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Molecular characterisation of the adiponectin receptors, AdipoR1 and AdipoR2

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Abstract

The increasing global prevalence of cardiovascular and metabolic diseases necessitates the development of more effective therapeutic strategies that, in turn, require a greater understanding of the regulatory networks involved. Research over the last decade has increased our appreciation of the key role of the adiponectin axis as a major regulator of metabolic, cardiovascular and inflammatory tone, thereby establishing it as a province of therapeutic opportunity. The receptors for adiponectin, AdipoR1 and AdipoR2, are distant relatives of the largest single class of drug targets, the G-protein coupled receptor (GPCR) family. However, unlike GPCRs they have intracellular N-termini and extracellular C-termini and signal via atypical pathways. Our current understanding of AdipoR1 and AdipoR2 is rudimentary, constraining our ability to target these receptors effectively. The aim of this thesis was to characterise molecular features of AdipoR1 and AdipoR2 that facilitate adiponectin signal transduction to advance our understanding and identify strategies to enhance adiponectin's beneficial effects.

We have begun to characterise basic properties of AdipoR1 and AdipoR2, focusing on molecular factors that drive cell-surface expression (CSE) of the receptors using a range of C-terminal, epitope-tagged AdipoR1 and AdipoR2 constructs. Surprisingly, under steady-state conditions (no serum starvation) only AdipoR1 was readily detected on the cell-surface (cell-surface ratio of AdipoR1 vs AdipoR2 is 0.6±0.1 vs 0.15±0.1, $p<0.05$). Generation and characterisation of a series of chimeric and truncated constructs demonstrated that a non-conserved, intracellular, N-terminal region of AdipoR2 ($R2_{(1-81)}$) restricted its CSE whilst the same region in AdipoR1 ($R1_{(1-70)}$) promoted its CSE. We also confirmed that AdipoR1 and AdipoR2 form heterodimer and that co-expression of these receptors increase the CSE of AdipoR2. Subsequently, we provided evidence that the subcellular localisation of AdipoR1 and AdipoR2 is governed by multiple motifs across their non-conserved and conserved cytoplasmic domains. For instance, two highly conserved motifs, an ER exit motif (FxxxFxxxF) and Di-Leucine motif (DxxxLL), in the conserved N-terminal domain are required for the proper CSE of both AdipoR1 and AdipoR2, whilst different parts of the non-conserved domain of AdipoR2 inhibits its CSE.

Moreover, we demonstrated that in HEK-293 cells over-expressing AdipoR1 adiponectin activated downstream signalling networks (AMPK, AKT, ERK & P38MAPK) acutely (peaking at 15 min) whereas signal transduction via AdipoR2 was relatively chronic (peaking at 24 h). This difference was also underpinned by the non-conserved N-terminal domains of AdipoR1 and AdipoR2. We also demonstrated that a number of conserved and non-conserved cysteines in the N-terminal domain of

AdipoR1 and AdipoR2 are subject to palmitoylation and that palmitoylation of a conserved cysteine, situated in the juxta-membrane region of the N-termini of AdipoR1 and AdipoR2 in a position analogous to that observed in GPCRs, plays a key role in the CSE of both receptors. Mutation of these sites inhibits CSE and signal transduction of full-length receptors *in vitro* and *in vivo*. Furthermore, palmitoylation of these 'canonical cysteines' promotes enrichment of N-terminal, cytoplasmic AdipoR1($R1_{(1-127)}$) and AdipoR2 ($R2_{(1-138)}$) constructs under the PM. Our further investigation revealed the differential effects of electrotransfer-mediated overexpression of AdipoR1 or AdipoR2 in the Tibialis Anterior (TA) muscle of lean (chow) or obese (10 wk HFD) mice (n=6/group). In lean mice, overexpression of AdipoR1 or AdipoR2 increased phosphorylation of downstream effectors AMPK, Akt and ERK (all $p<0.05$), but not p38MAPK. The magnitude of these effects was reduced in obese mice; consistent with the development of adiponectin resistance (circulating adiponectin was not reduced after 10 wk HFD). Both AdipoR1 and AdipoR2 increased *glut-4* mRNA (2-fold, $p<0.05$) and this was also affected by obesity. In contrast, only AdipoR2 increased *pparα* and a downstream target gene *Acox1* (all $p<0.05$) and this effect was blunted by obesity. Surprisingly, exclusive overexpression of AdipoR2 in TA muscle of obese mice resulted in marked systemic effects which included increased circulating adiponectin levels, decreased body weight gain and reduced epididymal fat mass and markers of adipose tissue inflammation (all $p<0.05$).

Collectively these results indicate that there are (i) fundamental differences between AdipoR1 and AdipoR2 and demonstrate that (ii) there are specific motifs in the intracellular N-terminal region of both AdipoR1 and AdipoR2 regulating the subcellular trafficking, (iii) both receptors need palmitoylation for efficient cell-surface expression and signal transduction. Also (iiii) Muscle-specific overexpression of AdipoR1 or AdipoR2 gives rise to differential local and systemic effects. Further studies are required to extend these novel observations and elaborate the complex mechanisms governing AdipoR trafficking and signalling to determine whether alterations in these processes contribute to the aetiology of human disease and or can be targeted therapeutically.

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 during candidature

Peer-reviewed papers:

1- Kim, Y.H. *, Barclay J.L. *, He J., Luo X., O'Neill H.M., **Keshvari S.**, Webster J.A., Ng C., Hutley L.J., Prins J.B., and Whitehead J.P., Identification of carboxypeptidase X (CPX)-1 as a positive regulator of adipogenesis. *FASEB J*,2016.

2- Barclay J.L.*, **Keshvari, S.*,** Whitehead J.P., Inder W.J., ANNALS EXPRESS: Development of an enzyme-linked immunosorbent assay for thrombospondin-1 and comparison of human plasma and serum concentrations. *Ann Clin Biochem,* 2016.

3- Barclay J.L., Petersons C.J., **Keshvari S**, Sorbello J., Mangelsdorf B.L., Thompson C.H., Prins J.B., Burt M.G., Whitehead J.P., Inder W.J., Thrombospondin-1 is a glucocorticoid responsive protein in humans. *Eur J Endocrinol,* 2015. EJE-15-0964.

4- **Keshvari, S.** and Whitehead J.P., Characterisation of the adiponectin receptors: Differential cell-surface expression and temporal signaling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks. *Mol Cell Endocrinol,* 2015. 409: p. 121-9 (Incorporated as Chapter 2)

5- Yang, M., Kimura, M., Ng, C., He, J., **Keshvari, S.**, Rose, F. J., Barclay, J. L., Whitehead, J. P., Induction of heme-oxygenase-1 (HO-1) does not enhance adiponectin production in human adipocytes: Evidence against a direct HO-1 - Adiponectin axis. *Mol Cell Endocrinol*, 2015. 413: p. 209-216

6- **Keshvari, S.,** Rose, F. J., Charlton, H. K., Scheiber, N. L., Webster, J., Kim, Y. H., Ng, C. Parton, R. G., Whitehead, J. P., Characterisation of the adiponectin receptors: The non-conserved N-terminal region of AdipoR2 prevents its expression at the cell-surface. *Biochem Biophys Res Commun,* 2013. 432(1): p. 28-33. (Incorporated as Chapter 3)

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3- **Keshvari, S.** and Whitehead J.P., Characterisation of the Adiponectin Receptors: Palmitoylation of AdipoR1 and AdipoR2 is required for Efficient Cell-Surface Expression and Down-Stream Signalling. *Australian Diabetes Society*, Melbourne, 2014.

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Contributions by others to the thesis

The publications submitted as part of this thesis have had contributions from other authors. The nature and extent of these contributions are detailed above.

- The electron microscopy analysis in chapter 2 figure 1E was performed by Dr Nicole L. Scheiber.

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Keywords

AdipoR, adiponectin, receptor, seven-transmembrane receptor, cell-surface expression, signal transduction, palmitoylation, electrotransfer.

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Fields of Research (FoR) Classification

FoR code: 0601, Biochemistry and Cell Biology, 80%

FoR code: 0699, Other Biological Sciences, 20%

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List of Figures

List of Abbreviations used in the thesis

7TM	Seven transmembrane
ABE	Acyl-biotinyl exchange
ACC	Acetyl-CoA carboxylase
AdipoR	Adiponectin receptors
Akt	Protein kinase B (PKB) also known as Akt
AKT2	oncoprotein-serine/threonine kinase
AMPK	Adenosine monophosphate- activated protein kinase
AMP	Adenosine monophosphate
APPL1	Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding and leucine zipper motif
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
C-NTD	Conserved N-terminal Domain
CD11b	Cluster of differentiation molecule 11B
CD68	Cluster of differentiation 68
CHO	Chinese Hamster Ovary
CSE	Cell Surface Expression
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
eNOS	Endothelial NO synthase
ECL	Extracellular loop
ER	Endoplasmic reticulum
ERK1/2	signal-regulated kinases 1 and 2
ERp46	Endoplasmic reticulum protein 46
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FOXO1	Forkhead box protein O1
gAD	Globular adiponectin
GFP	Green Fluorescent Protein
GLUT4	Glucose transporter type 4
GPCR	G-protein coupled receptor
HA	Hemagglutinin
HEK	Human Embryo Kidney
HFD	High fat diet

HMW	High Molecular Weight
HSP	High speed pellet
ICL	Intracellular loop
IF	Immunofluorescent microscopy
IL-6	Interleukin-6
IRS	Insulin receptor substrate
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
KO	Knockout
LMW	Low Molecular Weight
µg (ug)	Microgram
µl (ul)	Microlitre
min	Minute
mg	Milligram
ml	Millilitre
MW	Molecular Weight
MCP-1	Monocyte chemotactic protein-1
NC-NTD	Non-conserved N-terminal Domain
P38MAPK	p38mitogen-activated protein kinase
PAQR	Progestin and AdipoQ receptor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinases
PM	Plasma membrane
PPARα	Peroxisome proliferator-activated receptor α transcription factor
PTB	Phosphotyrosine binding
PTMs	Post translational modification
qRT PCR	quantitative real time Polymerase Chain Reaction
RACK	Receptor for activated protein kinase C1
RNA	Ribonucleic acid
SCD	Standard chow diet
SDS	Sodium dodecyl sulphate

siRNA	Small interfering RNA
SIRT1	Sirtuin 1
STAT3	Signal transducer and activator of transcription 3
TAM	Tibialis anterior muscle
TM	Transmembrane
TNFα	Tumour necrosis factor α
TXVDC5	Thioredoxin domain containing protein 5 precursor
UCP	Uncoupling Protein

Chapter One:

General Introduction

1.1 Obesity

Obesity, abnormal or excessive fat accumulation that may impair health[1], is one of today's most challenging public health problems which threatens to overwhelm health systems in both developed and developing countries. According to the World Health Organization (WHO), in 1995 there were an estimated 200 million obese adults worldwide. As of 2000, the number of obese adults has increased to over 300 million[2, 3]. In Australia, adult obesity rose from 7.1% in 1980 to approximately 65% for men and 50% for women in 2011[4].

Obesity is a complex condition which can lead to a wide range of cardiometabolic disorders including coronary heart disease, hypertension and stroke, type-2 diabetes, dyslipidaemia, certain types of cancer, gallbladder disease, osteoarthritis, gout and pulmonary diseases including sleep apnoea[2].

Adipose tissue, which was considered as a simple fat store, is now known to be a highly metabolically active endocrine organ that secretes hormones and cytokines collectively termed adipokines[5]. Adiponectin is one of the most intensively investigated adipokine. It has anti-diabetic (insulin sensitising), anti-inflammatory, anti-atherogenic, cardio-protective and anti-cancer properties, regulating glucose and fatty acid metabolism.

1.2 Adiponectin

In 1995, Scherer *et al.* identified a novel adipokine which they named Acrp30 (adipocyte complement-related protein of 30 kDa) due to its structure and weight[6]. Three independent groups each identified the same protein and named it Apm1 (adipose most abundant gene transcript 1)[7], AdipoQ[8] and GBP28 (gelatin-binding protein of 28 kDa)[9] respectively. It is now called adiponectin[10].

1.2.1 Structure and multimerisation

Human adiponectin is a protein of approximately 28 kDa (244 amino acids) which belongs to the complement factor C1q family of proteins. It is made up of four domains including: an amino-terminal signal peptide; a variable region with no homology to other known proteins; a collagenous domain involved in triple-helix formation; and the carboxyl-terminus comprising the globular domain which shows sequence homology with the C1q subunit of complement protein and the globular domains of collagens type VIII and X[11-13] (Figure 1.1A). Adiponectin is synthesised in monomeric form which oligomerises to form a variety of stable multimeric forms including trimers and hexamers, known as low molecular weight (LMW) multimers and larger complexes up to 18-mers, termed as high molecular weight (HMW) multimers.

Generation of trimers occur upon the formation of a triple helix by hydrophobic interaction within the collagenous domains[14]. Further multimerisation of adiponectin depends upon a series of post translational modification (PTMs)[15, 16] and is important for the pleiotropic biological functions of adiponectin. Early studies demonstrated the role of a conserved cysteine 36 (Cys 39 in mouse adiponectin) in the formation of disulphide bond, which is essential for the stable generation of multimers larger than trimers[17-19]. A number of conserved proline (shown to be hydroxylated) and five conserved lysine (subject to hydroxylation and subsequent glycosylation) residues situated in the collagenous domain of adiponectin also play a significant role in the formation of trimers and efficient production and secretion of HMW multimers respectively[19, 20] (Figure 1.1B). Consequently, given that bacteria are unable to perform PTMs (with the exception of disulphide bonds), bacterially produced adiponectin contain only trimers and hexamers[21].

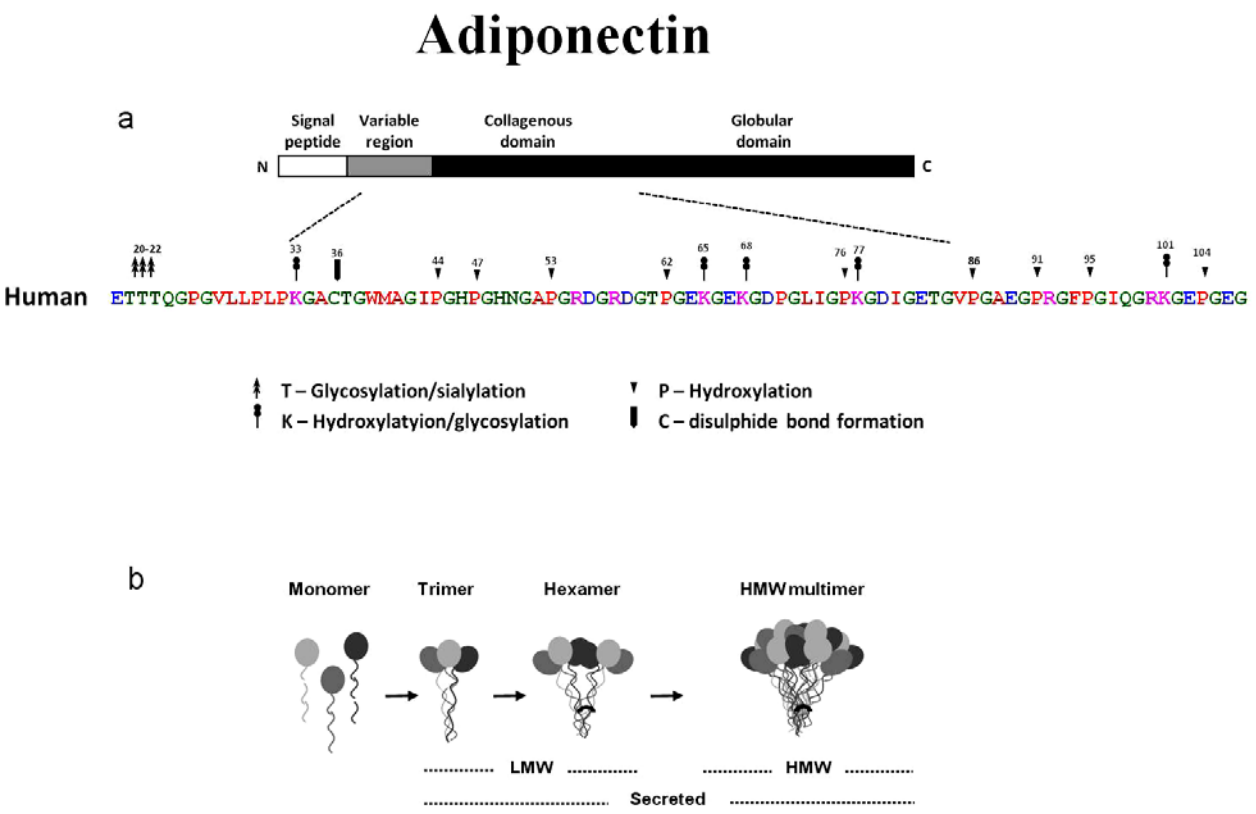

Figure 1.1. Adiponectin Post Translational Modifications and Multimerisation. (A) Adiponectin domains and post translational modification of the collagenous domain involved in multimerisation. **(B)** Adiponectin multimerisation and secreted forms. Adapted from (Richards *et al.*, 2006 and Richards *et al.*, 2010)[19, 22]

1.2.2 Adiponectin expression and its association with disease

Adiponectin is produced almost exclusively by adipocytes and is present in the circulation at a concentration of 2-30 ug/ml in human[23]. Although the adipocyte is the primary site of production, low levels of adiponectin, both at the mRNA and protein level, have been detected in other cell types such as osteoblasts[24], hepatocytes[25, 26] and myotubes[27] following treatment with inflammatory cytokines or other stressors. However, such 'ectopic' adiponectin production is unlikely to affect the circulating levels, due to the relatively low levels, and is proposed to act in an autocrine/paracrine manner[12]. In serum, adiponectin circulates as trimers, hexamers and HMW complexes[18]. In disease states such as obesity and type-2 diabetes, despite increased fat mass, circulating adiponectin levels decrease[28-30] with a selective reduction in HMW multimers[31, 32].

Similar reduction in adiponectin levels have been observed in other obesity-related cardiometabolic disorders such as cerebrovascular[33] and coronary artery disease[34, 35]. In patients with type-2 diabetes a reduction in adiponectin glycosylation has been also observed[36], providing further support of the importance and role of hydroxylation and glycosylation of conserved lysine residues in the formation and secretion of HMW multimers. Beside disease states, population studies demonstrated that adiponectin levels are higher in females than males[37] and increases by age in both genders[38]. Finally, in obesity and other related disorders elevated levels of pro-inflammatory cytokines have been observed and these cytokines have been shown to reduce adiponectin mRNA expression and protein secretion[39]. Overexpression of adiponectin from adipose tissue results in improved systemic insulin sensitivity and fatty acid oxidation[40, 41] whereas loss of function of adiponectin or its receptors results in decreased insulin sensitivity. Support for this contention comes from studies demonstrating adiponectin resistance at the level of the adiponectin receptors[42-46]. Collectively, these observations demonstrate that processes regulating adiponectin expression, multimerisation and secretion represent potential therapeutic targets.

1.3 Adiponectin Receptors

As described above, adiponectin has several beneficial properties such as insulin sensitising, anti-diabetes and cardioprotective effects. Adiponectin signalling networks appear to be complex and cell-type specific, however, a large body of evidence supports the view that most of adiponectin's pleiotropic effects are mediated through two cognate receptors termed AdipoR1 and AdipoR2[47] which represent the main focus of this project. Two additional adiponectin binding proteins, T-cadherin and PAQR3, will also be briefly described.

1.3.1 AdipoR1 and AdipoR2

In 2003, Kadowaki and colleagues performed a functional screen of a cDNA library from human skeletal muscle to identify proteins that bound globular adiponectin[48]. The isolated cDNA encoded for a protein termed AdipoR1 that showed 96.8% homology between human and mouse. A second, homologous reading frame was identified using bioinformatics and the gene product was also shown to bind adiponectin. The encoded protein exhibited 68% identity to AdipoR1 and was named AdipoR2. Further, independent structural/bioinformatics studies established AdipoR1 and AdipoR2 as prototypical members of the progestin and AdipoQ receptor (PAQR) superfamily of receptors that is unified by (predicted) structural homology. All PAQR members are predicted to have at least seven transmembrane domains (TMD), an ExxNxxxH motif that precedes TM1, an SxxxHxnD motif that spans the end of TM2 and the beginning of TM3, and an HxxxH motif that precedes

TM7. So far 11 members of human PAQR family are defined by UPF0073 motif which can identify PAQR proteins but cannot conjugate to other proteins[49]. Based on the sequence comparisons, the PAQR family is grouped to three main subclasses and AdipoR1 and AdipoR2 belong to the class I subfamily[49, 50]. More detailed discussion of the key features of AdipoR1 and AdipoR2 are discussed below, following the brief sections on T-cadherin and PAQR3.

1.3.2 T-cadherin

In 2004, Lodish and colleagues reported that T-cadherin, a member of the large family of cadherin cell surface proteins, bound hexameric and high molecular weight adiponectin multimers[51]. Cadherins are a class of cell adhesion molecule involved in calcium-dependent cell-cell interactions. T-cadherin is a unique member of this family as it lacks transmembrane and intracellular domains. It is anchored to the plasma membrane (PM) via a glycosyl phosphatidyl inositol (GPI) anchor[52]. T-cadherin is highly expressed in the vasculature and has been shown to be critical for adiponectin-mediated cardioprotection in mice demonstrating physiological relevance and a positive role[53]. In addition, expression of T-cadherin is upregulated in parallel with adiponectin accumulation at the sites of vascular injury[54]. Only hexamer and HMW adiponectin binds to T-cadherin which further demonstrate the importance of multimerisation and, indirectly, the post translational modifications (PTMs) that are required to facilitate hexamer/HMW multimer formation[51, 55]. Additional studies supported the interaction of adiponectin and T-cadherin, showing adiponectin in mammary tumours from wild-type (T-cadherin expressing) mice but not in T-cadherin deficient mice[56]. Beside the mentioned studies highlighting a positive role for T-cadherin, there is evidence to support a negative role. For instance, it is demonstrated that reduction of T-cadherin mRNA, using siRNA, increased adiponectin stimulated phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2)[57]. However, this could reflect limitations of the in vitro system such as differences between cell types and still support that T-cadherin plays a role mediating adiponectin effects. Furthermore, several genomic studies suggest that changes in T-cadherin, encoded by *Cdh13*, are associated with variation in circulating adiponectin levels in humans and a propensity to develop or be protected from cardiovascular and metabolic disease[56, 58-60]. Although T-cadherin lacks the intracellular domain needed for signal transduction, it can participate in intracellular signalling cascade by competing with AdipoR1 and AdipoR2 for adiponectin binding[57]. Little information is available concerning the regulation of T-cadherin expression. However, it has been demonstrated that the addition of progesterone and epidermal growth factor to human osteosarcoma cells results in an increase in T-cadherin mRNA expression[61].

