitations associated with CHKA, this study offers new biology into the process of AT_1R-EGFR transactivation, and may provide new therapeutic strategies to combat cardiovascular hypertrophy and dysfunction. Indeed, new molecules that target various components of the RAS have been described recently (ACE2 activators (Ferreira, Shenoy, Qi et al., 2011; Prada, Ferreira, Katovich et al., 2008), Ang1-7 peptide and non-peptide analogs (Cunha, Lima, Silva et al., 2013; Savergnini, Beiman, Lautner et al., 2010)). The next generation of RAS modifying agents will provide increased fidelity and efficacy by targeting

specific molecules (i.e., BMX, CHKA or TRIO) in contrast to blocking the RAS or AT_1R completely.

A major observation of my thesis was that AT_1R-EGFR transactivation, as measured by Grb2 recruitment, was independent of $G_{q/11}$ and β-arrestins, which was not expected (especially given our previous work (Smith, Chan, Qian et al., 2011; Thomas, Brandenburger, Autelitano et al., 2002). To date, most studies of EGFR transactivation have used various inhibitors and/or antagonists to better understand the signalling pathways that underlie AT_1R-EGFR transactivation. The rapid development of genome editing, based largely on CRISPR/Cas9 technology, provides a powerful platform to eliminate the expression of protein(s) and explore downstream signalling consequences. During my canditure, Grundmann et al., 2018 used CRISPR/Cas9 technology to systematically dissect G protein- from arrestin-driven ERK1/2 phosphorylation for a broad set of GPCRs (Grundmann, Merten, Malfacini et al., 2018). In concert with our studies, they show that arrestins do not initiate AT_1R-mediated ERK1/2 signalling, instead emphasising the vital role of G proteins as genuine drivers of GPCR-mediated signal transduction. Although this paper may seem to contradict my conclusion (by clearly illustrating the importance of G protein signalling), an obvious discrepancy is the readout used to measure GPCR signal transduction. Through the use of pharmacological inhibitors, mutant forms of the AT_1R and CRISPR HEK293 cells depleted of $G_{q/11}$ and β-arrestins, I clearly demonstrate that AT_1R-EGFR transactivation as measured by Grb2 recruitment, is not $G_{q/11}$ mediated. In support of this observation, other groups have shown that GPCR-induced ectodomain shedding is predominantly a $G\alpha_{12/13}$ or $G\alpha_q$ mediated event, raising the possibility that other Gα family member and/or Gβγ subunits may be involved (Inoue, Ishiguro, Kitamura et al., 2012).

A final factor in considering AT_1R-EGFR transactivation was the existence of AT_1R-EGFR heterodimers. The use of BiFC and Receptor-HIT assays provided evidence that the AT_1R and EGFR exist as constitutive preformed complexes that are responsive to agonist stimulation. The development of RET-based assays has been especially popular methods to demonstrate the existence of receptor oligomers. Criticism has been raised regarding the use of these approaches, suggesting that random proximity can generate easily detectable RET signals between proteins that are restricted to the cellular membrane (Bouvier & Hébert, 2014;

Felce, Latty, Knox et al., 2017; Lambert & Javitch, 2014). I would argue that AT_1R-EGFR heteromers are unlikely to merely reflect chance co-localisation at the membrane and/or endosome because the association of the EGFR with a membrane marker, K-ras, was decreased following EGF-stimulation, but not with AngII-stimulation. Modulation of EGFR trafficking differed depending on whether it was modulated by activation of the AT_1R or direct activation of the EGFR, emphasising that the BRET signal (whether it be Receptor-HIT or direct tagging of the AT_1R or EGFR) was not due to chance localisation.