Given that T-cadherin, directly or indirectly, mediates adiponectin specific actions many people consider T-cadherin to be an adiponectin receptor.

1.3.3 PAQR3

In 2009 Garitaonandia *et al* implicated PAQR3 or Raf Kinase Trapping to Golgi (RKTG), a member of the class I PAQR superfamily, as another adiponectin receptor[50]. They showed that wild type yeast did not respond to adiponectin but expression of PAQR3 conferred adiponectin sensitivity. In this study a functional assay was performed in yeast *Saccharomyces cerevisiae* that showed when PAQR receptors, from diverse sources, were heterologously expressed in yeast, they could activate the same downstream signal transduction pathway that repressed *FET3-lacZ* expression in response to their respective agonist ligands. It is demonstrated that addition of 100 pM adiponectin caused the repression of *FET3-lacZ* in cells expressing AdipoR1, AdipoR2, and PAQR3 but not the other members of the PAQR family such as PAQR4[50].

PAQR3 is a Golgi-localized membrane protein that modulates intracellular signalling by sequestering proteins onto the Golgi apparatus through spatial regulation of Raf kinase and G-subunit of G protein coupled receptors[62-64]. Given that PAQR3 is intracellular may explain why it was not recognized in earlier studies that used classical (binding) screening approaches[48, 51]. Overexpression of PAQR3 causes it to interact with Raf-1 kinase and sequesters Raf-1 kinase in the Golgi leading to suppression of carcinogenesis[50]. It was also showed that PAQR3 has a tumour suppressor activity in the development of colorectal cancers[65]. These data have led the author to the suggestion that the anticancer effects of adiponectin may be through effects on Raf-1 kinase via regulation of PAQR3. However, there is no evidence supporting that adiponectin modulates raf-1 kinase. Recently Wang *et al*. demonstrated that mice with deletion of PAQR3 are resistant to HFD-induced obesity and hepatic steatosis[66]. This study revealed that PAQR3 deletion in HFD mice improves insulin signalling, accompanied by increased energy expenditure and physical activity. Moreover, overexpression of PAQR3 reduced leptin signalling while down-regulation of PAQR3 enhanced leptin signalling in the hypothalamus[66]. There is evidence showing that PAQR3 regulates insulin signalling by tethering the p110α subunit of PI3K to the Golgi apparatus to modulate insulin sensitivity[67].

Collectively, it is demonstrated that PAQR3 plays an important role in regulating obesity and energy homeostasis accompanied by modulation of leptin signalling. Also there is evidence showing that PAQR3 has anti-cancer properties. However, the mechanism of its action and putative links with adiponectin remain obscure and require further study.

1.3.4 Adiponectin receptor structure

Human AdipoR1 is located on chromosome 1 (q32.1) and encodes a protein (AdipoR1) of 375 amino acids with a molecular weight of 42 kDa. Human AdipoR2 is located on chromosome 12 (p13.33) and encodes a protein (AdipoR2) of 386 amino acids with a molecular weight of 43 kDa. AdipoR1 and AdipoR2 are conserved across species, but they show no or very limited homology to other proteins at the primary amino acid level, with homology restricted to the PAQR family members[49, 68]. AdipoR1 and AdipoR2 are predicted to have 7 transmembrane (7TM) domains. Experimental findings demonstrate that AdipoR1 and AdipoR2 have intracellular N-termini and extracellular C-termini[48, 69] which are reverse to most other 7TM proteins, including the GPCRs. Overall amino acid homology across AdipoR1 and AdipoR2 is 68%. However, the first 70/81 amino acids of AdipoR1 and AdipoR2 show only 17% homology[70] whilst the rest of the sequences are 95% identical (Figure 1.2).

Recently, Kadowaki and colleagues[71] generated and solved the crystal structure of human adiponectin receptors[71]. In this study truncated form of AdipoR1 and AdipoR2 was used (residues 89-375 and 100-386 respectively). This means that the non-conserved and part of the conserved N-terminal domains were deleted and it was suggested that the truncated forms exhibited better expression and purification and displayed the same extents of adiponectin-stimulated downstream signalling[71]. However, it is well known that APPL1, the best characterised interacting protein for AdipoR1 and AdipoR2[72], and other interacting proteins[70, 73, 74] mediate most of the downstream signalling through interaction with the cytoplasmic domain of AdipoR1 and AdipoR2 (Detailed introduction of interacting proteins in section 1.3.4). It was demonstrated that the truncated form of AdipoR1 and AdipoR2 (residues 89-375 and 100-386 respectively) contains a short intracellular helix (residues 121-129; helix 0), seven transmembrane domain (helices 1-7), three intracellular loops (ICL 1-3) and three extracellular loops (ECL 1-3)[71]. Furthermore, it was demonstrated that the seven transmembrane domains form an enclosed large cavity where three conserved histidine residues coordinate a zinc ion[71]. Further functional studies indicated that the zinc-binding structure plays a role in adiponectin-stimulated AMPK phosphorylation and uncoupling protein 2 (UCP2) upregulation[71]. It was demonstrated that if the zinc-binding domain was compromised/mutated the receptor's capacity to transduce the adiponectin signalling, including AMPK activation and UCP2 expression, was reduced[71].

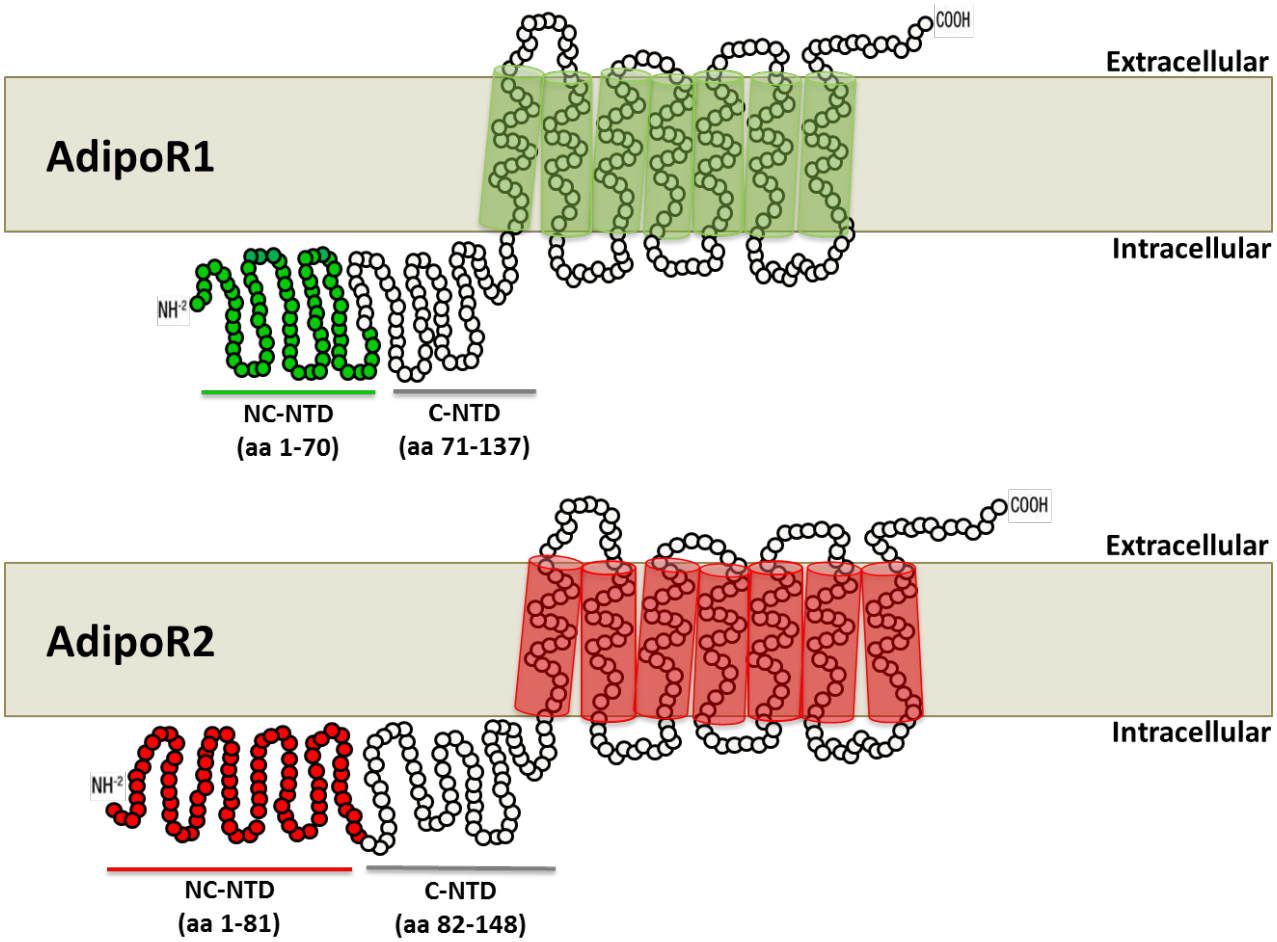

Figure 1.2. Schematic representations of AdipoR1 and AdipoR2 structure. Non-conserved N-terminal domain (NC-NTD) and conserved N-terminal domain (C-NTD).

1.3.5 Adiponectin receptor expression and regulation

Given the scarcity of good quality commercial antibodies for adiponectin receptors early studies reporting adiponectin receptor expression levels were based upon mRNA expression, which does not always correlate with protein levels. Indeed, diversity between expression of adiponectin receptor proteins and corresponding mRNA in monocytes of type 2 diabetic patients has been shown[75]. The studies exploring AdipoR1 and AdipoR2 expression demonstrated that they are fairly ubiquitously expressed with high levels in metabolically active tissues such as skeletal muscle[48, 76, 77] liver[48, 77, 78], heart[79-81] and adipose tissue[82, 83]. They are also expressed in other adiponectin responsive cells and tissues such as osteoblasts[84], pancreas[85, 86], leukocytes[75, 87] and the brain[88]. AdipoR1 is more highly expressed than AdipoR2 in all tissues except the liver in mice[48], however

such differences were less marked in human tissues with AdipoR2 mRNA being more ubiquitously expressed in human compared to mice[48]. The relative protein level in the various tissues, which is arguably more useful information, is currently unknown. Emerging evidence indicates that obesity-related diseases are characterised not only by hypoadiponectinemia but also by adiponectin resistance at the level of the adiponectin receptors [42-46]. In several mouse models of insulin-resistance, reduced AdipoR1 and AdipoR2 mRNA expression has been reported[89, 90]. Levels of free fatty acids and insulin are elevated in states of obesity and insulin-resistance and both have a negative effect on AdipoR1 and AdipoR2 expression, providing a potential explanation[76, 78, 83, 89, 91]. Reduced AdipoR1 and AdipoR2 mRNA is also observed in healthy humans with a family history of type-2 diabetes[92] who are predisposed to type-2 diabetes. This reduction in AdipoR1 and AdipoR2 expression may be, at least partly, one of the reasons for being predisposed to type-2 diabetes. Therapeutic treatments for insulin-resistance, like metformin and fibrates, increase adiponectin receptor expression[87, 93, 94]. Finally, up-regulation of AdipoR2, but not AdipoR1, has been demonstrated with growth hormone[82] while long-term exercise can specifically up-regulate AdipoR1 expression[95, 96]. The latter could contribute to enhanced insulin sensitivity in response to exercise[97], while the concentration of circulating adiponectin does not change following exercise[98].

1.3.6 Adiponectin receptor signalling

Adiponectin is thought to transduce many of its pleiotropic effects via AdipoR1 and AdipoR2 through the activation of a number of intracellular kinases including adenosine monophosphate (AMP)-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38MAPK) as well as activation of Peroxisome proliferator-activated receptor α transcription factor (PPARα)[99] (Figure 1.3). Early studies, involving suppression of AdipoR1 and AdipoR2 expression using siRNA suggested that AdipoR1 is a high-affinity receptor for globular adiponectin and low-affinity receptor for full-length adiponectin, whereas AdipoR2 has intermediate affinity for both the globular and full-length adiponectin[48]. However in these studies recombinant forms of adiponectin were used which does not resemble endogenous adiponectin. Our understanding of the molecular pathways connecting adiponectin receptors to their downstream effectors was increased with the discovery of adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif (APPL1) as a key signalling intermediate[100]. APPL1 is an adaptor protein of 79 kDa and is composed of multiple domains. The protein includes an N-terminal Bin–Amphiphysin–Rvs (BAR) domain, a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain[101]. The interaction of APPL1 with AdipoR1 and AdipoR2 is constitutive, is increased around two-fold upon adiponectin treatment, and is mediated by the PTB domain of

APPL1, interacting with the cytoplasmic domains of AdipoR1 and AdipoR2, although this appears to be independent of any tyrosine phosphorylation events[82,83]. The C-terminal containing PTB domain has also been shown to be critical for binding of APPL1 to a number of other proteins including oncoprotein-serine/threonine kinase (AKT2)[102], follicle stimulating hormone receptor (FSHR) and the tumour suppressor Deleted in Colorectal Carcinoma (DCC), and these interactions are also tyrosine phosphorylation independent[103].

APPL1 is a key mediator of adiponectin's effects as demonstrated in siRNA knockdown and overexpression studies[72]. APPL1 is required for adiponectin-induced activation of AMPK, Acetyl-CoA carboxylase (ACC) and p38MAPK[72]. Moreover, APPL1 lacking the PTB domain cannot interact with the AdipoR1 and AdipoR2 and caused a reduction in adiponectin- and insulin-stimulated glucose transporter type-4 (GLUT4) translocation[72]. It was suggested that the $APPL1^{\Delta PTB}$ mutant may function as a dominant negative inhibitor of adiponectin-mediated downstream events, probably by interaction and sequestration of endogenous APPL1[72]. As mentioned, APPL1 interacts with AKT2 and also with the p110 catalytic subunit of Phosphatidylinositol 3-kinase (PI3K)[102]. These two molecules are key elements in the insulin signalling pathway leading to the suggestion that APPL1 represents a link between adiponectin and insulin signalling[72]. The N terminal-BAR domain of APPL1 has been shown to interact with the GTP-bound (active) form of Rab5[72], a key regulator of endocytosis[101]. Interestingly, adiponectin-induced GLUT4 translocation in L6 myoblasts was blocked by overexpression of Rab5 and this was associated with a selective inhibition of p38MAPK activation[72] (Figure 1.3).

Endoplasmic reticulum protein 46 (ERp46; also called endo-protein disulphide isomerase (EndoPDI), plasma cell thioredoxin-related protein (PC-TRP) and Thioredoxin domain containing protein 5 precursors (TXVDC5)[104]) was identified as an AdipoR1-specific interacting protein[70]. ERp46 was shown to interact with amino acids 1-70 in the N-terminus of AdipoR1, which is highly conserved in AdipoR1 across species but non-conserved with AdipoR2[70]. ERp46 is a member of the thioredoxin family of proteins. It has thioreductase activity and is expressed in a range of tissues and cell-types including liver[104], plasma and endothelial cells[105]. It was originally identified in 3 proteomic screens[104-106], one of which demonstrated considerable enrichment in the ER[104]. Knockdown of ERp46 affected the distribution of AdipoR1, and AdipoR2, and adiponectin signalling with increased AdipoR1 and AdipoR2 levels at the PM and increased adiponectin-stimulated AMPK phosphorylation but reduced adiponectin-stimulated p38MAPK phosphorylation[70].

As mentioned above, adiponectin mediates its beneficial effects by activating a number of key signalling molecules such as AMPK, which plays a prominent role. AMPK is a ubiquitously

expressed intracellular energy sensor which regulates glucose and lipid homeostasis, body weight and food intake by responding to hormonal and nutrient signals and is activated by an increase in the intracellular AMP/adenosine triphosphate (ATP) ratio[107]. AMPK is a heterotrimer consisting of a catalytic α-subunit and regulatory β and γ subunits, each with different isoforms. Both AMP and ADP activate AMPK after binding to its γ-subunit[108-110], however, reversible phosphorylation at Threonine 172 within the activation loop of the α-subunit by upstream kinases is the most potent activator of AMPK [107, 111, 112].

AMPK activates mechanisms to increase production of ATP while reducing ATP-consumption. Once AMPK is phosphorylated by upstream kinases, AMPK phosphorylates ACC. Phosphorylation off ACC results in reduced malonyl CoA production and consequently inhibits carnitine palmitoyltransferase 1 (CPT-1), an enzyme controlling the transfer of long chain fatty acids to the mitochondria for their subsequent oxidation. Therefore, by phosphorylation of ACC more long chain fatty acids will be transferred to mitochondria thereby fatty acid oxidation increases[113]. Furthermore, AMPK promotes fatty acid uptake and oxidation by increasing the expression of genes regulated by PPARα such as CPTs and uncoupling protein 2 (*UCP2*) and 3 (*UPC3*)[113]. AMPK also reduces the expression of enzymes involved in the production of fatty acid, such as fatty acid synthase, thus reducing the accumulation of fatty acids in the liver[114].

AMPK also plays a critical role in glucose homeostasis by increasing glucose uptake through glucose transporters (GLUT4 and GLUT1) in muscle[115] and adipocytes[116] as well as decreasing expression of genes involved in the gluconeogenic pathway and consequently reducing hepatic glucose production[117].

The liver expresses both AdipoR1 and AdipoR2, whereas skeletal muscle expresses predominantly AdipoR1[48]. Importantly, obesity decreases not only plasma adiponectin but also AdipoR1 and AdipoR2 in liver and skeletal muscle to cause adiponectin resistance[118] and overexpression of AdipoR1 and AdipoR2 improves insulin resistance[90]. In 2007, Kadowaki and colleagues demonstrated that adenovirus-mediated restoration of AdipoR1 increased the activation of AMPK in the liver by adiponectin, whereas overexpression of AdipoR2 did not. However, overexpression of AdipoR2 significantly increases the expression of gene encoding PPARα itself as well as its target genes in the liver. These results suggested that in the liver, AdipoR1 is more involved in the activation of AMPK and subsequently reduces the expression of genes encoding hepatic gluconeogenic enzymes and therefore suppresses gluconeogenesis and lipogenesis. Conversely,

AdipoR2 activates the PPARα pathway to stimulate fatty acid oxidation and suppress inflammation and oxidative stress[90].

In skeletal muscle, insulin resistance has been reported to be associated with mitochondrial dysfunction[119]. In 2010, Kadowaki and colleagues used AdipoR1-knockout (muscle-R1KO) mice to determine whether decreased adiponectin/AdipoR1 signalling results in mitochondrial dysfunction[120]. Phosphorylation of AMPK was decreased in muscle-R1KO mice as were the levels of molecules involved in mitochondrial biogenesis, such as Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), at both mRNA and protein level. Further investigations, in C2C12 myocytes, revealed that adiponectin induces Ca^{2+} influx by AdipoR1, thereby activating CaMKKβ, which lead to increased PGC-1α expression. On the other hand, adiponectin/AdipoR1 activates AMPK and Sirtuin 1 (SIRT1), by this means inducing PGC1-α deacetylation and activation. Collectively these results indicate that adiponectin and AdipoR1 stimulate increases in both the expression and activation of PGC1-α, in a similar fashion to exercise, and decreased adiponectin/AdipoR1 signals in muscle in pathophysiological conditions such as obesity and type-2 diabetes may have roles in the development of PGC1-α dysregulation and mitochondrial dysfunction[120].

Aside from key metabolic target organs of liver and muscle, adiponectin also activates signalling pathways in a range of other tissues and cell lines. Adiponectin activates c-Jun N-terminal kinases (JNK) and p38MAPK pathways in human osteoblasts to stimulate proliferation and differentiation[121]. Such actions are mediated by AdipoR1 because suppression of AdipoR1 by siRNA leads to decreased effects of adiponectin on proliferation and differentiation. It has also been reported that proliferation of 3T3-L1 cells is stimulated by adiponectin and these cells only express AdipoR1[24]. In synovial fibroblasts the sequential activation of AMPK and p38MAPK has been demonstrated to be mediated by AdipoR1[122]. Also, in the hypothalamus AdipoR1 knockdown inhibits adiponectin-mediated phosphorylation of Insulin receptor substrate 1 (IRS1), IRS2, AKT and Forkhead box protein O1 (Foxo1) as well as phosphorylation of JAK2 and Signal transducer and activator of transcription 3 (STAT3)[88]. Furthermore, globular adiponectin acts through AdipoR1 to inhibit leptin-induced proliferation of an oesophageal adenocarcinoma cell line[123]. One of the important effects of adiponectin appears to be to stimulate production of nitric oxide (NO) in endothelial cells which contributes to vasodilation[124]. It also reduces rupturing of atherosclerotic plaques in vessel walls[23]. These data provide evidence that adiponectin has various effects on a number of cell types and that these are typically mediated by AdipoR1. Adiponectin stimulates ERK1/2 phosphorylation in primary vascular smooth muscle, vascular endothelial cells, hepatocytes

and Human Embryonic Kidney 293 (HEK 293) cells through a Src/Ras-dependent pathway[57]. Interestingly, by downregulation of AdipoR1 and AdipoR2 it was demonstrated that, at least in HEK 293 cells, either receptor is sufficient to mediate the stimulation of ERK1/2 by adiponectin[57].

Recently, an orally active small-molecule was synthesised by Kadowaki and colleagues[125] as AdipoR agonist named AdipoRon. AdipoRon binds to both AdipoR1 and AdipoR2 in vitro and shows very similar effects to adiponectin in muscle and liver, activates AMPK and PPARα pathways, and ameliorates insulin resistance and glucose intolerance in high fat diet fed mice[125]. Other groups also generated different peptides which mimic adiponectin actions[126, 127]. Collectively, these findings provide promising novel therapeutic approach for treating obesity-related disorders.

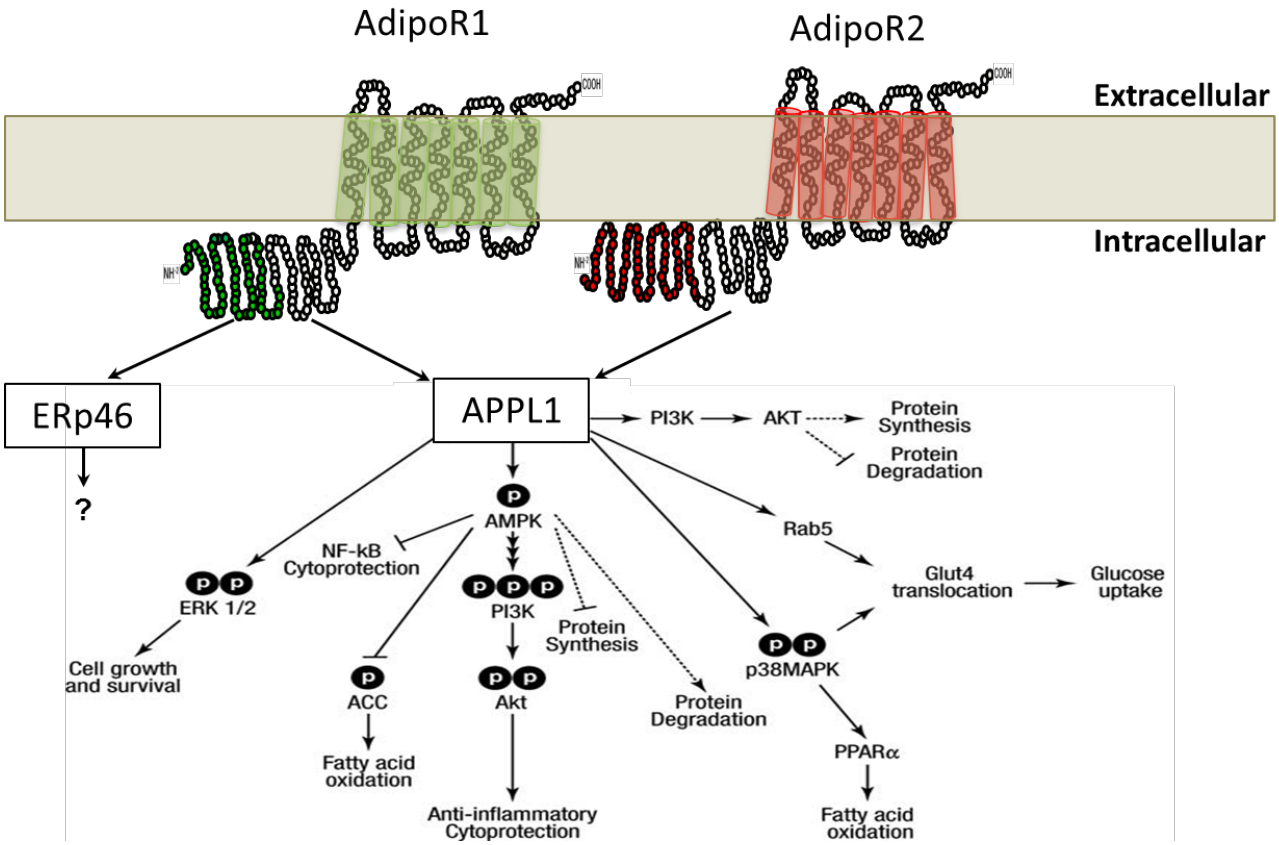

Figure 1.3. Schematic presentation of adiponectin signal transduction pathway. Both AdipoR1 and AdipoR2 interact with APPL1 via their intracellular N-terminal domain. Only AdipoR1 interacts with ERP46 through its non-conserved N-terminal domain. AdipoR1 and AdipoR2 activate various signalling pathways such as AMPK, p38 MAPK, PPARα, PI3K, Akt, and ERK1/2. Adopted and modified from (Brochu-Gaudreau *et al.* 2010)[128].

1.3.7 Adiponectin receptors trafficking

Subcellular localisation, trafficking and degradation of many receptors have been shown to affect function and signalling. Thus, the function and signalling of the AdipoR1 and AdipoR2 is also likely to be regulated through changes in subcellular localisation (see above). Although GPCRs have opposite topology and employ different signal transduction pathways to the adiponectin receptors, we have used understanding gained from extensive studies of the GPCRs to provide a framework to study mechanisms regulating the subcellular localisation and trafficking of AdipoR1 and AdipoR2. As such, I will provide some background to the GPCRs, which represent the largest family of drug targets and support the rationale of understanding molecular details of the adiponectin receptors, may reveal novel therapeutic strategies.

1.3.8 G protein-coupled receptors (GPCRs)

GPCRs are divided into 6 classes (A-F) based on their sequence homology and functional similarity[129]. Each GPCR is also composed of a number of functional domains that control receptor properties such as subcellular localisation, agonist binding and endocytosis. Chimeric, truncated and mutant receptors have been employed to identify and investigate the functions of these domains. It is apparent from these studies that while GPCR proteins possess the same overall TM topology, similar domains may perform different functions in different receptors. For example, the list of functions assigned to the intracellular carboxyl terminal (C-terminal) tail of GPCRs is extensive. Deletion of this entire C-terminal tail in the histamine H2 receptor demonstrated a potential role in determining cell surface expression[130] while in the angiotensin II and the Bradykinin B2 receptors, C-terminal truncations did not affect cell surface expression but compromised the internalisation of each receptor[131, 132]. The C-terminal tail has also been implicated in agonist-induced receptor phosphorylation in GPCRs such as the delta opioid receptor and the α1B-Adrenergic receptor[133, 134] and may also function in agonist induced receptor desensitisation as shown for the α1B-Adrenergic receptor[133]. Moreover, the intracellular C-terminus mediates binding of G-proteins and accessory proteins to GPCRs such as the angiotensin II receptor and rhodopsin[135, 136]. The C-terminus, however, is not the only intracellular domain of GPCRs involved in G-protein coupling and the binding of non-G-proteins. Binding can also occur in the intracellular loops as demonstrated in the angiotensin II and dopamine D2 receptors[135, 137]. Proteins found to interact with these loops in GPCRs include those of cytoskeletal-associated proteins and calcium-binding proteins which have been shown to regulate PM trafficking and G-protein activation, respectively[135]. Extracellular domains of GPCRs, encompassing both the amino terminal tail and the extracellular loops also

participate in a range of functions. In class B GPCRs (including the calcitonin and parathyroid hormone receptor), correct translocation of the receptor to the PM is governed by the extreme N-terminal signal sequence[138]. In contrast, other GPCRs (such as the formyl peptide receptor and angiotensin I receptor) rely upon N-glycosylation modifications of ectodomain asparagine residues for correct protein folding and cell surface expression[139, 140]. Not surprisingly, the ectodomains of GPCRs mediate ligand binding although the exact site is receptor specific. In some GPCRs, ligand binding occurs in either the 1st, 2nd or 3rd extracellular loops (ECL1, ECL2 and ECL3, respectively)[141-143], whereas in Class C GPCRs, binding occurs via the Venus flytrap structure in their large extracellular amino terminus[144]. Studies have also shown that ligand binding is not only determined by extracellular domains but may also depend upon receptor-specific residues buried in TM domains. The binding of nicotinic acid to its receptor, for example, occurs via the ECL1 and ECL2 however, site-directed mutagenesis and chimeras with a closely related receptor have identified residues in the 3rd and 7th TM domains that are critical for binding[143]. Studies investigating ligand binding in the angiotensin II receptor identified critical residues in the ECL2 as well as in the 7th TM domain[145]. In fact, these studies led the authors to propose that the ligand (angiotensin II) orients itself in such a way that its N-terminus interacts with the ECL2 while its C-terminus interacts with specific residues in the 7th TM domain. Therefore, the binding of ligands to some GPCRs may not only depend upon the ectodomains but may require interaction with specific residues that are buried in the membrane layer. In 2006 it was demonstrated that there is no exact binding site for adiponectin in the C-terminal of AdipoR1 and AdipoR2 and a region which includes transmembrane and extracellular domains interacts with adiponectin[72]. Recently, crystal structure analysis of AdipoR1 and AdipoR2 revealed that the three extracellular loops and the final 13-residues of the adiponectin receptors are required for adiponectin binding and subsequent signal transduction of AdipoR1 and AdipoR2[71]. Collectively, these suggest that the receptors bind adiponectin deep within the membrane, with the interaction involving several of the TM domains.

1.3.9 Receptor dimerisation

For a long time, it was generally accepted that GPCRs existed as monomers. However, this dogma has been superseded and current understanding favours the concept that most GPCRs exist and function as either dimers or oligomers[146]. Several observations indicate that GPCR dimerisation occurs in the early stages of the biosynthetic process, most likely in the ER[147]. Since the ER plays a central role in the quality control of protein synthesis, dimerisation or oligomerisation[148] might be a common requirement for GPCRs to pass the quality control checkpoints[149]. Receptor-receptor interactions may be mediated by covalent and/or non-covalent interactions between extracellular

domains, intracellular domains and the transmembrane domains. Several motifs have been described to be involved in the dimerisation of GPCRs. For instance, the glycophorin A like GxxxG motif has been identified in a range of GPCRs and it is shown that mutation of this motif can reduce the dimerisation of neurotransmitter transporter proteins[150] and yeast pheromone receptors[147]. Dimerisation of adiponectin receptors is well established[48, 70, 151]. In 2010 it was demonstrated that a GxxxG in the 5th TM of R1 is required for the dimerisation. Using bimolecular fluorescence complementation (BiFC) it was shown that mutation of two glycine residues to glutamic acid in the cell membrane (G269E and G273E) leads to intracellular accumulation of the receptor leading to the suggestion that dimerization of AdipoR1 is critical for correct plasma membrane delivery[151]. Using BiFC it was also suggested that adiponectin treatment prevents the formation of dimers[151]. A limitation of this approach is that the acute effects of adiponectin treatment were not determined. However, the effect of adiponectin on receptors dimerisation was confirmed later in 2013 by Almabouada *et al.* which demonstrated that 30 min treatment with adiponectin resulted in reduced AdipoR1 and AdipoR2 oligomerisation[152].

1.3.10 Receptors intracellular trafficking and cell surface expression

Intracellular trafficking and precise targeting to the functional destinations of GPCRs plays a crucial role in controlling the physiological functions of the receptors. It has been known that cell-surface expression of GPCRs is coordinated by many regulatory factors. First, GPCR export to the cell surface is regulated by multiple proteins, such as receptor activity modifying proteins (RAMPs), ER chaperones, and accessory proteins. These proteins may stabilise receptor conformation, facilitate receptor maturation, and promote receptor delivery to the plasma membrane[153-155]. Second, recent studies have indicated that the exit of GPCRs from the ER may be directed by specific motifs embedded within the receptors[155-158]. Third, post-translational modifications, such as N-linked glycosylation, have long been known to be required for the delivery of some GPCRs to the cell surface[159]. Fourth, GPCR cell-surface targeting depends on the microtubule networks[160] and GPCRs may directly interact with tubulin to control their cell-surface movement[161]. Fifth, GPCR dimerisation may influence proper receptor folding/assembly and the ability of receptors to pass through the ER quality-control system[162]. There are a number of known specific motifs for protein export from the ER to Golgi and from Golgi to plasma membrane such as, tyrosine based motifs (NPxY and YxxxØ, where x can be any residue and Ø is a hydrophobic residue), di-leucine-based motifs ([D/E]xxxL[L/I] and DxxLL)[158, 163] and motifs composed of hydrophobic amino acids such as $F(x)_3F(x)_3F$, $F(x)_6LL$ and $FN(x)_2LL(x)_3L$[164-166]. During the course of this project Beck-Sickinger and colleagues demonstrated that two different motifs in the intracellular domain of AdipoR1 play a

significant role in the proper cell surface targeting of this receptor[167]. They demonstrated that although these two motifs ($F(x)_3F(x)_3F$ and $D(x)_3LL$) have different roles, both regulate the expression of AdipoR1 on the plasma membrane[167]. In this study the effects of these motifs, which are highly conserved, were not investigated because preliminary studies suggested that AdipoR2 was not expressed at the cell surface[168, 169].

1.3.11 Receptor palmitoylation

Palmitoylation is a post-translational lipid modification in receptors, and many other proteins, which regulates diverse aspects of protein trafficking and function. Palmitoylation is a reversible addition of saturated 16-carbon palmitic acid to specific cysteine residues through the formation of a labile thioester bond[170]. Palmitoylation increases the hydrophobicity of the protein that typically facilitates membrane interaction and trafficking and may also regulate protein-protein interactions and/or enzymatic activity. Furthermore, palmitoylation may help anchor a protein into a membrane and increase its stability[171]. Two mechanisms have been proposed with regards the mechanism of protein palmitoylation. The first is through the action of an enzyme generally referred to as protein acyl-transferase (PAT). The second mechanism is non-enzymatic involving spontaneous auto-acylation in the presence of long-chain acyl-coenzyme As (CoAs)[172-174]. Many GPCRs are palmitoylated. Palmitoylation normally occurs at a conserved cysteine residue in the cytoplasmic C-terminal tail, 13-14 amino acids distal to the 7th TM domain. Other, additional cysteine residues, including some situated in intracellular loops, may also be palmitoylated[175]. As described above, palmitoylation may affect localisation, function and signalling of the GPCRs[176-178].

1.3.12 Receptors endocytosis

Following the cell surface expression, some signal may occur while receptors are at the PM, whilst some other signalling happens following internalisation. Therefore, some receptors internalise in response to agonist stimulation to transduce specific signals. An important role for β-arrestin 1 and 2 in mediating internalisation of some GPCRs has been established[177, 179]. In 2008, overexpressed tagged AdipoR1 and AdipoR2 was used to demonstrate the endocytosis of adiponectin receptors[168]. It was identified that Rab5, a member of GTPase large family that regulate vesicle transport in cell[180], is involved in the endocytosis of AdipoR1[168].

Collectively, our understanding of AdipoR1 and AdipoR2 is rudimentary relative to other 7TM proteins. So far understanding gained from other 7TM proteins such as GPCRs has led to some

insights about the adiponectin receptors. Further investigations will help to define key features of AdipoR1 and AdipoR2 and may reveal possible therapeutic opportunities.

1.4 Project aims

AdipoR1 and AdipoR2 are members of a 7TM receptor family, PAQR, of which little is known. Evidence suggests that adiponectin mostly, if not totally, exerts its beneficial effects such as insulin sensitizing and cardio-protection through these two receptors. Considering the importance of adiponectin as a therapeutic target, it is critical to understand receptor function at both a molecular and physiological level to combat obesity and associated disorders. The *in vivo* models have provided contradictory results regarding the potential therapeutic benefits of targeting AdipoR1 and/or AdipoR2 and the *in vitro* data, particularly for AdipoR2, does little to help explain these discordant findings. Understanding the structure and factors regulating the subcellular localisation and signal transduction of AdipoR1 and AdipoR2 will increase our knowledge and help to facilitate targeted therapeutic strategies.

1.4.1 Specific hypothesises and aims:

1- ***Hypothesis:*** The subcellular localisation and temporal signal transduction of the adiponectin receptors, AdipoR1 and AdipoR2, is different, and these differences are underpinned by non-conserved N-terminal domain (NC-NTD).

Aim 1-1: Compare the subcellular localisation of AdipoR1 and AdipoR2, particularly the cell surface expression.

Aim 1-2: Establish whether the non-conserved N-terminal domains are responsible for different expression at the cell surface by generating and characterising cell surface expression of chimeric receptors (combining the intracellular N-terminal domain of AdipoR1 with the transmembrane and extracellular C terminal domain of AdipoR2, and vice versa).

Aim 1-3: Compare the temporal signalling profile of AdipoR1 and AdipoR2 (and chimeric constructs) by examining adiponectin stimulated signalling pathways.

2- ***Hypothesis****:* AdipoR1 and AdipoR2 are subjected to palmitoylation and palmitoylation is required for CSE and function of AdipoR1 and AdipoR2.

Aim 2-1: Determine the palmitoylation of AdipoR1 and AdipoR2 and discover the potential palmitoylation sites by generation of different cysteine mutants.

Aim 2-2: Examine the effects of palmitoylation on AdipoR1 and AdipoR2 CSE and function using wild-type and palmitoylation mutant receptors.

3- ***Hypothesis:*** Muscle specific overexpression of AdipoR1 and AdipoR2 enhance adiponectin signalling *in vivo.*

Aim 3-1: Identify the effects of overexpression of AdipoR1 and AdipoR2 in mouse skeletal muscle using *in vivo* electrotransfer system.

Chapter Two:

“Characterisation of the adiponectin receptors: The non-conserved N-terminal region of AdipoR2 prevents its expression at the cell-surface”

2.1 Introduction to this publication

This Chapter was published as an original article in *Biochemical and Biophysical Research Communications* journal. In this study, we demonstrate that:

- There are fundamental differences between AdipoR1 and AdipoR2, with only the former showing constitutive cell-surface expression.
- The non-conserved, N-terminal residues of AdipoR2 (residues 1-81) act as a 'brake', restricting its cell-surface expression.
- We also show (in line with previous reports) that AdipoR1 and AdipoR2 can homo- and hetero-dimerise. The latter is important as we demonstrate that co-expression of AdipoR1 with AdipoR2 results in cell-surface expression of both AdipoR1 and AdipoR2.

Collectively these results demonstrate that there are fundamental differences between AdipoR1 and AdipoR2 and suggest that the trafficking and signalling properties of AdipoRs are likely to be determined by receptor composition (homo- or hetero-dimer/oligomer) and interacting proteins.

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Characterisation of the Adiponectin Receptors: The non-conserved N-terminal region of AdipoR2 prevents its expression at the cell-surface

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Keywords
Adipokine, Seven-transmembrane receptors, AdipoR, PAQR, Cell-surface Expression

2.2 Abstract

Adiponectin is a beneficial adipokine with insulin-sensitizing, anti-inflammatory and anti-atherogenic effects. These effects are mediated by two poorly characterised, closely related, atypical seven-transmembrane receptors. In the current report we have used C-terminal, epitope-tagged AdipoR1 and AdipoR2 constructs to monitor cell-surface expression by indirect immunofluorescence microscopy and quantitative plate-based analysis. We demonstrate that only AdipoR1 is constitutively expressed on the cell-surface. Further investigations, involving characterisation of a number of chimeric and truncated constructs, show the non-conserved region of AdipoR2 (residues 1–81) restricts its cell-surface expression. Introduction or deletion of this region, into AdipoR1 or AdipoR2, resulted in inhibition or promotion of cell-surface expression, respectively. We also confirmed that AdipoR1 and AdipoR2 can form heterodimers when co-expressed and that co-expression leads to the cell-surface expression of AdipoR2. Collectively these studies demonstrate that the non-conserved region of AdipoR2 restricts its cell-surface expression and raise the possibility that the majority of cell-surface AdipoR2 may be present in the form of heterodimers.

2.3 Introduction

Since their discovery in 2003[48] the adiponectin receptors, AdipoR1 and AdipoR2, have been the subject of extensive investigations. A large body of evidence has accumulated which indicates that these receptors mediate many of the salutary effects of adiponectin, a key adipokine produced by adipocytes[12], and thereby defining them as attractive therapeutic targets[181]. Early characterisation suggested the receptors represented an atypical form of seven-transmembrane domain receptor (7TMR) that showed reverse topology to the classic GPCRs, with intracellular and extracellular N-termini and C-termini respectively[48]. They were subsequently recognised as prototypical members of a 7TMR family, termed the PAQR family, that show conserved structural and topological organisation with some, albeit limited, invariant intracellular amino acids[49]. Recent evidence suggests the receptors may have intrinsic ceramidase activity[182].

Classic loss and gain of function studies showed AdipoR1 and AdipoR2 transduce the effects of adiponectin to activate a number of intracellular signalling networks including AMPK and PPARα[48, 90]. Evidence from knockout mice suggests that both receptors are important for metabolic homeostasis however there are striking differences between the different mouse lines generated by three independent groups making detailed mechanistic interpretation difficult[90, 183, 184].

Perhaps surprisingly our understanding of the basic biology of AdipoR1 and AdipoR2 is relatively limited. Like GPCRs[185], they have been reported to form homo- and hetero-dimers[48, 57, 151] although the functional and physiological significance of this is unclear. A dimerization motif ($G(X)_3G$) has been identified in the fifth transmembrane domain of AdipoR1[151] and a recent report has also identified two motifs ($D(X)_3LL$ and $F(X)_3F(X)_3F$) within the intracellular N-terminal region of AdipoR1 that are required for anterograde trafficking to the cell-surface[167]. Interestingly, all three motifs are conserved in AdipoR2. In addition, a number of proteins have been shown to interact with the intracellular N-terminal regions of AdipoR1 and or AdipoR2 and modulate signal transduction[186]. The best characterised of these is APPL1[72], which interacts with both AdipoR1 and AdipoR2[100]. We recently identified ERp46 as an AdipoR1-specific interacting protein and demonstrated that knockdown of ERp46 increased the enrichment of AdipoR1, and AdipoR2, in the plasma membrane (PM) and altered adiponectin signalling[70]. In the current report we have extended these studies by further characterisation of AdipoR1 and AdipoR2 at the level of cell-surface expression.

2.4 Materials and Methods

2.4.1 Reagents and antibodies

Reagents were from Sigma-Aldrich (Castle Hill, Australia) unless otherwise stated. Tissue culture reagents were from Invitrogen (Mount Waverley, Australia). Primary antibodies against FLAG (Sigma Aldrich), HA (Covance), and Calnexin (Affinity Bioreagents) were from the indicated suppliers. AdipoR1 and AdipoR2 antibodies were as described[70]. Secondary antibodies were from Molecular Probes (Invitrogen) or Rockland (PA, USA). Molecular biology reagents were from New England Biolabs (Ipswich, NA, USA) or Promega Corporation (Madison, WA, USA).

2.4.2 Molecular biology

Original constructs encoding C-terminally epitope-tagged (HA or FLAG) human AdipoR1 and AdipoR2 were as described[70]. Standard PCR-based approaches were used to generate chimeric and truncated receptor constructs. Integrity of all constructs was confirmed by direct sequencing. Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney (HEK) cells were transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Cells were typically analysed 24 h after transfection.

2.4.3 Generation of plasma membrane (PM) and ER fractions

Preparation of cell lysates for biochemical analysis of the subcellular distribution of AdipoR1 and AdipoR2 was essentially as described[70].