As already mentioned in Chapter 4.0, the existence of AT_1R-EGFR heteromers is consistent with previous data (Olivares-Reyes, Shah, Hernandez-Aranda et al., 2005), with a number of GPCRs shown to complex with the EGFR (Jensen, Godfrey, Niklas et al., 2013; Maudsley, Pierce, Zamah et al., 2000; Tilley, Kim, Patel et al., 2009; Zajac, Law, Cvetkovic et al., 2011). Although we show that modulation of AT_1R-EGFR heteromerisation differed depending on whether the complex was modulated by activation of the AT_1R or the EGFR, it is still unknown how this interaction effects downstream signalling of each receptor, or the mechanisms/binding domains that facilitate their interaction. To understand the functional significance of AT_1R-EGFR heterodimers requires the identification of heterodimer selective ligands or the development of molecular strategies that selectively disrupt AT_1R-EGFR complexes. Early studies have shown that peptides corresponding to GPCR transmembrane domains target protein-protein interaction interfaces and interfere with GPCR dimerisation (Hebert, Moffett, Morello et al., 1996; Jastrzebska, Chen, Orban et al., 2015), meanwhile antibodies have been described that sterically block EGFR domain rearrangement and receptor dimerisation (Li, Schmitz, Jeffrey et al., 2005; Schmiedel, Blaukat, Li et al., 2008; Yang, Yang, Pike et al., 2010), however neither approaches have been explored in the context of preventing GPCR-RTK dimers.

The spatial distribution of receptors following their activation is a critical determinant of downstream signal propagation. The development of single-molecular microscopy methods has allowed for the direct visualisation of individual membrane proteins and has provided the means to investigate the spatiotemporal organisation of receptors. Single molecule measurements have shown that receptor activation leads to the compartmentalisation of receptors with cytosolic effector and adaptor molecules, leading to higher-order signalling

clusters (Wu, 2013; Yu, 2016). Indeed, studies have revealed that GPCRs and G proteins transiently interact with each other and form dynamic signalling nanodomains or 'hot spots' at the plasma membrane (Sungkaworn, Jobin, Burnecki et al., 2017). In the case of RTKs, EGF stimulation significantly reduces EGFR mobility in a kinase-dependent manner, consistent with the formation of signalling clusters (Chung, Akita, Vandlen et al., 2010; Low-Nam, Lidke, Cutler et al., 2011). In concordance with earlier studies (Didion, 2016; Olivares-Reyes, Shah, Hernandez-Aranda et al., 2005), a common theme that has emerged from single-molecule studies is that the structural components of the plasma membrane play an important role in regulating receptor interaction.

Live-cell, single-molecule studies have also challenged the classical view that GPCR signalling occurs exclusively at the cellular membrane (or the endosomal membrane in the case of β-arrestin-dependent activation of MAPK), having demonstrated that GPCRs can signal at Golgi/trans-Golgi network (Calebiro, Nikolaev, Gagliani et al., 2009; Ferrandon, Feinstein, Castro et al., 2009; Irannejad, Tomshine, Tomshine et al., 2013). Internalised thyroid stimulating hormone receptor (TSHR) trafficks retrogradely to the trans-Golgi network, where it activates endogenous G_s-protein, leading to late phase cAMP/PKA activation and gene transcription (Godbole, Lyga, Lohse et al., 2017). This newly discovered modality of intracellular GPCR signalling might be particularly relevant for AT_1R-EGFR transactivation. Future studies are required to not only further investigate the nature, size and composition of AT_1R-EGFR complexes but whether these complexes also form at intracellular sites.

While most of this thesis focused on information flowing from the AT_1R to the EGFR, a control experiment revealed an unanticipated observation, whereby EGF-mediated Ca^{2+} signalling was dependent on a component emanating from the AT_1R and its capacity to couple to G proteins, representing the first overt example of what we describe as 'reverse transactivation'. Using a BRET-based approach, I demonstrate that EGF-stimulation activates pathways downstream of the AT_1R, whereby EGFR activation can mediate β-arrestin recruitment to the activated and phosphorylated AT_1R. Using AT_1R conformation-sensitive biosensors, I observed that EGF-stimulation promoted conformational changes that were distinct from those produced by AngII. This difference was also reflected in the fact that

in contrast to AngII, which causes a rapid internalisation of the AT_1R from the cell surface to endosomes, EGF-stimulation lead to a retention of the AT_1R at the plasma membrane. A recent study that examined AT_1R heterologous phosphorylation and arrestin recruitment, also provided evidence that EGF stimulation promotes β-arrestin2 binding to the AT_1R in a PKC-dependent manner (Tóth, Prokop, Gyombolai et al., 2018). Although this study did not directly focus on this observation, they suggest a mechanism whereby arrestin recruitment does not require the activate state of the receptor. Given that the significant proportion of the EGF signalling is blocked by candesartan, we would argue the possibility exists that the EGFR can directly activate the AT_1R.