2 4.4 Immunofluorescence microscopy

Immunofluorescence microscopy of permeabilised cells was performed as described[19]. For microscopy of non-permeabilised cells, cells were washed in ice-cold PBS then blocked in 0.2% BSA and 0.2% fish skin gelatin in CO_2-independent medium for 35 min on ice. Cells were incubated with primary antibody in blocking solution for 45 min followed by 4 x 5 min washes in PBS on ice. After washing, cells were fixed in 4% PFA in PBS on ice for 20 min and quenched in 0.3M glycine for 15 min. Following this step cells were processed as described[19]. The percentage of cells expressing detectable levels of total or cell-surface expression of AdipoR1 or AdipoR2 was determined by scoring HA-positive permeabilised (total) or non-permeabilised (cell-surface) cells respectively. For each independent experiment at least 100 cells were counted per condition.

2.4.5 Plate-based determination of cell-surface expression of AdipoR1 and AdipoR2

Quantitative measurement of total and cell-surface expression of AdipoR1 and AdipoR2 was performed using a plate-based assay, which was based on the methods outlined above for immunofluorescence microscopy. Signals were detected using the Odyssey infrared imaging system (LICOR).

2.4.6 Electron Microscopy

CHO cells were fixed with 0.2% glutaraldehyde / 2% PFA in 0.1 M phosphate buffer and processed for EM as described[187]. Sections were labelled with α-HA antibodies followed by 10 nm protein A-gold.

2.4.7 Statistical analysis

Data are presented as mean ± SEM. Significance was determined using a Student's t test with statistical significance defined as $p<0.05$.

2.5 Results

2.5.1 AdipoR1 but not AdipoR2 is expressed at the cell-surface

We previously observed that the biochemical subcellular fractionation properties of AdipoR1 and AdipoR2 differ, with the bulk of AdipoR1 resident in the PM whilst the majority of AdipoR2 is present in the ER in HeLa cells[70]. We confirmed this differential subcellular distribution following subcellular fractionation of HEK cells (Figure 2.1A), suggesting this is a general phenomenon.

In order to investigate this further we employed indirect immunofluorescence microscopy. Attempts to detect total cellular and cell-surface expression of endogenous AdipoR1 and AdipoR2 proved unsuccessful in a range of cell types and this probably reflects the relatively low levels of expression of these proteins. To circumvent this problem and allow detailed mechanistic studies we performed experiments involving transient transfection of C-terminal, HA-tagged AdipoR1 and AdipoR2 constructs (Figure 2.1B). Cell-surface expression of the C-terminal-tagged constructs results in exposure of the epitope-tag, providing a straightforward method for determination of cell-surface expression. In permeabilised cells, the distribution of AdipoR1-HA and AdipoR2-HA appeared similar, with both showing reticular staining patterns characteristic of the ER (Figure 2.1C). Immunoelectron microscopy also revealed similar intracellular distribution profiles for AdipoR1-HA and AdipoR2-HA, with both proteins found in the rough ER as well as a range of

morphologically-diverse membranous structures including tubular and vesicular profiles (Figure 2.1E). In contrast, in non-permeabilised cells AdipoR1-HA was readily detected at the cell-surface but AdipoR2-HA was undetectable (Figure 2.1C). Semi-quantitative analysis was performed by scoring cells expressing detectable total (permeabilised) or cell-surface (non-permeabilised) expression of AdipoR1-HA or AdipoR2-HA. Around 50% of cells expressed detectable levels of AdipoR1-HA or AdipoR2-HA (Figure 2.1D). Cell-surface expression of AdipoR1-HA was evident in around 45% of cells (Figure 2.1D). A plate-based assay was employed to provide more quantitative analysis (Figure 2.1F & G). This approach revealed that around 50% of AdipoR1-HA was present at the cell-surface, whilst cell-surface AdipoR2-HA was undetectable (Figure 2.1F & G). These complementary approaches indicate that around 90% of cells expressing AdipoR1-HA have readily detectable cell-surface expression and that this represents around 50% of total cellular AdipoR1-HA. Similar results were obtained using AdipoR1-FLAG and AdipoR2-FLAG tagged constructs (data not shown). These results reveal a surprising difference in steady-state, cell-surface expression of AdipoR1 and AdipoR2.

2.5.2 Characterisation of adiponectin receptor chimera suggests a key role for the non-conserved N-terminal region of the AdipoRs.

We next sought to determine the molecular basis for the observed differences. AdipoR1 and AdipoR2 share 68% identity at the amino acid level. However, the cytoplasmic domains can be split into two regions that differ in their degree of sequence homology. The N-terminal regions, consisting of AdipoR1$_{(1–70)}$ and AdipoR2$_{(1–81)}$, show only 17% homology whilst the remaining sequences, comprising AdipoR1$_{(71–136)}$ and AdipoR2$_{(82–147)}$, show 95% homology (Figure 2.2A). To test whether the non-conserved, N-terminal regions underpinned the differences between the AdipoRs we generated two chimeras, swapping the non-conserved, N-terminal domains to produce an AdipoR2$_{(1–81)}$/R1$_{(71–375)}$-HA chimera (R2/R1) and an AdipoR1$_{(1–70)}$/R2$_{(82–386)}$-HA chimera (R1/R2) that expressed at similar levels (Figure 2.2B). Semi-quantitative microscopy indicated that 40-45% of permeabilised cells expressed detectable levels of the two chimeras but only the R1/R2-HA chimera was detectable at the cell-surface, and this was present in around 40% of cells (Figure 2.2C). Quantitative plate-based analysis confirmed these results, with around 40% of total cellular R1/R2-HA detected at the cell-surface (Figure 2.2D). These results indicate that the difference in cell-surface expression of AdipoR1 and AdipoR2 can be explained entirely by the non-conserved, N-terminal regions.

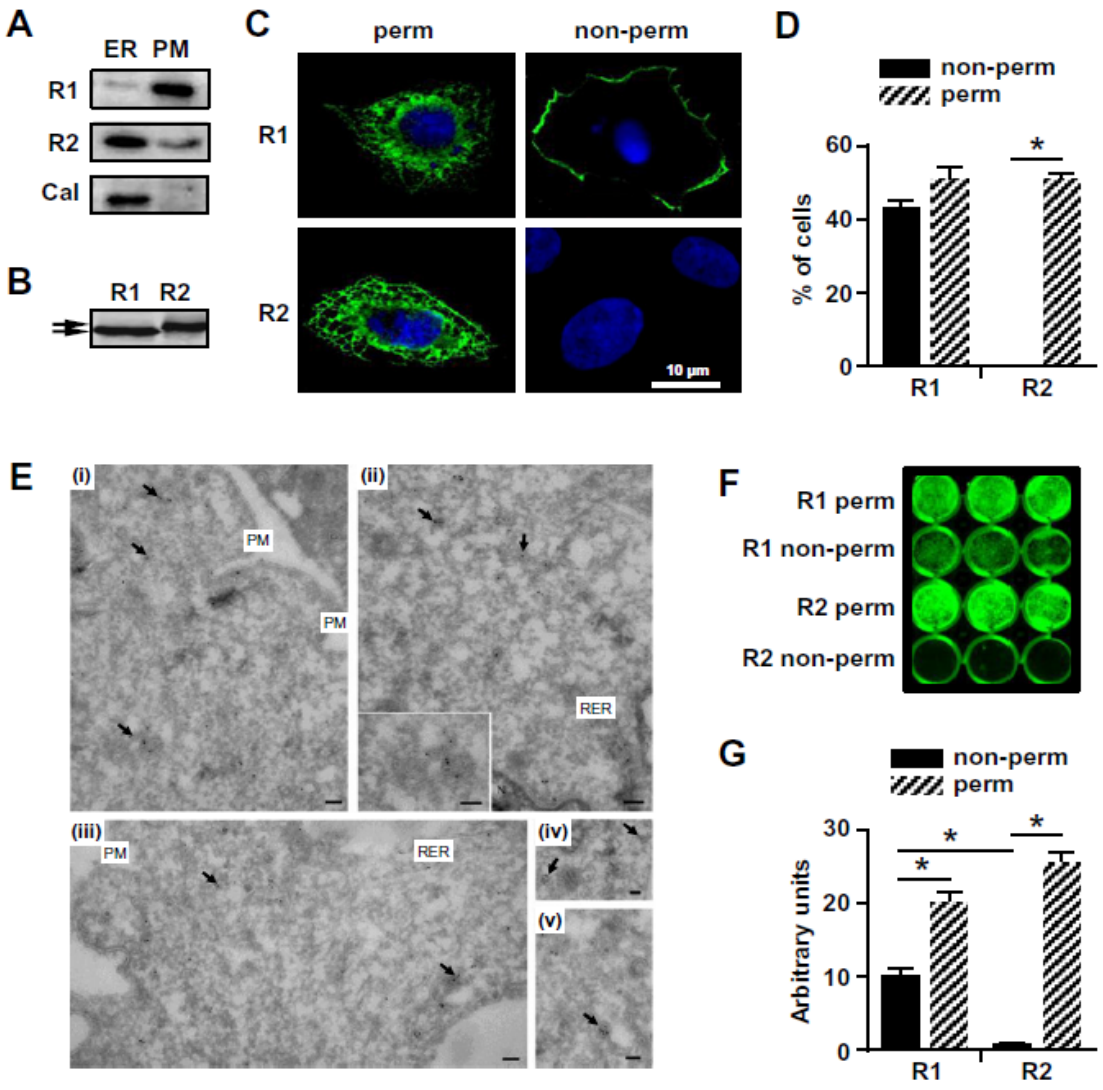

Figure 2.1. Differences in cell-surface expression of AdipoR1 and AdipoR2. (A) HEK cells were fractionated and the distribution of endogenous AdipoR1, AdipoR2 and Calnexin in the ER and PM fractions was determined by Western blot. **(B)** Western blot of CHO cells transiently transfected with AdipoR1-HA or AdipoR2-HA probed with α-HA antibody. **(C)** Immunofluorescence microscopy of transiently expressed AdipoR1-HA or AdipoR2-HA constructs in permeabilised and non-permeabilised CHO cells. Nuclei are counterstained with DAPI. **(D)** Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with ≥ 100 cells counted for each condition, per experiment; *$p<0.05$). **(E)** Immunogold localization showing intracellular distribution of transiently expressed AdipoR1-HA (i, ii, iv & v) and AdipoR2-HA (iii). Note the labelling of the RER as well as a range of morphologically-diverse membranous structures including tubular and vesicular profiles (arrows); inset shows higher magnification of labelled elements in panel i). PM, plasma membrane; N, nucleus. Bars, 100 nm. **(F)** Plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression. **(G)** Quantitation of plate-based analysis from four independent experiments (*$p<0.05$).

2.5.3 Characterisation of truncated adiponectin receptors demonstrates that AdipoR2$_{(1-81)}$ inhibits cell-surface expression.

We next examined whether AdipoR1$_{(1-70)}$ drives expression at the cell-surface or AdipoR2$_{(1-81)}$ prevents cell-surface expression by generating and characterising truncated receptors lacking the non-conserved regions, $_{(\Delta\ 1-70)}$AdipoR1-HA (ΔR1) and $_{(\Delta\ 1-81)}$AdipoR2-HA (ΔR2). Western blot showed the truncated constructs were expressed at similar levels (Figure 2.2E). Semi-quantitative microscopy and quantitative plate-based analysis demonstrated that ΔR1 and ΔR2 expressed at the cell-surface with similar efficiency (Figure 2.2F & G). These results indicate that residues 1-81 in AdipoR2 interfere with cell-surface expression. It is also noteworthy that the efficiency of cell-surface expression of ΔR1 and ΔR2 was lower than that observed for full-length AdipoR1-HA, or the R1/R2-HA chimera, at around 20% of total cellular ΔR1 and ΔR2. This may reflect a positive role for residues 1-70 of AdipoR1 or simply a limitation of the truncation approach (akin to an artefact). Further truncation of the entire cytoplasmic domains $_{(\Delta 1-127)}$AdipoR1 or $_{(\Delta 1-138)}$AdipoR2 abolished cell-surface expression of either construct (data not shown).

2.5.4 Co-expression of AdipoR1 with AdipoR2 promotes cell-surface expression of AdipoR2

Next we investigated the effects of co-expression of AdipoR1 and AdipoR2 on cell-surface expression. Co-transfection followed by co-immunoprecipitation demonstrated that AdipoR1 and AdipoR2 can form homo- and hetero-oligomers (Figure 2.3A), consistent with previous reports[48, 57, 151]. Around 50% of AdipoR2 was present in hetero-oligomers when co-expressed with AdipoR1. Control experiments, where lysates of singly transfected cells were mixed prior to immunoprecipitation, indicated that formation of such oligomers was dependent on co-expression and did not reflect an artefact of the approach. Cell-surface expression of AdipoR1-HA was unaffected by co-expression with either AdipoR1-FLAG or AdipoR2-FLAG (Figure 2.3B). Importantly, cell-surface expression of AdipoR2-FLAG was readily detected in cells co-transfected with AdipoR1-HA (Figure 2.3B) demonstrating that co-expression of AdipoR1 promotes cell-surface expression of AdipoR2.

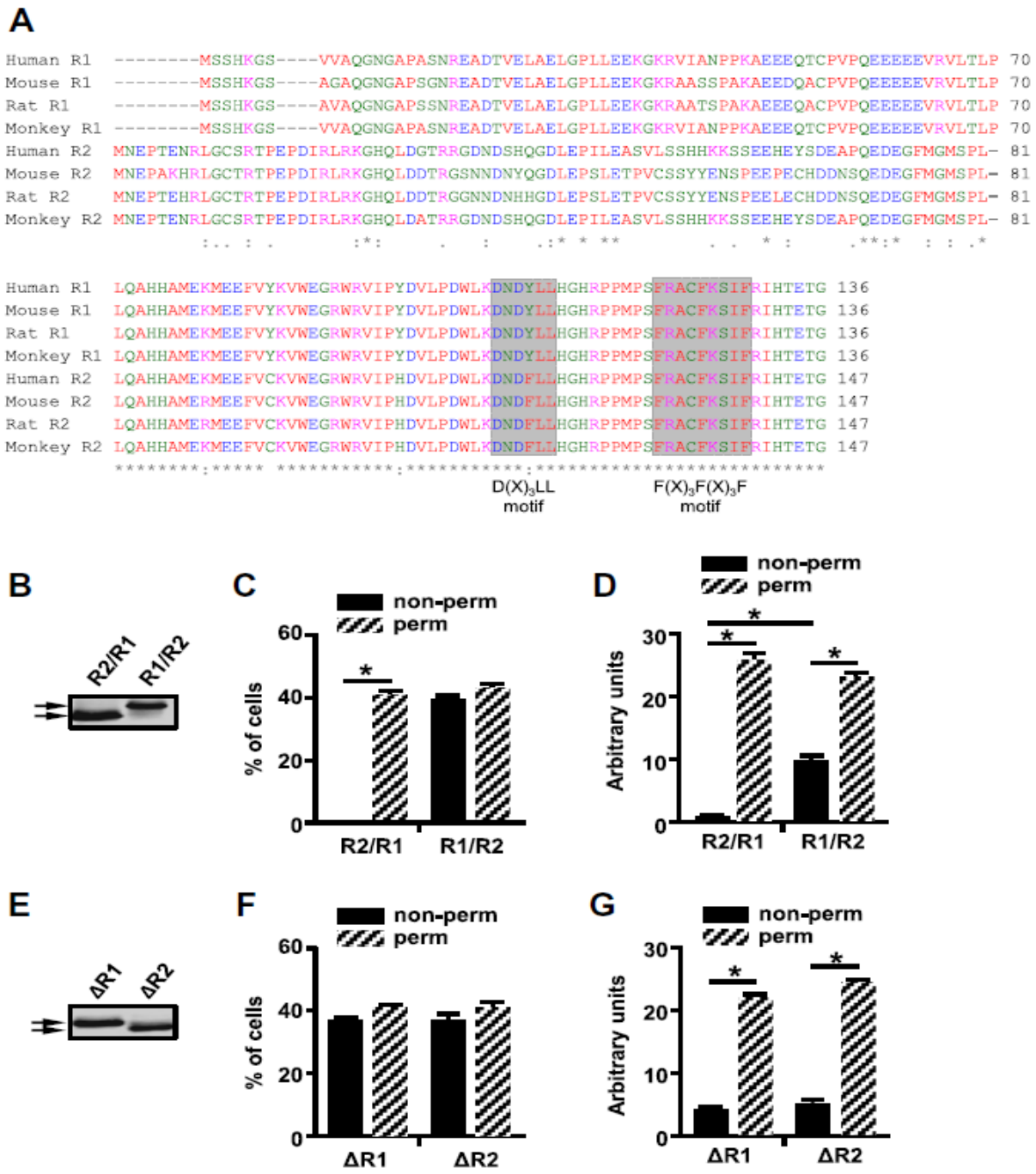

Figure 2.2. Characterisation of cell-surface expression of AdipoR1 and AdipoR2 chimera and truncated constructs. (A) Alignment of the cytoplasmic, N-terminal domains of AdipoR1 and AdipoR2 showing the non-conserved (residues 1-70 and 1-81) and conserved regions (residues 71-136 and 82-147) respectively. The recently described anterograde trafficking motifs ($D(X)_3LL$ and $F(X)_3F(X)_3F$) are highlighted. **(B)** Western blot of transiently expressed $AdipoR2_{(1–81)}/R1_{(71–375)}$-HA chimera (R2/R1) or $AdipoR1_{(1–70)}/R2_{(82–386)}$-HA chimera (R1/R2). **(C)** Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with ≥ 100 cells counted for each condition, per experiment; *$p<0.05$). **(D)** Quantitative, plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression from four

independent experiments (*p<0.05). **(E)** Western blot of transiently expressed $_{(\Delta 1\text{-}70)}$AdipoR1-HA (ΔR1) or $_{(\Delta 1\text{-}81)}$AdipoR2-HA (ΔR2). **(F)** Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with ≥ 100 cells counted for each condition, per experiment). **(G)** Quantitative, plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression from four independent experiments (*p<0.05).

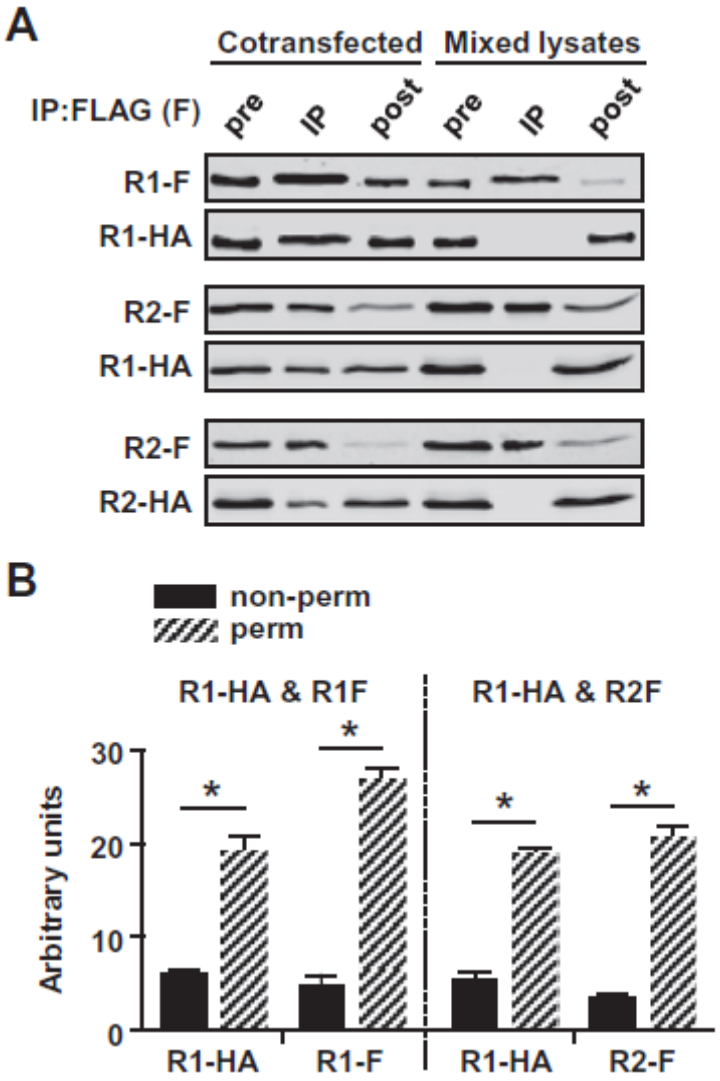

Figure 2.3. Co-expression of AdipoR1 promotes cell-surface expression of AdipoR2. (A) CHO cells were either co-transfected or singly transfected with AdipoR constructs as indicated and lysates were immunoprecipitated using α-FLAG antibody. Western blots show AdipoRs in starting material (pre), immunopreciptated material (IP) and post-IP supernatant (post) probed with α-FLAG (F) and α-HA antibodies respectively. **(B)** Cells were co-transfected with AdipoR1-HA and either AdipoR1-FLAG (F) or AdipoR2-F. Cell-surface (non-permeabilised) and total (permeabilised) AdipoR1-HA and AdipoR1-F or AdipoR2-F were measured using quantitative, plate-based analysis. The graph shows results from four independent experiments (*p<0.05).

2.6 Discussion

This report establishes major differences in the steady-state, cell-surface levels of the adiponectin receptors, AdipoR1 and AdipoR2, and indicates that these differences can be explained entirely by differences in the non-conserved, N-terminal cytoplasmic domains. Cell-surface expression of AdipoR2 is restricted by amino acids 1-81. Co-expression with AdipoR1 can overcome this. These findings have major implications, in particular when considering cell-surface accessibility of the receptors and the potential for enhancing adiponectin sensitivity by increasing cell-surface expression of AdipoR2.

Our observations showing restricted cell-surface expression of AdipoR2 under steady-state conditions may appear somewhat surprising. However, it has been proposed that the AdipoRs and PAQR3 may be derived from a shared evolutionary protein[49, 169]. PAQR3, which has been reported to bind adiponectin and referred to as AdipoR3[50, 188], is also known as Raf kinase trapping to Golgi (RKTG) and is a Golgi-resident membrane protein with a cytoplasmic N-terminus that is known to interact with, and provide spatial regulation of, Raf kinase[63, 189]. Collectively, these findings highlight our limited understanding of the basic biology of the AdipoRs. Although the original description and preliminary characterisation of transiently expressed AdipoR1 and AdipoR2 proteins suggested both receptors presented at the PM with exposed C-termini[48], subsequent anecdotal evidence from independent groups suggested AdipoR2 may not be expressed at the cell-surface[168, 169]. We have shown that endogenous AdipoR1 and AdipoR2 display different subcellular fractionation properties, with AdipoR1 enriched in the PM and AdipoR2 enriched in the ER in HEK cells (in this report) and in HeLa cells[70]. Moreover, in this study we have provided rigorous quantitative assessment of cell-surface expression of transiently expressed, epitope-tagged AdipoR1 and AdipoR2 and a range of chimeric and truncated constructs which provide evidence that the cell-surface expression of AdipoR2 is restricted by the non-conserved residues 1-81. Indeed, our observations showing cell-surface expression of a $_{(\Delta 1\text{-}81)}$AdipoR2-HA (ΔR2) construct are entirely consistent with those from the original report by Kadowaki and colleagues, who inadvertently characterised a truncated form of AdipoR2 lacking this N-terminal region[48, 49].

We also demonstrated that co-expression of AdipoR1 with AdipoR2 results in increased cell-surface expression of AdipoR2. Several groups have shown that AdipoR1 and AdipoR2 can form homo and hetero-dimers[48, 57, 151]. Our data suggests that formation of hetero-oligomers occurs with the expected frequency (around 50%) when AdipoR1 and AdipoR2 are co-expressed, prompting us to speculate that the appearance of AdipoR2 at the cell-surface when co-expressed with AdipoR1

reflects the cell-surface expression of AdipoR1/AdipoR2 dimers. Such a model is also consistent with our previous observations, showing that ERp46 restricts the localisation of endogenous AdipoR1 and AdipoR2 at the PM[70]. ERp46 interacts specifically with AdipoR1, via the non-conserved, N-terminal region of AdipoR1[70], suggesting that this interaction precludes the AdipoR1/AdipoR2 interaction. Thus, knockdown of ERp46 would be expected to facilitate increased interactions between endogenous AdipoR1 and AdipoR2, hence increased cell-surface expression of both receptors. The importance of dimerization, particularly hetero-dimerization, and interacting proteins in the regulation of GPCR trafficking and signalling is now widely recognised[185, 190, 191] and it seems likely that similar principles will apply to the AdipoRs.

A major determinant of the extent of hetero-dimerization is the relative expression levels of AdipoR1 and AdipoR2, as well as other modulators such as ERp46. Current information detailing the relative levels of AdipoR1 and AdipoR2 is restricted to mRNA. Whilst this is a clear limitation, such information provides a basis to estimate the likely extent of hetero-dimerization in different tissues. For example, in mice the mRNA levels of AdipoR1 are 5-6 fold higher than AdipoR2 in skeletal muscle whereas expression of the two receptors is comparable in liver[89]. Although it would follow that a greater proportion of AdipoR2 would be present in hetero-dimers in muscle, the hetero-dimers would still represent a minor species in this tissue where AdipoR1 appears to be functionally dominant[120]. In contrast, the hetero-dimers represent a potentially major species in the liver, and macrophages, where AdipoR2 has been shown to be of functional importance[192, 193]. Intriguingly, recent evidence suggests the formation of AdipoR1 dimers is reduced by adiponectin[151]. Considering the above, future, more-comprehensive studies to characterise the effects of adiponectin on the dynamics of both homo- and hetero-AdipoR dimers are warranted.

Emerging evidence supports the notion that adiponectin resistance contributes to the aetiology of obesity related disease[12, 89]. Increasing cell-surface expression of the receptors, most notably AdipoR2, may provide a novel therapeutic approach to help improve adiponectin sensitivity. Indeed, several lines of evidence suggest that increased transduction of the adiponectin signal from the PM leads to enhanced coupling to AMPK[70, 72, 168], which itself represents a major target for therapeutic intervention[194].

The current report reveals fundamental differences between AdipoR1 and AdipoR2 and, in combination with earlier studies of AdipoRs and GPCRs, suggest that the trafficking and signalling properties of AdipoRs are likely to be determined by receptor composition (homo- or hetero-dimer/oligomer) and interacting proteins. Our current findings suggest that the majority of cell-

surface AdipoR2 may be present in the form of hetero-dimers, which are likely to have unique characteristics when compared with AdipoR1 or AdipoR2 homo-dimers respectively. Increased understanding may provide new opportunities to selectively enhance adiponectin's key, beneficial effects.

Acknowledgments

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Chapter Three:

"Characterisation of the adiponectin receptors: Differential cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks"

3.1 Introduction to this publication

This chapter was published in *Molecular and Cellular Endocrinology* Journal as an original investigation.

In this report we confirm and extend our previous report showing that the differential cell-surface expression of AdipoR1 and AdipoR2 is determined by the non-conserved, N terminal domains of AdipoR1 and AdipoR2 (residues 1-70 and 1-81 respectively) and demonstrate that:

- serum starvation increases cell-surface expression of both receptors
- both receptors display ligand-dependent internalisation
- the non-conserved, N-terminal trunks contain multiple regions that have positive and negative effects on cell-surface expression
- AdipoR1 and AdipoR2 display different temporal signalling profiles
- AdipoR1 mediates effects acutely (peak at 15 min)
- AdipoR2 mediates effects more chronically (peak at 24 h)
- the non-conserved, N-terminal trunks determine the temporal signalling profiles of AdipoR1 and AdipoR2

Collectively these results highlight the importance of the non-conserved, N-terminal trunks of these atypical 7TM receptors. This is the first report to compare the signalling properties of full-length AdipoR1 and AdipoR2 and, hence, the first to identify such fundamental differences. As discussed in the manuscript, Yamauchi et al, Nature, 2003, cited > 1500 times, performed somewhat similar experiments but the results and conclusions are compromised because they inadvertently used a truncated form of AdipoR2 that lacks the non-conserved, N-terminal trunk of AdipoR2.

Keshvari, S. and Whitehead J.P., Characterisation of the adiponectin receptors: Differential cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks. *Mol Cell Endocrinol*, 2015. **409**: p. 121-9

Characterisation of the Adiponectin Receptors: Differential cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks.

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Keywords: Signal Transduction, Receptor, Adiponectin

3.2 Abstract

The adiponectin axis regulates cardiometabolic and inflammatory tone making it an attractive therapeutic focus. Rudimentary understanding of the adiponectin receptors, AdipoR1 and AdipoR2, constrains our ability to target these atypical seven trans-membrane proteins. Here, we aimed to further elaborate the molecular details governing cell-surface expression and signal transduction by transient expression of AdipoR1 or AdipoR2 in HEK293 cells. Following serum starvation, adiponectin reduced cell-surface expression of both receptors, consistent with internalisation, and promoted phosphorylation of downstream effectors. Temporal phosphorylation profiles differed with AdipoR1 and AdipoR2 transduced signals peaking at 15 min and 24 h. Analysis of receptor chimeras showed that the non-conserved N-terminal trunks ($AdipoR1_{(1-70)}$ and $AdipoR2_{(1-81)}$) define the temporal signalling profiles and contain multiple regions that promote or inhibit cell-surface expression, respectively. These findings highlight the importance of the non-conserved N-terminal trunks and demonstrate that cell-surface expression of AdipoR1 and AdipoR2 is required for effective coupling to downstream effectors.

3.3 Introduction

Adiponectin is a key adipokine with demonstrated anti-diabetic, anti-inflammatory and anti-atherogenic properties[195]. Adiponectin's pleiotropic effects are mediated through two atypical 7 transmembrane (7TM) domain receptors termed AdipoR1 and AdipoR2[12, 48]. Empirical evidence demonstrates that AdipoR1 and AdipoR2 have intracellular N-termini and extracellular C-termini[48] unlike most other 7TM proteins including the G-protein coupled receptors (GPCRs). *In vivo* and *in vitro* studies have demonstrated that AdipoR1 and AdipoR2 mediate the effects of adiponectin via activation of a number of signalling molecules such as AMPK, PPARα, ERK and P38MAPK[90, 100, 196-198]. Coupling to these downstream effectors has been shown to be modulated by proteins that interact with the cytoplasmic, N-terminal domains of both AdipoR1 and AdipoR2, such as APPL1, RACK and protein kinase CK2[73, 74, 100], or with the non-conserved N-terminal trunk of AdipoR1, namely ERp46[70].

Emerging evidence indicates that obesity-related diseases are characterised not only by hypoadiponectinemia but also by adiponectin resistance at the level of the adiponectin receptors[42-46, 199]. Thus, a greater understanding of the molecular processes required to facilitate efficient adiponectin receptor coupling to intracellular signalling pathways may be expected to provide new insights into pathophysiological events and the identification of novel therapeutic approaches.

We recently reported that under steady-state conditions (no serum starvation) the cell-surface expression of AdipoR1 and AdipoR2 differs. AdipoR1 is enriched in the plasma membrane whilst AdipoR2 is more abundant in the ER[200]. We also demonstrated that this difference is due to the non-conserved N-terminal trunks of AdipoR1 and AdipoR2[200]. In the current report we have extended these studies by performing further characterisation of the molecular features governing the cell-surface expression and subsequent coupling to downstream signalling effectors of AdipoR1 and AdipoR2. Our results demonstrate that the non-conserved N-terminal trunks dictate the cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2.

3.4 Materials and methods

3.4.1 Reagents and antibodies

Reagents were from Sigma–Aldrich (Castle Hill, Australia) unless otherwise stated. Tissue culture reagents were from Invitrogen (Mount Waverley, Australia). Primary antibodies against HA and Sodium Potassium ATPase were from Covance (Washington, USA) and Abcam (Melbourne, Australia) respectively. Primary antibodies against AdipoR1 and AdipoR2 were as described[70].

Secondary antibodies were from Life Technology (Invitrogen). Molecular biology reagents were from New England Biolabs (Ipswich, NA, USA) or Promega Corporation (Madison, WA, USA). Serum from adult WT and adiponectin knockout mice [201] was collected in accordance with ethics approval from the animal ethics committee of the University of Queensland.

3.4.2 Molecular biology

Original constructs encoding C-terminally epitope-tagged (HA) human AdipoR1 and AdipoR2 were as described[70]. Chimeric and truncated receptor constructs were generated as described[200]. AdipoR1 and AdipoR2 mutants were generated by QuikChange site-directed mutagenesis (Agilent Technology, CA, USA). Mutations were confirmed by DNA sequencing (Sanger method). Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney 293 (HEK293) cells were transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was typically around 70% and cells were analysed 24-48 h post-transfection.

3.4.3 Flow cytometry analysis

Flow cytometry was performed to determine the percentage of transfected cells that were expressing AdipoR1 or AdipoR2 at the cell-surface. Flow cytometry was carried out using a CyAn™ ADP Analyser (Beckman Coulter, Sydney, Australia) and FlowJo software. Briefly, HEK293 cells were washed with cold PBS and stained with HA antibody. For analysis of permeabilised cells, cells were incubated in 0.1% Saponin for 15 min prior to blocking. After washing with PBS, cells were stained with an AlexaFluor 488-conjugated secondary antibody (Invitrogen). Cells were then lifted non-enzymatically and 80,000 events were analysed by flow cytometer. For all experiments, mean fluorescence intensity (MFI) values were calculated by subtracting secondary only staining from specific anti-HA staining.

3.4.4 Plate-based determination of cell-surface expression of AdipoR1 and AdipoR2

Quantitative measurement of total and cell-surface expression of AdipoR1 and AdipoR2 was performed, in permeabilised and non-permeabilised cells respectively, using a plate-based assay as described[200]. Briefly, parental HEK293 cells or transfected HEK293 cells were incubated in either the presence or absence of serum at 37°C overnight. Cells were incubated in 100% ice-cold methanol for 5 min to permeabilise (for measurement of total receptor levels) or left non-permeabilised (for determination of receptors at the cell-surface). Cells were then stained with HA antibody followed by fixation with 4% Paraformaldehyde. Cells were stained with an AlexaFluor

488-conjugated secondary antibody. Signals were detected using the POLARstar Omega plate-reader (BMG Labtech, Offenburg, Germany).

3.4.5 Immunofluorescence microscopy

Immunofluorescence microscopy of permeabilised and non-permeabilised cells was performed as described[200] affording details of the subcellular distribution of the receptors and the cell-surface expression respectively. Images were taken using a Delta Vision OMX microscope (Applied Precision, GE Healthcare, Washington, USA).

3.4.6 Akt, ERK and p38MAPK phosphorylation assays

Parental HEK293 cells or transfected HEK293 cells were serum-starved overnight then stimulated with recombinant human globular adiponectin (Prospec Protein Specialists, USA) or vehicle for 15 min, 1 h or 24 h. Phosphorylation of Akt, ERK and p38MAPK was measured using AlphaScreen SureFire kits essentially as described (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Plates were read using a POLARstar Omega plate reader. Background signals were determined by treatment of cells with the Akt1/2 kinase inhibitor, U0126 (ERK inhibitor) or SB203580 (p38MAPK inhibitor) and were subtracted to give specific phospho-signals for Akt, ERK and p38MAPK respectively.

3.4.7 SDS-PAGE/Western blotting of AdipoR1 and AdipoR2

Western blotting of particulate fractions (enriched for the ER and PM that contain greater than 90% of total cellular AdipoR1 and AdipoR2) was performed on parental and transfected cells as described[70].

3.4.8 Statistical analysis

Data are presented as mean ± SEM. Significance was determined using one way ANOVA followed by Tukeys test with statistical significance defined as $p < 0.05$.

3.5 Results

3.5.1 Serum starvation increases the cell-surface expression of AdipoR1 and AdipoR2.

We previously reported that under steady-state conditions the subcellular localisation of AdipoR1 and AdipoR2 differed with around 50% of AdipoR1 present on the cell-surface whilst AdipoR2 was localised predominantly at the ER[200]. We subsequently demonstrated that the non-conserved, N-

terminal trunks of AdipoR1$_{(1-70)}$ and AdipoR2$_{(1-81)}$ underpinned these differences[200]. To extend these studies we first examined the cell-surface expression of transiently expressed, C-terminally HA-tagged AdipoR1 and AdipoR2[200] in serum-starved or non-starved HEK cells using flow cytometry and plate-based assays as well as high resolution microscopy. Flow cytometry was used to determine the number of cells with detectable cell-surface expression of AdipoR1 or AdipoR2. Serum starvation did not affect the number of cells with AdipoR1 on the cell-surface however the number of cells with detectable cell-surface expression of AdipoR2 was significantly increased following serum starvation (Figure 3.1A). A complementary plate-based assay was used to determine total and cell-surface levels of AdipoR1 and AdipoR2. This approach revealed significantly increased cell-surface expression of both AdipoR1 and AdipoR2 following serum starvation (Figure 3.1B). Finally, qualitative analysis by high resolution confocal microscopy suggested that the cell-surface expression of AdipoR1 was increased in cells following serum starvation. We were unable to detect cell-surface expression of AdipoR2 in non-permeabilised cells in steady-state or serum-starved cells which probably reflects a limitation of this approach (Figure 3.1G-J). These results extend our previous findings[200] by showing that serum starvation results in an increase in the proportion of AdipoR1 or AdipoR2 that is expressed on the cell-surface with the latter resulting in an increase in the number of cells with detectable cell-surface levels of AdipoR2. Notwithstanding, the levels of AdipoR2 on the cell-surface of serum starved cells are still relatively limited compared to those of AdipoR1.

3.5.2 Adiponectin reduces cell-surface expression of AdipoR1 and AdipoR2.

We next went on to investigate the effect of serum, and more specifically adiponectin, on receptor cell-surface expression. Following overnight serum starvation cells overexpressing either AdipoR1 or AdipoR2 were incubated with 10% fetal bovine serum (FC) for 30, 60, 90, 120 and 240 min. Analysis by flow cytometry (Figure 3.2A-B) and microscopy (Figure 3.2C-J) indicated that cell-surface expression of both AdipoR1 and AdipoR2 was reduced by 60% and 90% after 30 min. To investigate the role of adiponectin more specifically we then used serum from wild-type (WT) or adiponectin knockout (Adn$^{-/-}$) mice. Serum from WT mice promoted similar effects as the FC, whilst serum from Adn$^{-/-}$ mice was without effect (Figure 3.2A-B). Similar results were obtained following treatment with 2.5 µg/ml globular adiponectin (gAd) (Figure 3.2A-B). These results are consistent with the adiponectin-mediated internalisation of AdipoR1 and AdipoR2 reported previously[152], and suggest this is a specific, ligand-mediated event.

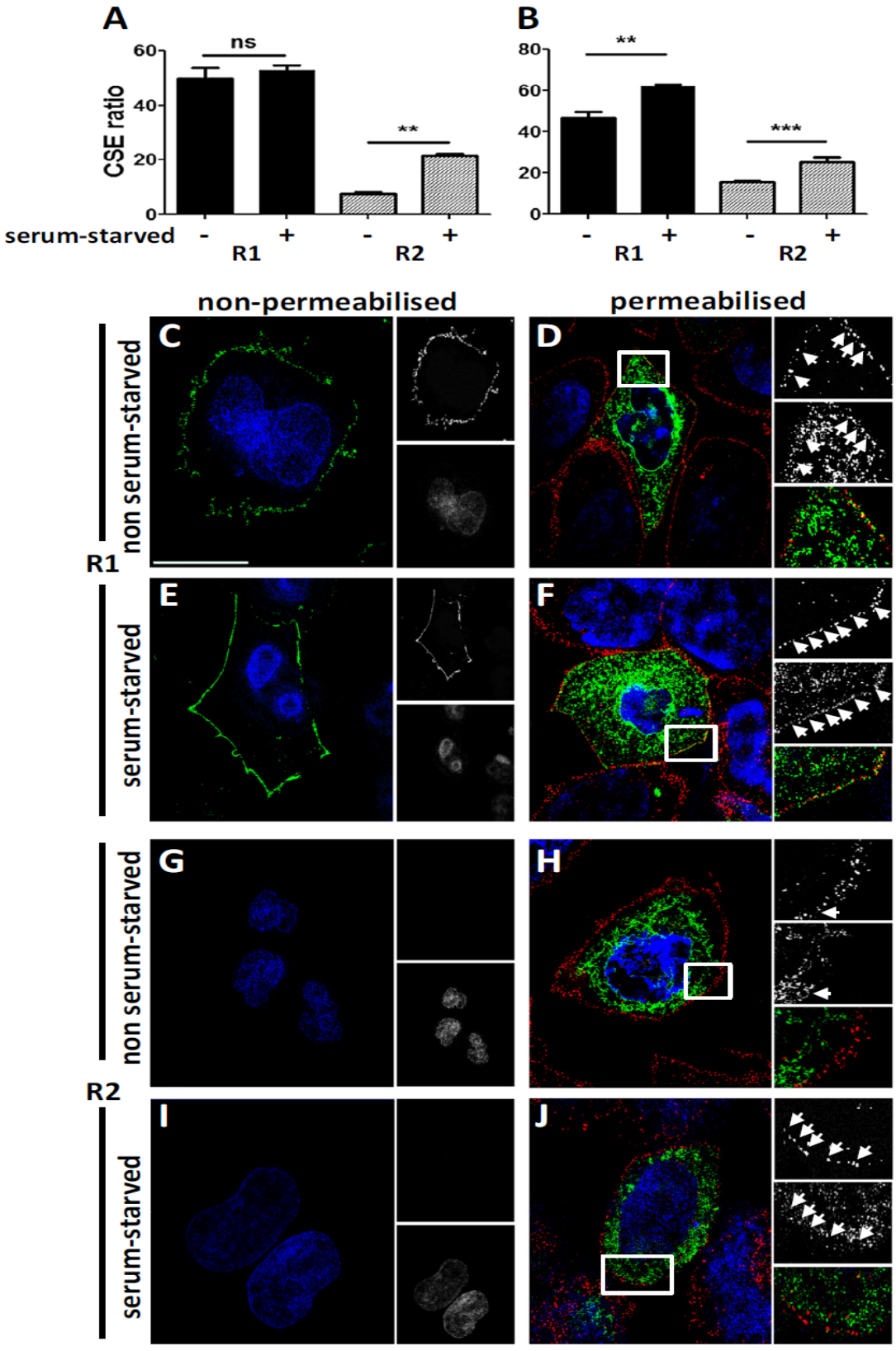
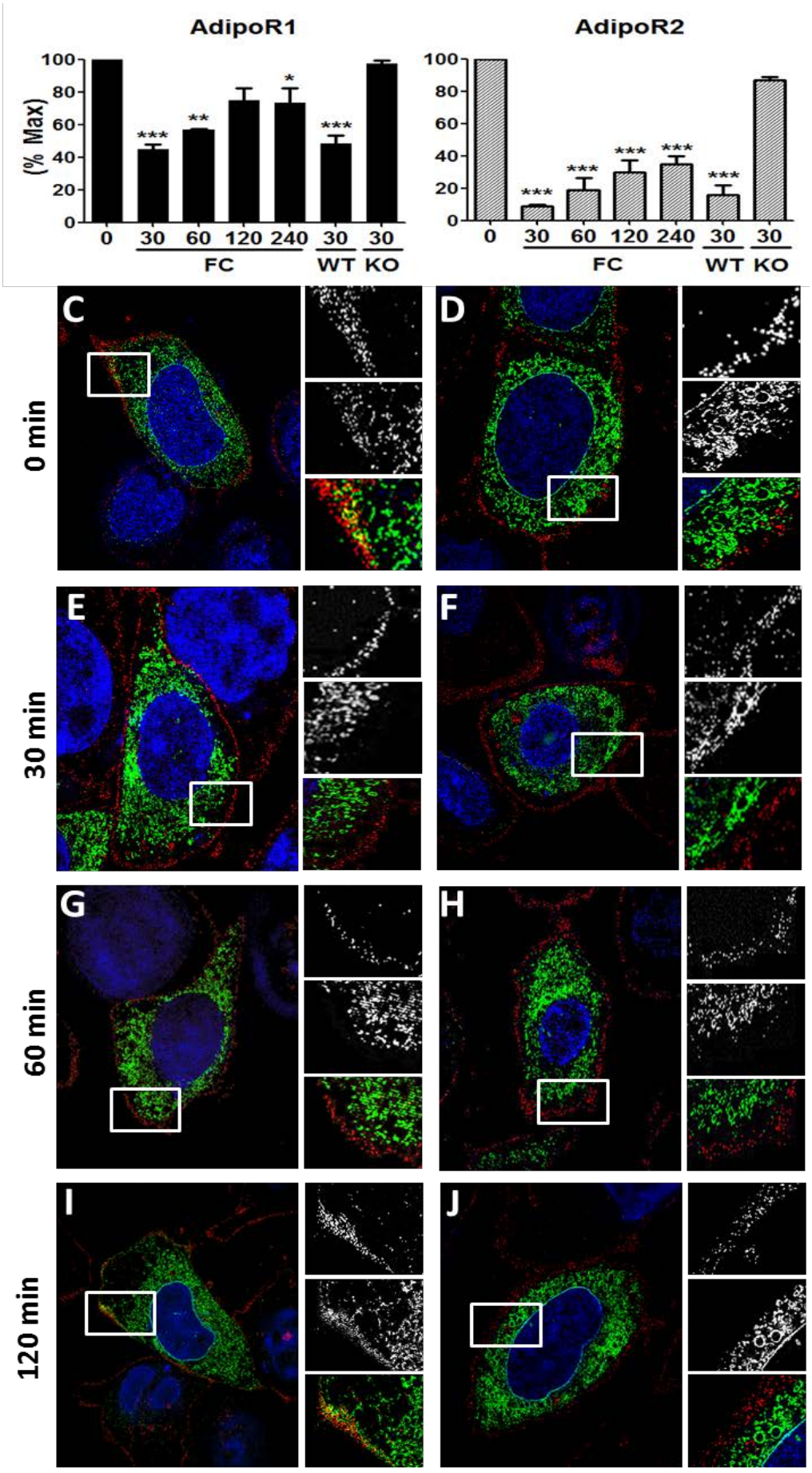


Figure 3.1. Effects of serum starvation on cell surface expression of AdipoR1 and AdipoR2. (A) Flow cytometry and (B) plate-based analysis of HEK293 cells transiently expressing AdipoR1-HA and AdipoR2-HA constructs. The cell-surface expression (CSE) ratio shows the (A) percentage of transfected cells

expressing AdipoR1 or AdipoR2 at the cell-surface and (B) the ratio of cell-surface to total receptor levels Results are from four independent experiments (*p<0.05). (C-J) immunofluorescent microscopy of transiently expressed AdipoR1-HA or AdipoR2-HA constructs (green) in permeabilised or non-permeabilised CHO cells with or without 16 h serum starvation. Plasma membrane is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue).