How this novel transactivation process occurs (a RTK can transactivate a GPCR) remains an open question. As already mentioned in Chapter 5.0, some groups have suggested that RTK-induced GPCR transactivation is mediated exclusively through intracellular signalling, while others have proposed that growth factor receptors trigger the release of GPCR ligands (El-Shewy, Johnson, Lee et al., 2006; Hobson, Rosenfeldt, Barak et al., 2001; Mira, Lacalle, González et al., 2001). Interestingly, the formation of GPCR-RTK complexes is also thought to mediate cross-communication between both receptor systems (Akekawatchai, Holland, Kochetkova et al., 2005). This alternative route in RTK signalling is still in its infancy, with further research required to determine the exact mechanisms that allow for EGFR to influence AT_1R activity.

This new concept of GPCR activation may have important implications for the development of future therapeutics. The EGFR is a validated therapeutic target for a number of different cancers; however there is emerging evidence that the RAS may also be involved in the ontogeny of selective cancers (Li, Zhang, Zhao et al., 2017; Wegman-Ostrosky, Soto-Reyes, Vidal-Millán et al., 2015; Wen, Dunne, O'Reilly et al., 2017; Xu, Fan, Wu et al., 2017). In 1998, a retrospective study conducted by Lever and colleagues, showed a decreased risk of lung and breast cancer in patients taking ARBs and ACE inhibitors (Lever, Hole, Gillis et al., 1998). This seminal study was the first to implicate the RAS as a potential component of cancer progression. Subsequent retrospective studies have been performed, however the results have been varied (Chae, Valsecchi, Kim et al., 2011; Christian, Lapane, Hume et al., 2008; Collaboration, 2011; Fryzek, Poulsen, Lipworth et al., 2006; Li, Malone, Weiss et al.,

2003; Wang, Liu, Chao et al., 2013). The most conclusive evidence to date is a large scale Cancer Outline Profile Analysis of gene expression-profiling datasets that comprised of nearly 3,200 microarray experiments (Rhodes, Ateeq, Cao et al., 2009). One of the most consistently amplified genes was AGTR1, which was overexpressed approximately 100-fold higher in 10-20% of breast tumours. Interestingly, AGTR1 over-expression was mutually exclusive with HER2 over-expression. More recently, in vitro studies have shown that perturbation of various RAS components reduce the growth of many tumour cells including breast cancer cells (Coulson, Liew, Connelly et al., 2017), non-small cell lung cancer cells (Aydiner, Ciftci & Sen, 2015), pancreatic cancer cells (Reddy, Baskaran & Molteni, 1995) and gynaecologic cancer cells (Ino, Shibata, Yamamoto et al., 2011).

While the use of therapeutics that targets the activities of EGFR and HER family members are commonplace for many cancers, resistance to these treatments remain a problem. One potential explanation is that some of the actions of the EGFR may necessitate the concomitant activation of the AT_1R and may provide an alternative avenue by which cancer cells can signal. Interestingly, it has been shown that the use of RAS blockers, particularly ARBs, significantly improves the overall survival of patients with metastatic non-small cell lung cancer (Aydiner, Ciftci & Sen, 2015). In fact, the survival benefit of ARBs was most beneficial when used concurrently with erlotinib treatment (an EGFR tyrosine kinase inhibitor). While these studies demonstrate the association of AT_1R activity with malignancy, there has been very little progress made in better understanding the molecular mechanisms underlying this. In this regard, identifying how the EGFR may activate the AT_1R remains part of ongoing studies. We are currently in collaboration with Professor Gregory Monteith to determine if EGFR signalling and motility, in a breast cancer cell line, is regulated by AT_1R signalling.

7.1 Summary

In summary, in this thesis I report an alterative approach to monitoring AT_1R-EGFR transactivation in live cells, which provides a more direct and proximal view of this process. Data presented here suggests that a re-evaluation of this paradigm might be required, providing evidence that AT_1R-EGFR transactivation may be independent of $G\alpha_{q/11}$ and β-arrestin activity. Instead, I demonstrate that both AngII- and EGF-stimulation promotes

AT_1R-EGFR heteromerisation. At present, it is difficult to reconcile whether this complex formation subserves transactivation. The existence of complexes may facilitate the novel concept of 'reverse transactivation.' I was able to demonstrate that EGFR actions may be mediated in part by the coincidental activation of the AT_1R, an observation that may have ramifications for our understanding of these receptors and drugs that target them, in cardiovascular disease and cancer.

Chapter Eight

List of References.

9.0 Reference list:

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Chapter Nine

Appendices.

9.0 Appendices

Appendix A: Solutions & Buffers

LB Media (1L)

10g	Bactotyrptone
5g	Yeast
10g	NaCl

pH to 7.2-7.4

+/- 15g Agar

10x ADS Buffer

68g	Sodium Chloride
47.6g	HEPES ($C_8H_{18}N_2O_4S$)
1.2g	Sodium dihydrogen Orthophosphate (NaH_2PO_4) (1.37g $NaH_2PO_4.H_2O$)
10g	Glucose
4g	Potassium Chloride
1g	Magnesium Sulfide ($MgSO_4$) OR (1.97g $MgSO_4$.7H2O)
900ml	UHP Water (Autoclaved)

pH media to 7.35 +/- 0.05 using 1M Sodium Hydroxide (NaOH)

1x ADS Buffer

100mL	10x ADS Buffer
900mL	UHP Water (Autoclaved)

PBS

1 packet	Dulbecco Phosphate Buffered Saline
1L	UHP Water (Autoclaved)

4xTris-HCl/SDS (pH 6.8)

0.125M	Tris-HCl
0.1%	SDS

4xTris-HCl/SDS (pH 8.8)

0.375M	Tris-HCl
0.1%	SDS

DMEM + 10% FBS

1 packet	Dulbecco's modified Eagle Media (containing high glucose, L-glutamine and sodium bicardbonate) (Invitrogen, 12100-038)
50mL	FBS (heat inactivated)

Enzyme Digest Buffer (EDB)

110-140U/mL	Collagenase II (Worthington Biochemical Corporation, 4176)
0.8mg/mL	Pancreatin (Sigma, P-3292)
Variable	Enough 1x ADS buffer to make a total of 300uL EDB/Heart

DMEM CCT SS2 (1L)

1 packet	DMEM powder (Invitrogen, 12100-038)
800ml	UHP Water (autoclaved)
50mL	7.5% Sodium Bicarbonate (final concentration 4.4mM)
20mL	50x Essential Amino Acids (Invitrogen, 11130-051)
10mL	100x Non-Essential Amino Acids (Invitrogen, 11140-050)
10mL	100x Antibiotic/Antimyotic (Invitrogen, 15240-062)
10mL	100x Sodium pyruvate (Invitrogen, 11360-070)
10mL	100x Vitamins (Invitrogen, 11120-052)
400uL	4.9mg/mL Insulin (final concentration 2μg/mL)
30mG	BrDu
1mL	10mg/mL Apo-transferrin
25.64mL	2M Potassium Chloride

pH to 7.2 using 1M HCl

MEM + 10% NBCS (1L)

1packet	MEM powder to make 1L (Invitrogen, 11900-016)
800ml	UHP Water (autoclaved)
29ml	7.5% Sodium Bicarbonate solution
20mL	50x Essential Amino Acids (Invitrogen, 11130-051)
10mL	100x Non-Essential Amino Acids (Invitrogen, 11140-050)
10mL	100x Antibiotic/Antimyotic (Invitrogen, 15240-062)
10mL	100x Vitamins (Invitrogen, 11120-052)
100ml	NBCS heat inactivated (Invitrogen, 16010-159)

pH to 7.2 using 1M HCl

Percoll Stock gradient

900mL	Percoll (Sigma, P-1644)
100mL	10x ADS Buffer

0.1% Gelatin

1g	Gelatin (Sigma, G1890)
1L	UHP Water (Autoclaved)

2M Potassium Chloride

37.27g	Potassium Chloride
200mL	UHP Water (autoclaved)

BrDu (10.257mM, 100x)

31.5mg	BrDu (Sigma, B5002)
10mL	MEM + 10% NBCS

Insulin (4.9mg/mL)

100mg	Insulin (Sigma, I6634)
200mL	UHP Water (autoclaved)
400uL	1M HCl Acid

Apo-Transferrin (10mg/mL)

100mg	Apo-transferrin (Sigma, T1147**)**
10mL	UHP Water (autoclaved)

RIPA Buffer (with protease and phosphatase inhibitors)

86.5mL	ddH_2O
2.5mL	2M Tris-HCl (pH7.5)
2mL	5M NaCl
0.4mL	0.5M EDTA pH 8.0
5mL	1M NaF
1mL	10% SDS
2.5mL	20% Deoxycholic acid (sodium salt)
OR	
0.5g	Deoxycholic acid