3.5.3 Overexpression of AdipoR1 and AdipoR2 enhances adiponectin stimulated Akt, ERK and P38MAPK phosphorylation.

We next examined the effects of AdipoR1 and AdipoR2 overexpression on adiponectin-stimulated phosphorylation of key signalling molecules implicated in mediating adiponectin's effects, namely Akt[198, 202], ERK[57, 198] and P38MAPK[70, 122]. In the parental HEK293 cells endogenous levels of AdipoR1 and AdipoR2 were undetectable by standard Western blot whereas transfected AdipoR1 and AdipoR2 were readily detected (Figure 3.3A & B). Parental cells or cells overexpressing AdipoR1 or AdipoR2 were treated with recombinant gAd for 15 min or 24 h (based on preliminary timecourse experiments - data not shown) to determine acute and long-term effects. Under these experimental conditions there was no detectable adiponectin-stimulated phosphorylation of Akt, ERK, or p38MAPK in the parental cells. However, significant and maximal phosphorylation of Akt, ERK, and p38MAPK occurred after 15 min in AdipoR1 expressing cells, and after 24 h in AdipoR2 overexpressing cells (Figure 3.3C-E). We then went on to perform dose response studies, treating cells with increasing concentrations of gAd (0.5 – 5.0 µg/ml) for either 15 min or 24 h. After 15 min, the phospho-Akt dose response was similar in AdipoR1 and AdipoR2 overexpressing cells (Figure 3.3F), although AdipoR1 typically mediated 10-20% higher phosphorylation than AdipoR2. However, after 24 h the phospho-Akt dose response was markedly different. AdipoR2 promoted robust Akt phosphorylation with as little as 0.5 µg/ml gAd whilst AdipoR1 was without effect at concentrations up to 1.5 µg/ml gAd (Figure 3.3I). Coupling to ERK and p38 MAPK also showed different characteristics with AdipoR1 exhibiting greater transduction than AdipoR2 at 15 min, especially at gAd concentrations of 2.0 µg/ml or higher (Figure 3.3G & H). These differences became less marked after 24 h (Figure 3.3J & K) with phosphorylation of P38MAPK being constitutively higher in AdipoR2 cells compared with AdipoR1 cells (Figure 3.3K). Collectively these results provide evidence that there are fundamental differences between signalling emanating from AdipoR1 and adipoR2, most notably the difference in temporal profiles with AdipoR1 acting more acutely than AdipoR2.


AdipoR1
AdipoR2
(% Max)
100
80
60
40
20
0
0 30 60 120 240 30 30
FC
WT KO
***
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*
0 min
30 min
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120 min
C
D
E
F
G
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J

Figure 3.2. Adiponectin reduces the cell-surface expression of AdipoR1 and AdipoR2. Flow cytometry analysis of cell-surface expression ratio of HEK293 cells transiently transfected with (A) AdipoR1-HA or (B) AdipoR2-HA. Cells were incubated with fetal bovine serum (FC) for 0, 30, 60, 120, 240 min or with serum from wild-type mice (WT) or serum from adiponectin knockout mice (KO) or with gAd (2.5 µg/ml) for 30 min. The % MAX represents receptor cell-surface expression in cells without any addition and was set to 100% for AdipoR1 and AdipoR2 respectively. Results are from four independent experiments (*$p<0.05$). (C-J) immunofluorescent microscopy of AdipoR1-HA and AdipoR2-HA constructs transiently expressed in CHO cells following 0, 30, 60 or 120 min treatment with serum. Plasma membrane is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue).

3.5.4 The subcellular localisation of AdipoR1 and AdipoR2 is governed by multiple domains.

We previously reported that the non-conserved N-terminal trunks of AdipoR1$_{(1-70)}$ and AdipoR2$_{(1-81)}$ underpinned the observed differences in cell-surface expression[200]. To investigate this further we generated and characterised the cell-surface expression of a number of chimera (Figure 3.4A). Analysis by flow cytometry demonstrated a striking profile, with increasing inclusion of the non-conserved trunk of AdipoR2 reducing cell-surface expression whilst the converse was observed upon increasing content of the non-conserved trunk of AdipoR1 (Figure 3.4B). These findings suggest there is not a single region or motif within the non-conserved trunks that underpins the different cell-surface expression profiles of AdipoR1 and AdipoR2 but that multiple regions contribute to these differences.

Next we characterised the signalling properties of the two chimera in which the entire non-conserved N-terminal trunks had been swapped (termed R1$_{(70)}$R2 and R2$_{(81)}$R1, respectively) (Figure 3.4A). In cells overexpressing R2$_{(81)}$R1 chimera the temporal profiles of Akt, ERK and p38MAPK phosphorylation showed peak phosphorylation at 24 h whereas in cells overexpressing R1$_{(70)}$R2 chimera phosphorylation peaked at 15 min (Figure 3.4C-E). These profiles closely resembled those of AdipoR2 and AdipoR1 respectively (see Figure 3.3A-C), indicating that key differences in the temporal signalling profiles of AdipoR1 and AdipoR2 are dictated by the non-conserved N-terminal trunks.

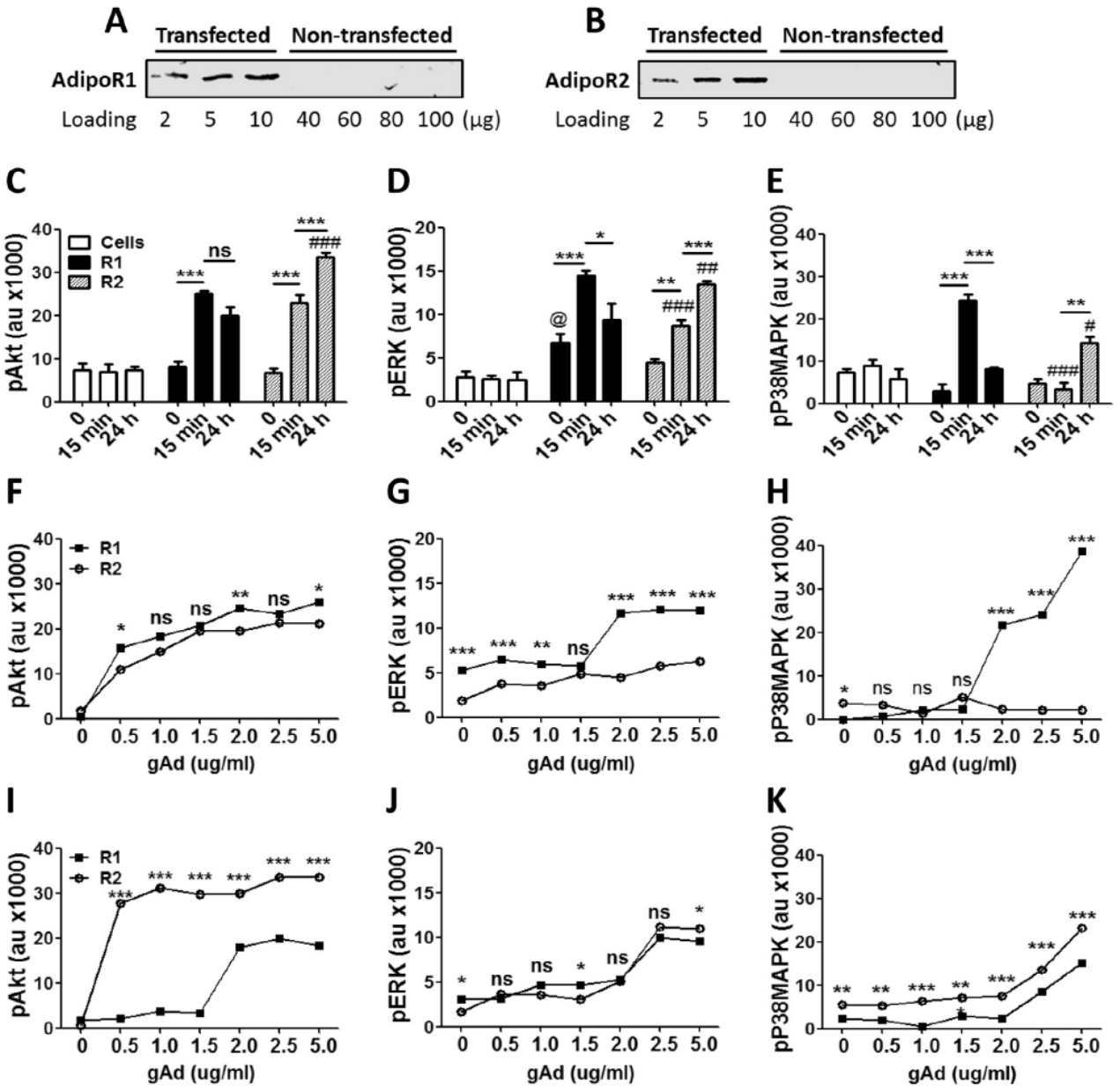

Figure 3.3. AdipoR1 and AdipoR2 exhibit different temporal activation profiles. Western blot showing levels of (A) AdipoR1 and (B) AdipoR2 in transfected and parental (non-transfected) HEK293 cells. Alphascreen analysis of (C) Akt, (D) ERK and (E) P38MAPK phosphorylation in HEK cells transiently expressing AdipoR1 or AdipoR2 constructs incubated with 2.5 µg/ml globular adiponectin (gAd) for 0, 15 min or 24 h. Alphascreen analysis of Akt, ERK and P38MAPK phosphorylation in HEK cells transiently expressing AdipoR1 or AdipoR2 constructs incubated with increasing concentrations of gAd (0 to 5 µg/ml) for 15 min (F-H) or 24 h (I-K). Results are from at least four independent experiments. In graphs C-E: *p<0.05, **p<0.01, ***p<0.001, comparing AdipoR1 or AdipoR2 at different timepoints; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, comparing AdipoR1 vs AdipoR2 at the same timepoint; $^{@}$p<0.05, comparing AdipoR1 vs parental cells at the same timepoint. In graphs F-I: *p<0.05, **p<0.01, ***p<0.001 comparing AdipoR1 vs AdipoR2.

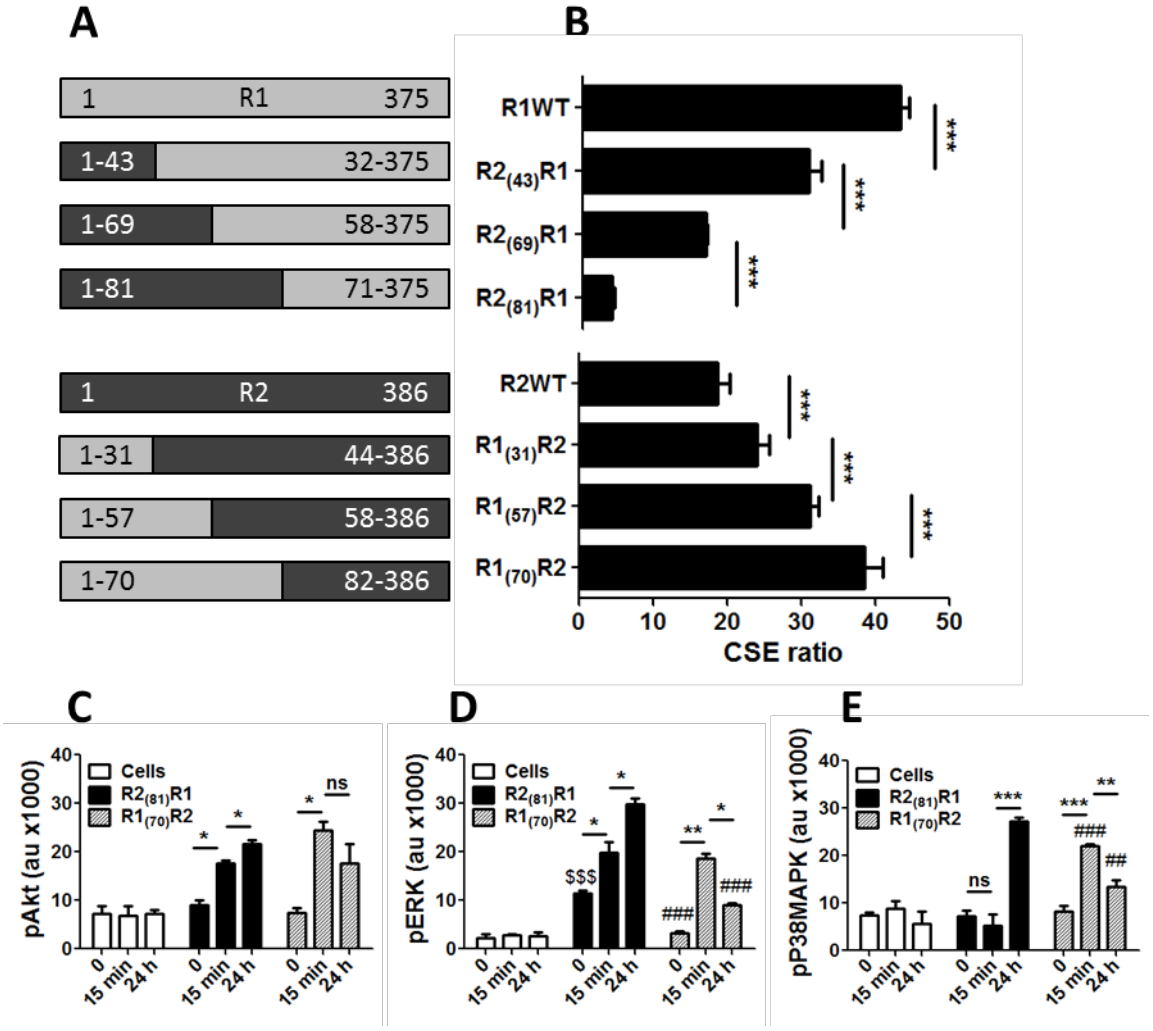

Figure 3.4. The non-conserved, N-terminal domain of AdipoR1 and AdipoR2 regulates cell-surface expression and signal transduction. (A) schematic representation of generated chimeric constructs. (B) Flow cytometry analysis of cell-surface expression ratio in HEK cells transiently expressing WT AdipoR1-HA, AdipoR2-HA and chimeric constructs, (AdipoR2$_{(1–43)}$/R1$_{(32–375)}$-HA, AdipoR2$_{(1–69)}$/R1$_{(58–375)}$-HA, AdipoR2$_{(1–81)}$/R1$_{(71–375)}$-HA, AdipoR1$_{(1–31)}$/R2$_{(44–386)}$-HA, AdipoR1$_{(1–57)}$/R2$_{(70–386)}$-HA and AdipoR1$_{(1–70)}$/R2$_{(82–386)}$-HA). Alphascreen analysis of (C) Akt, (D) ERK and (E) p38MAPK phosphorylation in HEK cells transiently expressing chimeric AdipoR2$_{(1–81)}$/R1$_{(71–375)}$-HA (R2/R1) or AdipoR1$_{(1–70)}$/R2$_{(82–386)}$-HA (R1/R2) constructs incubated with 2.5 µg/ml globular adiponectin (gAd) for 0, 15 min or 24 h. Results are from at least four independent experiments (*p<0.05). $^{\#}$comparing different genes, same timepoint. $^{\$}$comparing selected gene and parental cells, same timepoint.

3.5.5 Cell-surface expression and downstream signalling of AdipoR1 and AdipoR2 is regulated by conserved $F(x)_3F(x)_3F$ and $D(x)_3LL$ motifs.

Two different motifs within the intracellular N-terminal trunk of AdipoR1 have been shown to be essential for its efficient cell-surface expression[167]. Mutation of an acidic di-leucine motif ($_{106}D(x)_3LL$) or a putative ER exit motif ($_{121}F(x)_3F(x)_3F$), which are known to regulate trafficking of GPCRs[164, 203], resulted in inhibition of cell-surface expression of AdipoR1[167]. Alignment of the primary amino acid sequence of AdipoR1 and AdipoR2 from multiple species revealed conservation of these motifs between AdipoR1 and AdipoR2 (Figure 3.5A) leading us to speculate that these motifs would also be required for efficient cell-surface expression of AdipoR2. To test this we generated and characterised the expression of AdipoR2, and AdipoR1, mutants in which the key residues were mutated to alanine (termed R1/R2-FFF or R1/2-DLL where each of the residues was mutated to A). Flow cytometry of serum starved cells revealed a significant reduction in the cell-surface expression of AdipoR1-FFF and AdipoR1-DLL as well as AdipoR2-FFF and AdipoR2-DLL constructs compared to the WT receptors (Figure 3.5B & F). Furthermore, these constructs exhibited reduced adiponectin-stimulated phosphorylation of Akt (Figure 3.5C&G), ERK (Figure 3.5D&H) and P38MAPK (Figure 3.5E&I). These results indicate that efficient cell-surface expression of AdipoR1 and AdipoR2 is required for adiponectin signal transduction.

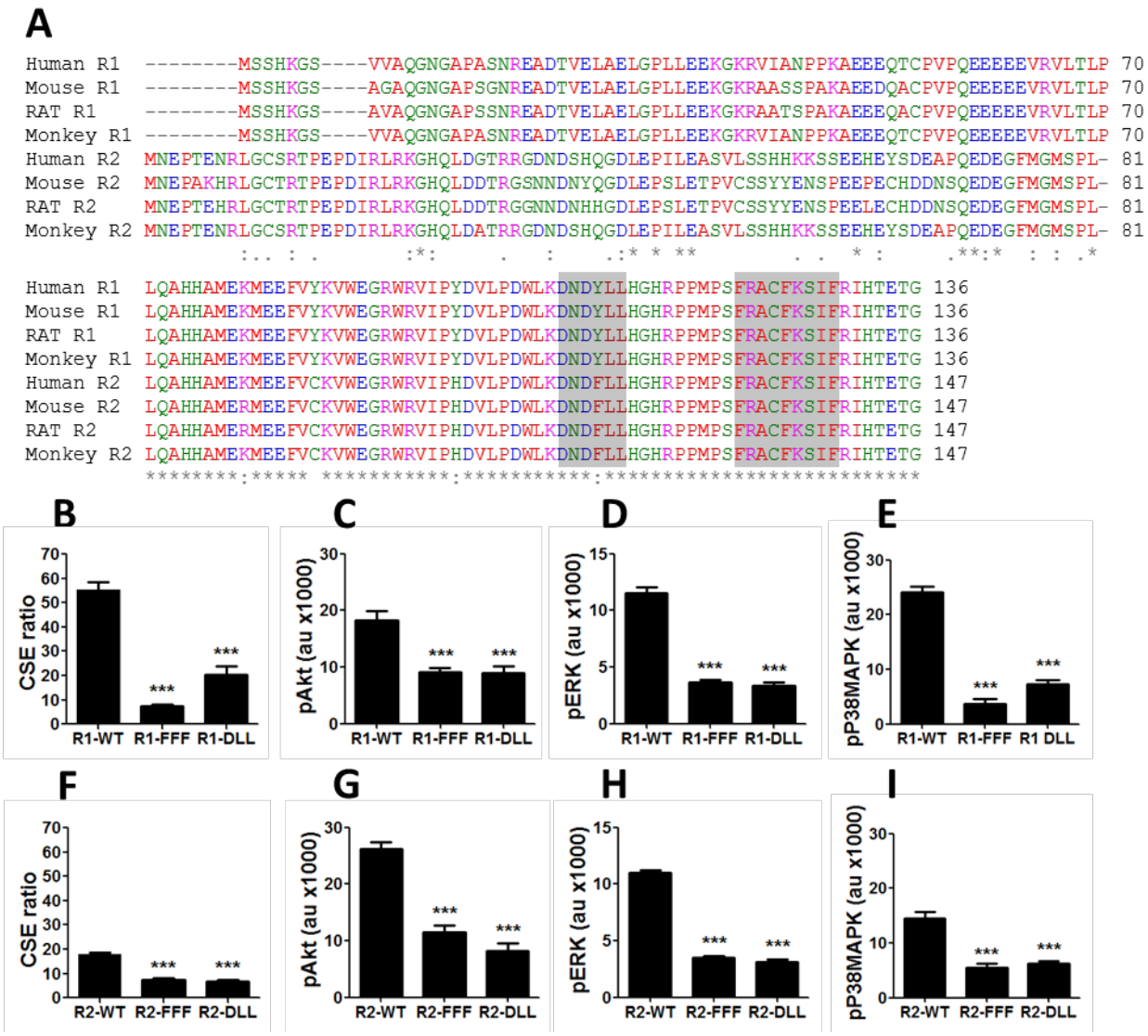

Figure 3.5. F(x)3F(x)3F and D(x)3LL motifs are required for efficient cell surface expression and signal transduction via AdipoR1 and AdipoR2. (A) Sequence alignment of the cytoplasmic N-terminal trunk of AdipoR1 and AdipoR2. Conserved F(x)3F(x)3F and D(x)3LL motifs are highlighted (in grey). (B) Flow cytometry analysis of cell-surface expression ratio in HEK293 cells transiently expressing WT AdipoR1, the F 121, 125, 129A (R1-FFF) and D106, L110, L111A (R1-DLL) mutants. Alphascreen analysis of (C) Akt, (D) ERK and (E) p38MAPK phosphorylation in HEK cells transiently expressing WT AdipoR1, R1-FFF or R1-DLL constructs incubated with 2.5 ug/ml gAd for 15 min. (F) Flow cytometry analysis of cell-surface expression ratio in HEK293 cells transiently expressing WT AdipoR2, the F 132, 136, 140A (R2-FFF) and D117, L121, L122A (R2-DLL) mutants. Alphascreen analysis of (G) Akt, (H) ERK and (I) P38MAPK phosphorylation in HEK293 cells transiently expressing WT AdipoR2, R2-FFF or R2-DLL constructs incubated with 2.5 ug/ml gAd for 24 h. Results are from at least four independent experiments (*** $p<0.001$).

3.6 Discussion

In the current report we have elaborated the molecular details governing differential cell-surface expression and downstream coupling of the adiponectin receptors, AdipoR1 and AdipoR2. We show that serum starvation increases cell-surface expression of both AdipoR1 and AdipoR2 and that, in contrast to serum from WT mice, serum from mice lacking adiponectin fails to reduce receptor cell-surface expression consistent with receptor internalisation. We also demonstrate that the temporal signal transduction profiles differ for AdipoR1 and AdipoR2. Furthermore, we show that these differences are intrinsically coupled to their cell-surface expression profiles and their non-conserved, N-terminal trunks which appear to contain multiple regions that promote or reduce cell-surface expression respectively.

Previously, we reported that AdipoR1, but not AdipoR2, interacted with ERp46 via its non-conserved N-terminal trunk[70]. Further investigations revealed that the non-conserved N-terminal trunks of AdipoR1 and AdipoR2 also dictated the cell-surface expression of AdipoR1 and AdipoR2, with robust cell-surface expression of AdipoR1 but not AdipoR2 observed under steady-state conditions in CHO cells[200]. To address the impact of this difference on coupling to downstream signalling events we first characterised the effects of serum starvation followed by serum replacement on cell-surface expression of the receptors. Serum withdrawal resulted in increased expression of both AdipoR1 and AdipoR2 although AdipoR2 still exhibited significantly lower cell-surface expression than AdipoR1. The addition of serum or gAd reduced cell-surface expression of both receptors and microscopy suggested that this decrease reflected classic ligand-dependent internalisation consistent with a previous report[152]. Our findings using serum from wild-type and adiponectin KO mice suggest that, at least under these experimental conditions, no other circulating factors, such as members of the CTRP family[204], are able to promote internalisation of the adiponectin receptors.

Since the seminal discovery of the adiponectin receptors by Kadowaki and colleagues[48] a large body of evidence has accumulated which indicates that adiponectin-stimulated activation of intracellular signalling pathways via the adiponectin receptors is highly variable across cell-types and tissues[12]. The precise mechanisms for this variability remain relatively poorly understood but a number of factors are likely to contribute. For example, the expression levels of AdipoR1 and AdipoR2 differ across tissues and cell-types[48] as do the expression levels of proteins that have been shown to interact with the receptors and modulate downstream signalling[169]. Moreover, the adiponectin receptors exhibit different binding properties[48] and investigators have used a range of

different recombinant multimeric or globular forms of adiponectin[12]. Whilst all of these factors are likely to contribute to differential activation of intracellular signalling pathways it is also noteworthy that relatively few studies have characterised signalling emanating specifically from AdipoR1 and AdipoR2 under tightly controlled conditions making this an area where our understanding remains particularly rudimentary. Moreover, inspection of the literature reveals several major caveats. For example, in the original report from Yamauchi and colleagues AdipoR2 was identified by sequence homology to AdipoR1 and this probably explains why a truncated form lacking the non-conserved N-terminal trunk was cloned and characterised[48]. We previously reported that a similar truncated construct exhibits increased cell-surface expression, compared with full-length AdipoR2[200], and, in light of the current findings, we predict that it would also show altered signalling profiles although this remains to be determined.

In the present report we used gAd to investigate signalling mediated via AdipoR1 or AdipoR2 constructs in HEK293 cells. This recombinant form of adiponectin has been used widely by others, as it represents a more homogenous, less variable product than full-length multimeric adiponectin [12] whilst HEK293 cells have been shown to be a suitable cell model for investigations into adiponectin signalling[57]. Interestingly, and in contrast to the findings from Lee and colleagues[57], we were unable to detect endogenous adiponectin receptors in our parental (non-transfected) HEK293 cells and, consistent with this, we did not observe any response upon treatment with gAd in the parental cells. As expected however, transfection of AdipoR1 or AdipoR2 conferred sensitivity to gAd. We found that acute (15 min) coupling to Akt was similar between AdipoR1 and AdipoR2 under conditions where cell-surface expression of AdipoR1 was typically three-fold higher than for AdipoR2. Moreover, sensitivity of AdipoR2-mediated Akt phosphorylation was significantly greater than that for AdipoR1 after chronic (24 h) treatment, indicating far-greater longevity of the AdipoR2 signal. These results were, at least to some extent, recapitulated when coupling to ERK and p38MAPK was analysed. For both, AdipoR1-mediated phosphorylation peaked at 15 min whilst AdipoR2-mediated phosphorylation peaked at 24 h. To our knowledge, this is the first time such differences in the temporal profiles of adiponectin signalling emanating from AdipoR1 and AdipoR2 have been described.

Detailed analysis of a series of chimeric receptors revealed that the differential cell-surface expression of AdipoR1 and AdipoR2 was defined by multiple regions within the non-conserved N-terminal trunks indicating that no single motif underpinned the observed differences. Moreover, functional investigations revealed that the temporal signalling profiles were also determined by these non-conserved N-terminal trunks.

Two motifs ($D(x)_3LL$ and F(x)3F(x)3F) were previously reported to play a role in anterograde trafficking of AdipoR1[167]. We demonstrated that these motifs are conserved within the juxtamembrane region of AdipoR2 and are required for the efficient cell-surface expression of both receptors. We found that mutation of either of these motifs inhibited cell-surface expression and adiponectin-stimulated phosphorylation of Akt, ERK and p38MAPK. These data further highlight the importance of efficient cell-surface expression of AdipoR1 and AdipoR2 for efficient downstream signalling and the complexity of adiponectin receptor trafficking. Further work is required to elaborate the molecular details governing the contribution of the non-conserved N-terminal trunks and the conserved sequence motifs (including $D(x)_3LL$ and F(x)3F(x)3F) and how these, in turn, contribute to the temporal signalling profiles of AdipoR1 and AdipoR2 respectively.

Adiponectin and its receptors are recognised as attractive potential targets for the treatment of cardiometabolic disease[12]. In the current report we have increased our understanding of processes governing cell-surface expression of the adiponectin receptors and demonstrate that efficient cell-surface expression is required to afford sensitivity to adiponectin. We have established that the non-conserved, N-terminal trunks of AdipoR1 and AdipoR2 serve as key determinants of the cell-surface expression and signalling profiles of the receptors. This work provides a foundation for future studies that may aim to enhance adiponectin sensitivity by increasing cell-surface expression of the receptors, particularly AdipoR2.

Acknowledgements

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Chapter Four:

“Palmitoylation of the adiponectin receptors, AdipoR1 and AdipoR2, is essential for function.”

4.1 Introduction to this chapter

This chapter was submitted to *Proceedings of the National Academy of Sciences* Journal as an original investigation.

In the current report we demonstrated a pivotal role for palmitoylation of the adiponectin receptors in receptor function and demonstrated that:

- Both AdipoR1 and AdipoR2 are palmitoylated.
- Palmitoylation of a conserved 'canonical' site (common to GPCRs) in the juxta-membrane region of both receptors is essential for efficient cell-surface expression of both receptors.
- Palmitoylation of the canonical site is required for adiponectin-stimulated phosphorylation of downstream effectors AMPK, AKT, ERK and p38MAPK.
- Overexpression of WT AdipoR1 or AdipoR2 in mouse skeletal muscle resulted in increased phosphorylation of downstream effectors whilst the palmitoylation-defective mutants were without effect.
- Palmitoylation of non-conserved cysteines in AdipoR2 contribute to the maintenance of AdipoR2 stability.

Collectively, these findings provided direct biochemical evidence that human AdipoR1 and AdipoR2 are palmitoylated at multiple sites and provide the first evidence of an important role for post-translational modification of the adiponectin receptors in receptor homeostasis and function and hence activity of the adiponectin axis.

Palmitoylation of the adiponectin receptors, AdipoR1 and AdipoR2, is essential for function.

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4.2 Abstract

Dysregulation of the adiponectin axis contributes to obesity-related cardiometabolic disorders making it an attractive therapeutic target. However, our understanding of the adiponectin receptors, AdipoR1 and AdipoR2, two atypical seven transmembrane domain (7TMD) proteins that have inverted membrane orientation compared with other 7TMD receptors such as GPCRs, is rudimentary restricting therapeutic opportunities. Here, we have begun to elaborate the dynamic molecular mechanisms that contribute to AdipoR1 and AdipoR2 function using *in-silico*, *in vitro* and *in vivo* approaches. Bioinformatics analysis revealed several putative palmitoylation sites including a conserved 'canonical' site (common to GPCRs) in the juxta-membrane region of both receptors as well as additional non-conserved sites. Palmitoylation of these sites was confirmed using acyl-biotinyl exchange chemistry and site-directed mutagenesis which also revealed rapid turnover of palmitoylation. Cell-based characterization of palmitoylation-defective receptor mutants, AdipoR1$_{(C124A)}$ or AdipoR2$_{(C135A)}$, demonstrated that palmitoylation of the canonical site is essential for efficient cell-surface expression (CSE) of both receptors and also for adiponectin-stimulated phosphorylation of downstream effectors AMPK, AKT, ERK and p38MAPK. Finally, we extended these investigations into a more physiological setting by employing *in vivo* electrotransfer (IVE) to examine the effects of overexpression of wild-type (WT) or palmitoylation-defective AdipoR1 or AdipoR2 constructs on downstream effectors in mouse skeletal muscle. Overexpression of WT AdipoR1 or AdipoR2 resulted in increased phosphorylation of downstream effectors whilst the palmitoylation-defective mutants were without effect. Collectively, these findings establish adiponectin receptor palmitoylation as a key regulator of receptor function, hence activity of the adiponectin axis.

Significance Statement

The adiponectin axis represents a therapeutic target for treatment of cardiometabolic disease. Evidence of adiponectin resistance is accumulating but rudimentary understanding of the adiponectin receptors R1 and R2 constrains therapeutic opportunities. Here we demonstrate a pivotal role for palmitoylation of the adiponectin receptors in receptor function. Deletion of a single highly conserved palmitoylation site resulted in failure of the receptors to express at the cell surface and transduce the adiponectin signal establishing receptor palmitoylation as a key determinant of adiponectin sensitivity. Future work is required to determine whether reduced receptor palmitoylation contributes to the development of adiponectin resistance and progression of cardiometabolic disease and or whether manipulating adiponectin receptor palmitoylation may serve as a means to enhance adiponectin sensitivity.

4.3 Introduction

The adiponectin axis represents an attractive therapeutic target. Adiponectin is an adipocyte-derived hormone that exhibits a range of predominantly beneficial cardiometabolic properties[6, 205]. Moreover, circulating levels of adiponectin are reduced in obesity and such hypoadiponectinemia is implicated in the aetiology of obesity-related diseases including type 2 diabetes and cardiovascular disease[205]. Adiponectin mediates many of its pleiotropic effects via two receptors, R1 and R2[48]. These receptors are atypical 7TMD receptors that are structurally distinct from the G-protein coupled receptor (GPCR) family, with intracellular N-termini and extracellular C-termini that couple to a number of intracellular downstream effectors including AMPK, AKT, ERK and p38MAPK[48, 71, 90]. Emerging evidence indicates that, in addition to the hypoadiponectinemia mentioned above, adiponectin resistance also contributes to the aetiology of cardiometabolic diseases[42, 43, 45, 46]. Thus, pharmacological approaches to increase circulating adiponectin levels and or enhance adiponectin sensitivity are of therapeutic potential.

Increased understanding of the molecular mechanisms that regulate the cellular properties of the adiponectin receptors will identify a framework to explore possible pathophysiological events and potential therapeutic approaches. Several studies have identified a number of adiponectin receptor-interacting proteins that modulate adiponectin coupling to intracellular signaling pathways[206]. The best characterized of these being APPL1, which interacts with both R1 and R2 to facilitate adiponectin signaling[72]. In addition, primary amino acid sequences and motifs that determine key cellular and functional characteristics including cell-surface expression and temporal signaling profiles have been identified within R1 and R2[167, 200, 207]. In contrast, our understanding of the role of post-translational modifications (PTMs) in the biology of the adiponectin receptors is limited. It was recently reported that induction of myocardial infarction (MI) promoted phosphorylation of R1, which was not phosphorylated in the normal heart, via GPCR kinase 2 which resulted in adiponectin resistance[208]. This finding highlights the potential pathophysiological relevance of PTM of the adiponectin receptors.

In the current study we set out to investigate the putative role of a ‘housekeeping’ PTM, S-palmitoylation, which has been shown to influence the cellular homeostasis and regulation of a variety of proteins and receptors at multiple levels[209, 210]. S-palmitoylation is a reversible lipid modification involving the addition of a saturated 16-carbon palmitate moiety to specific cysteines via a thioester linkage that may, like phosphorylation or ubiquitination, act as a switch. Palmitoylation of GPCRs commonly occurs on one or more cysteines found 10 to 14 residues

following the seventh TMD[211] and has diverse effects including regulation of cellular trafficking and downstream signaling[177, 209, 210]. Here we demonstrate that R1 and R2 are constitutively palmitoylated and that palmitoylation of a conserved 'canonical' cysteine, 13 amino acids prior to the first TMD, is required for efficient CSE and adiponectin-mediated activation of downstream effectors.

4.4 Materials and methods

4.4.1 Mice.

All experimental procedures were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee. Male WT C57Bl/6 mice (AMREP AS, Melbourne, VIC, Australia) were used for all experiments and commenced when mice were 16 weeks of age. Animals were maintained at 22.0±0.5°C under a 12-h day, 12-h night cycle and fed standard chow diet containing 5% of total energy from fat (Specialty Feeds, Glen Forrest, WA, Australia).

4.4.2 Reagents and antibodies.

Reagents were from Sigma–Aldrich (Castle Hill, Australia) unless otherwise stated. Tissue culture reagents were from Invitrogen (Mount Waverley, Australia). Primary antibodies against HA and Flag were from Covance (Washington, USA) and primary antibody against Sodium Potassium ATPase (Nak) was from Abcam (Melbourne, Australia). Secondary antibodies were from Life Technology (Invitrogen). Molecular biology reagents were from New England Biolabs (Ipswich, NA, USA) or Promega Corporation (Madison, WA, USA).

4.4.3 Molecular biology.

Original constructs encoding C-terminally epitope-tagged (HA) human R1 and R2 were as described[70]. R1 and R2 mutants were generated by QuikChange site-directed mutagenesis (Agilent Technology, CA, USA). Mutations were confirmed by DNA sequencing (Sanger method). Chinese Hamster Ovary (CHO), Human Embryonic Kidney 293 (HEK293) and INS-1 cells were transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Cells were routinely transfected with 200 ng of plasmid and analyzed 24 h post-transfection.

4.4.4 Real-time PCR.

Gene expression levels were quantified using real-time PCR. Mice tibialis anterior muscles were powdered under liquid nitrogen and homogenized using Fast prep-24 (MP Biomedicals, NSW,

Australia) in Trizol (Invitrogen, Australia). Total RNA was extracted as per the manufacturer's instructions and cDNA was synthesized from 1 mg total RNA. RT-PCR was performed using the SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life Technologies). Results were normalized using mRNA for TATA box.

4.4.5 Flow cytometry.

Flow cytometry was carried out as previously described[207]. A CyAn™ ADP Analyser (Beckman Coulter, Sydney, Australia) and FlowJo software were used to determine the number of cells expressing HA-tagged R1 or R2 at the cell-surface relative to the total number of cells expressing these receptors in permeabilised cells.

4.4.6 Detection of palmitoylation.

ABE methodology was performed essentially as described[177]. To prevent protein palmitoylation, cells were incubated with 2-bromopalmitate (2BP) for 1 or 4 h prior to collection of crude membrane. Following the indicated treatments/transfections, cells were washed with cold PBS and lysed in lysis buffer (5 mM Tris, 0.5 mM EDTA, 10 mM NaF and 10mM Na3Vo4) containing protease inhibitor cocktail (Roche). Lysis was improved by repeated passaging through a 26 gauge needle. For enrichment of membranes, lysates were depleted of nuclei via centrifugation at 800 g for 10 min. The supernatant was then centrifuged at 257,000 g for 1 h, and the pellet was resuspended in lysis buffer containing 1% Triton X-100. Total protein was quantified with a bicinchononic acid (BCA) assay (Pierce) using BSA as the standard. After undergoing a freeze-thaw cycle, the membrane fraction was incubated with 10mM N-Ethyl Malemide (NEM) for four hours to block free thiols followed by Chloroform-Methanol precipitation to purify the protein. Next, samples were incubated with a buffer containing hydroxylamine (0.7 M) and EZ-link HPDP-biotin (1 mM) for 1h for cleavage of thioester linkages, and capture of nascent thiols by biotin. Unreacted biotin was removed by Chloroform-Methanol precipitations. Biotinylated proteins were affinity purified using streptavidin beads (Pierce) by incubation at 4uC for 1 h. For immunoblot analysis, elution was performed and samples were separated via SDS-PAGE on a Mini-Gel apparatus (Bio-Rad). Palmitoylated AdipoR1 and AdipoR2 was detected by anti-HA Western blot analysis.

4.4.7 In vivo electrotransfer (IVE).

IVE was performed as described[212].

4.4.8 Microscopy.

Indirect immunofluorescence microscopy was performed as described[207].

4.4.9 Determination of AMPK phosphorylation.

Phospho-AMPK in INS-1 cells or in TAM was measured using PathScan Phospho-AMPKα (Thr172) Sandwich ELISA (Cell-Signaling technology, Danvers, Massachusetts) according to manufacturer's instruction. Parental/transfected INS-1 cells were serum-starved overnight then stimulated with human globular adiponectin (Prospec Protein Specialists, USA) or vehicle for 15 min or 24 h.

4.4.10 Measurement of AKT, ERK and p38MAPK phosphorylation.

Phospho-AKT, ERK and p38MAPK were determined using AlphaScreen SureFire kits (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) as described[207].

4.4.11 Statistical analysis.

Data are presented as mean ± SEM. Significance was determined using one way ANOVA followed by Tukeys test with statistical significance defined as $p < 0.05$.

4.5 Results

4.5.1 R1 and R2 are palmitoylated.

Using the CSS-palm v4.0 palmitoylation prediction algorithm[213] we identified a number of putative palmitoylation sites (based on prediction score and location) that were situated within the intracellular N-terminus of both R1 and R2. These included a highly conserved 'canonical' site located 13 amino acids prior to the first predicted TMD in R1 ($R1_{C124}$) and R2 ($R2_{C135}$) (Figure 4.1A), which mirrors the typical palmitoylation site found 10-14 amino acids after the seventh TMD in several GPCRs. In addition, there was one further site in R1 ($R1_{C54}$) and two in R2 ($R2_{C11}$ & $R2_{C96}$) that were not conserved between R1 and R2 but were conserved across species (Figure 4.1A). Subsequently, we used acyl-biotinyl exchange (ABE) to examine the palmitoylation status of transiently expressed C-terminally HA-tagged R1 and R2 in quiescent CHO and HEK293 cells treated with vehicle or an inhibitor of palmitoylation, 2-Bromo Palmitate (2BP). Our results indicated that both R1 and R2 were palmitoylated in control cells (Figure 4.1B & C) whilst

treatment with 2BP was sufficient to reduce palmitoylation of both by ≥ 95% after 1 h in CHO cells (Figure 4.1B) and ≥ 80% after 4 h in HEK cells (Figure 4.1C). These results show that both R1 and R2 are palmitoylated and suggest palmitoylation has a relatively high rate of turnover that exhibits variability across cell-types.

Palmitoylation of R1 and R2 is required for cell surface expression (CSE).

Having previously reported that CSE of R1 and R2 is around 60% and 20% in quiescent cells [200] we next sought to investigate a role for palmitoylation in modulation of CSE of R1 and R2, given the recognized role of palmitoylation in regulation of receptor trafficking[209]. We used flow cytometry to determine CSE of the receptors expressed transiently in CHO and HEK293 cells as above. Quantitative analysis revealed that treatment with 2BP significantly reduced the CSE of both R1 and R2 in a timeline consistent with the effects on palmitoylation (Figure 4.1D & E). These results suggest palmitoylation of the receptors is required for their efficient CSE.