10x Running Buffer

60.4g	Tris Base (Trizma base)
288g	Glycine
20g	SDS

Make up to 1L with water

10x Transfer Buffer

72.5g	Tris
36.6g	Glycine

Make up to 1L with water

1x Transfer Buffer

100mL	10x Transfer buffer
200mL	Methanol

Make up to 1L with water

Blocking Buffer

50% Odyssey blocking buffer

50% 1x PBS

50x TAE Buffer (1L)

242g Tris base

57.1mL Glacial acetic acid

18.6g EDTA

Make up to 1L with water and pH 8.5

Western Gels

12% Resolving Gel	**# of Gels**				
Item	1	2	3	4	5
40% Acrylamide/bis acrylamide 37:5:1	4.5mL	9mL	13.5mL	18mL	22.5mL
4x Tris-HCl/SDS pH 8.8	3.75mL	7.5mL	11.25mL	15mL	18.75mL
ddH_20	6.75mL	13.5mL	20.25mL	27mL	33.75mL
APS	75uL	150uL	225uL	300uL	375uL
TEMED	15uL	30uL	45uL	60uL	75uL

10% Resolving Gel	**# of Gels**				
Item	1	2	3	4	5
40% Acrylamide/bis acrylamide 37:5:1	3.75mL	7.5mL	11.25mL	15mL	18.75mL
4x Tris-HCl/SDS pH 8.8	3.75mL	7.5mL	11.25mL	15mL	18.75mL
ddH_20	7.5mL	15mL	22.5mL	30mL	37.5mL
APS	75uL	150uL	225uL	300uL	375uL
TEMED	15uL	30uL	45uL	60uL	75uL

8% Resolving Gel	**# of Gels**				
Item	1	2	3	4	5
40% Acrylamide/bis acrylamide 37:5:1	3mL	6mL	9mL	12mL	15mL
4x Tris-HCl/SDS pH 8.8	3.75mL	7.5mL	11.25mL	15mL	18.75mL
ddH_20	8.25mL	16.5mL	24.75mL	33mL	41.25mL
APS	75uL	150uL	225uL	300uL	375uL
TEMED	15uL	30uL	45uL	60uL	75uL

4% Stacking Gel	**# of Gels**	
Item	3	6
40% Acrylamide/bis acrylamide 37:5:1	975uL	1.95mL
4x Tris-HCl/SDS pH 8.8	2.5mL	5mL
ddH_20	6.43mL	12.85mL
APS	75uL	150uL
TEMED	15uL	30uL

Appendix B: Animals Ethics & Approval Certificate

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Office of Research Ethics
Director
Nicole Shively

Animal Ethics Approval Certificate

26-Apr-2018

Please check all details below and inform the Animal Ethics Unit within 10 working days if anything is incorrect.

Activity Details

Chief Investigator: Dr Dominic Ng, Biomedical Sciences

Title: Molecular and pharmacological regulation of cardiac myocyte cell death.

AEC Approval Number: SBMS/351/17

Previous AEC Number:

Approval Duration: 02-Sep-2017 to 02-Sep-2019

Funding Body:

Group: Anatomical Biosciences

Other Staff/Students: Yvonne Yeap, Dominic Richards, Shannon O'Brien, Uda Ho, Melissa Reichelt, Kevin Wathen-Dunn

Location(s): St Lucia Bldg 75 - AIBN

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Rats - non genetically modified	Sprague Dawley (neonate)	Juvenile / Weaners / Pouch animal	Unknown		2112	2016

Permits

Provisos

Approval Details

Description	Amount	Balance
Rats - non genetically modified (Sprague Dawley (neonate), Unknown, Juvenile / Weaners / Pouch animal,)		
2 Sep 2017 Initial approval	2112	2112
31 Dec 2017 Use in 2017 (from 2018 MAR; AEMAR37981)	-96	2016

Animal Ethics Unit
Office of Research Ethics
The University of Queensland

Cumbrae-Stewart Building
Research Road
St Lucia Qld 4072 Australia

+61 7 336 52925 (Enquiries)
+61 7 334 68710 (Enquiries)
+61 7 336 52713 (Coordinator)

animal.ethics@research.uq.edu.au
uq.edu.au/research