To rule out the possibility that 2BP inhibited CSE of the receptors by an indirect effect and investigate the potential contribution of palmitoylation at each site more specifically we generated a

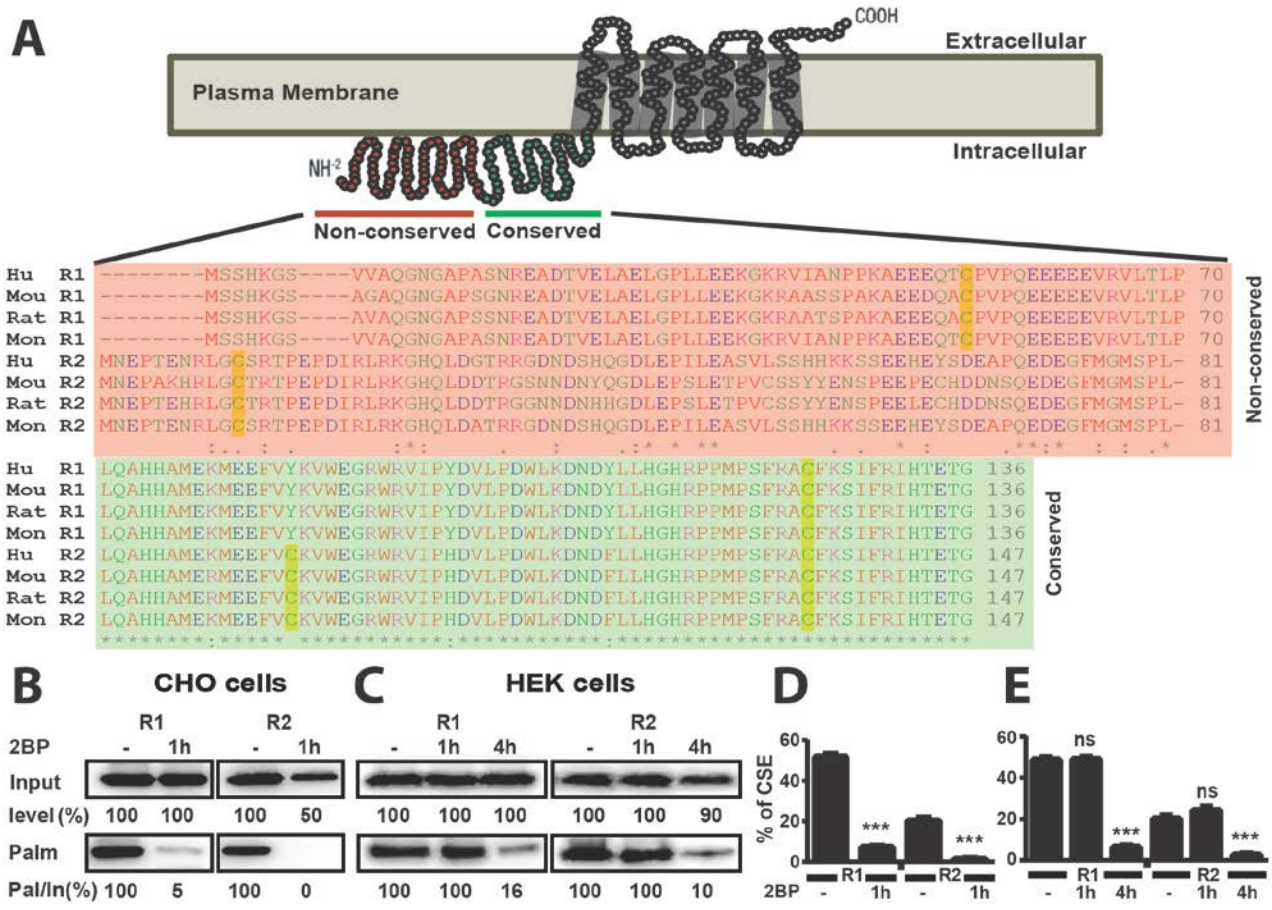

Figure 4.1. R1 and R2 are palmitoylated and palmitoylation is required for CSE. (A) Upper panel - Schematic representation of adiponectin receptors with the non-conserved N-terminal domain colored mauve and the conserved N-terminal region in green. Lower panel - Sequence alignment of the cytoplasmic N-terminal trunk of R1 and R2. Putative palmitoylation sites are highlighted in yellow. Western blots (using anti-HA) showing the expression (upper panel) and palmitoylation (lower panel) of transiently expressed C-terminal HA-tagged R1 or R2 in (B) CHO and (C) HEK 293 cells following treatment with either vehicle or 2BP. Protein expression levels (%) are shown beneath the top panel (Input) and normalized to control (without 2BP). Palmitoylation values are shown beneath the lower panel (Palm) and presented as the ratio of palmitoylation/protein (Pal/In (%)) normalized to the control (without 2BP). Flow cytometry analysis of (D) CHO and (E) HEK 293 cells transiently expressing R1 or R2. The % of CSE represents the percentage of cells exhibiting CSE of the receptors (non-permeabilised cells) relative to the total number of cells expressing the receptors (permeabilised cells) after treatment with vehicle or 2BP. Results are from 4 independent experiments (***p<0.001).

series of constructs where each of the cysteine residues was replaced by alanine ($R1_{C54A}$, $R1_{C124A}$, $R2_{C11A}$, $R2_{C96A}$ and $R2_{C135A}$) and characterized them following transient expression in CHO cells. Analysis of $R1_{C124A}$ revealed mutation of the canonical cysteine in R1 lead to a complete loss of palmitoylation and CSE (Figure 4.2A & B). In contrast mutation of the R1-specific cysteine, $R1_{C54A}$, reduced palmitoylation by around 50% whilst CSE was unaltered (Figure 4.2A & B). Mutation of the canonical cysteine in R2, $R2_{C135A}$, reduced palmitoylation relatively modestly, by only 30%, but significantly reduced CSE (Figure 4.2C & D). In contrast, mutation of either of the R2-specific cysteines, $R2_{C11A}$ & $R2_{C96A}$, reduced palmitoylation by around 60% but had no significant effect on CSE (Figure 4.2C & D). Interestingly, the expression levels of $R2_{C11A}$ and $R2_{C96A}$ were also markedly reduced (Figure 4.2C - upper panel). There are several potential explanations for these observations. In the case of R1 it seems reasonable to propose that palmitoylation at the canonical site is required for both CSE and subsequent palmitoylation at the non-conserved site. Palmitoylation of the canonical site in R2 appears to be a prerequisite for CSE but not for palmitoylation at the R2-specific sites. Moreover, palmitoylation at $R2_{C11A}$ and $R2_{C96A}$ appears necessary for the stability of R2. In support of this, it is noteworthy that treatment with 2BP resulted in reduced expression of R2, but not R1 (see Figure 4.1B & C).

To investigate the effect of deletion of the canonical palmitoylation site on subcellular distribution in more detail we employed high resolution (OMX) immunofluorescence microscopy. Compared to the WT receptors, both $R1_{C124A}$ and $R2_{C135A}$ exhibited altered subcellular distribution patterns showing reduced proximity to the plasma membrane as well as a loss of staining around the nucleus (Figure 4.2E).

Palmitoylation of the canonical cysteine promotes enrichment of the N-terminal cytoplasmic domain of R1 or R2 to the PM. To further examine the role of palmitoylation at the canonical sites we generated truncated WT and mutant constructs that encompassed the N-terminal cytoplasmic domains of R1 and R2 (spanning amino acids 1-127 and 1-138 respectively) termed $NR1_{WT}$, $NR1_{C124A}$, $NR2_{WT}$ & $NR2_{C135A}$. Palmitoylation of WT constructs and reduced palmitoylation of the $NR1_{C124A}$ and $NR2_{C135A}$ constructs was confirmed by ABE (Figure 4.3A). Immunofluorescence microscopy revealed striking enrichment of both the $NR1_{WT}$ and $NR2_{WT}$ constructs underneath the PM (Figure 4.3B). This was absent for the $NR1_{C124A}$ construct and reduced for the $NR2_{C135A}$ construct, both of which showed a more regular, diffuse distribution extending throughout the cytoplasm. Consistent with this altered distribution, biochemical analysis of subcellular fractions revealed enrichment of the WT constructs in the PM relative to the $NR1_{C124A}$ and $NR2_{C135A}$

constructs which were more enriched in the ER (Figure 4.3C). These results provide further evidence of an important role for palmitoylation of the canonical sites regulating the subcellular distribution of R1 and R2.

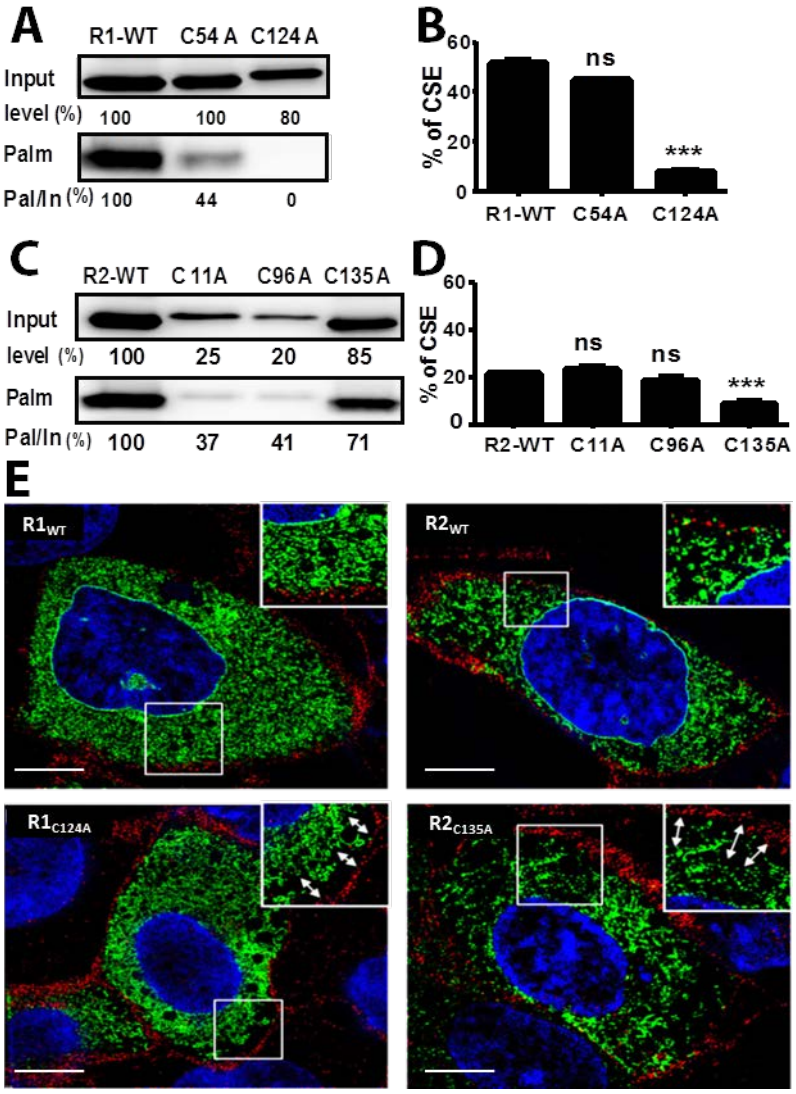

Figure 4.2. Deletion of the canonical palmitoylation site prevents CSE. (A & C) Western blots (using anti-HA) showing the expression (top panel) and palmitoylation (lower panel) of C-terminally HA-tagged receptor transiently expressed in CHO cells. Protein expression levels (%) are shown beneath the top panel (Input) and normalized to the WT control. Palmitoylation values are shown beneath the lower panel (Palm) and presented as the ratio of palmitoylation/protein (Pal/In (%)) normalized to the WT control. (B & D) Flow cytometry analysis of HEK cells transiently expressing receptor constructs as indicated. The % of CSE shows the percentage of cells exhibiting CSE of the receptors (non-permeabilised) relative to the total number of cells exhibiting receptor expression (permeabilised). Results are from 4 independent experiments (***$p<0.001$). (E) Immunofluorescent microscopy of $R1_{WT}$, $R1_{C124A}$, $R2_{WT}$ and $R2_{C135A}$ constructs transiently expressed in CHO cells. The PM is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue).

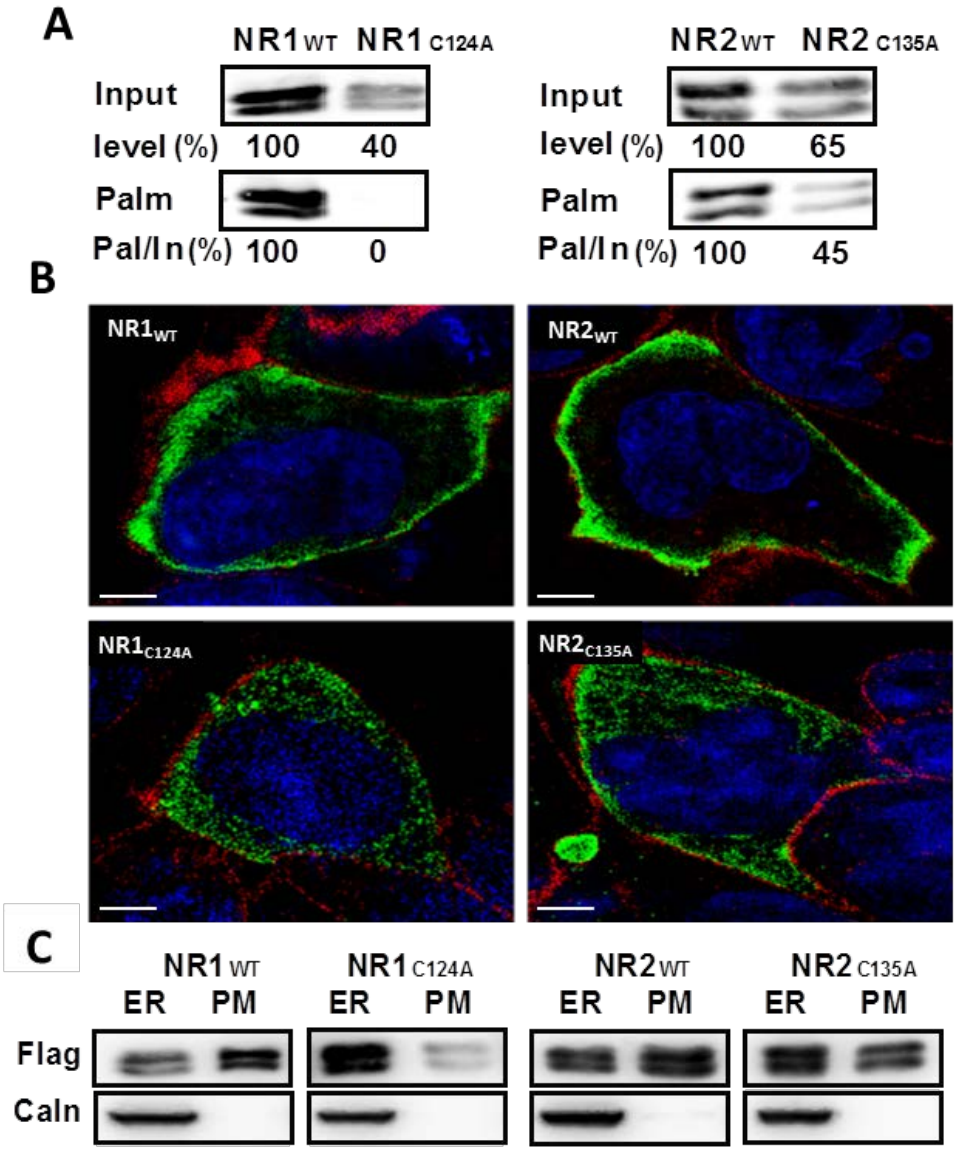

Figure 4.3. Palmitoylation of the canonical cysteine promotes enrichment of the N-terminal cytoplasmic domains with the PM. (A) Western blots (using αFlag) showing expression (top panel) and palmitoylation (lower panel) of N-terminally Flag-tagged receptor constructs $NR1_{WT}$, $NR1_{C124A}$, $NR2_{WT}$ and $NR2_{C135A}$ transiently expressed in CHO cells. Protein expression levels (%) are shown beneath the top panel (Input) and normalized to the WT control. Palmitoylation values are shown beneath the lower panel (Palm) and presented as the ratio of palmitoylation/protein (Pal/In (%)) normalized to the WT control. (B) Immunofluorescent microscopy of $NR1_{WT}$, $NR1_{C124A}$, $NR2_{WT}$ and $NR2_{C135A}$ constructs transiently expressed in CHO cells. The PM is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue). (C) Western blot showing distribution of $NR1_{WT}$, $NR1_{C124A}$, $NR2_{WT}$ and $NR2_{C135A}$ constructs following transient expression in HEK 293 cells and subcellular fractionation. Calnexin (Caln) serves as an ER marker to demonstrate the lack of contamination of the PM with ER.

Palmitoylation of R1 and R2 is required for downstream signaling.

Having established that deletion of canonical palmitoylation interfered with the CSE of both R1 and R2 we hypothesized that this would also result in compromised adiponectin-stimulated activation of downstream effectors implicated in mediating adiponectin's effects, namely AMPK, AKT, ERK and p38MAPK[198, 214]. We previously reported that R1 and R2 display different temporal signaling profiles[207], hence we analyzed adiponectin-stimulated phosphorylation of downstream effectors in HEK293 cells transiently expressing R1 or R2 constructs after 15 min or 24 h respectively. Overexpression of $R1_{WT}$ or $R1_{C54A}$ promoted a similar increase in adiponectin-stimulated phosphorylation of AKT, ERK and p38MAPK relative to parental cells (Figure 4.4A,C & E). In contrast, overexpression of $R1_{C124A}$ failed to enhance adiponectin-stimulated phosphorylation (Figure 4.4A, C & E). Similar results were seen in cells overexpressing R2 constructs, with $R2_{WT}$, $R2C_{11A}$ and $R2_{C96A}$ all showing enhanced adiponectin-stimulated phosphorylation relative to parental cells which were indistinguishable from cells overexpressing the $R2_{C135A}$ construct (Figure 4.4B, D & F). Surprisingly, overexpression of the receptors failed to promote adiponectin-stimulated AMPK phosphorylation in the HEK293 cells or in CHO cells (data not shown). However, overexpression of the WT receptors in INS-1 cells did result in a significant increase in adiponectin-stimulated AMPK phosphorylation (Figure 4.4G & H). As before, deletion of the canonical palmitoylation site inhibited this effect. The $R2_{C96A}$ construct also failed to promote a significant increase in AMPK phosphorylation (Figure 4.4H). Indeed, it was surprising that the $R2_{C11A}$ and $R2_{C96A}$ constructs enhanced adiponectin-stimulated phosphorylation of AKT, ERK and p38MAPK to a similar level as $R2_{WT}$ given they were expressed at only 20-25% of $R2_{WT}$. To address the possibility that this reflected receptor redundancy due to high levels of overexpression we performed a titration experiment, transfecting HEK293 cells with increasing amounts of $R2_{WT}$ or $R2_{C96A}$ constructs and assessing receptor expression levels (Figure 4.4I) and adiponectin-stimulated AKT phosphorylation (Figure 4.4J). Our results confirmed that $R2_{WT}$ was expressed around 4-fold higher than $R2_{C96A}$ (with comparable expression observed at 50 ng and 200 ng respectively) and that coupling to AKT phosphorylation showed a similar relationship, peaking with 50 ng of $R2_{WT}$ and 200 ng of $R2_{C96A}$ respectively (Figure 4.4I & J). Collectively, these data demonstrate that deletion of canonical palmitoylation blocks the ability of R1 or R2 to transduce the adiponectin signal and also suggests that reduced palmitoylation of the non-canonical sites, particularly in R2, may compromise receptor function as a result of reduced stability.

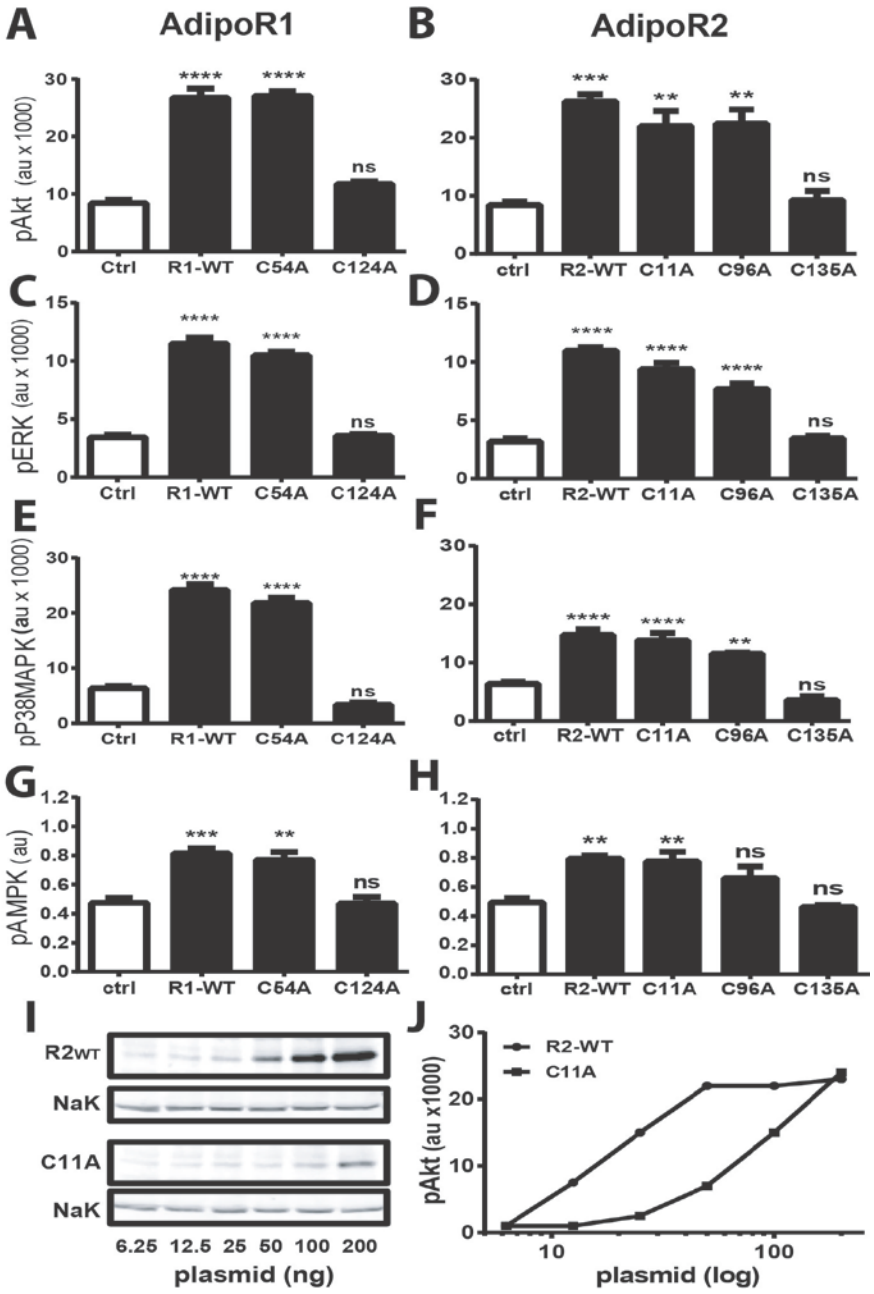

Figure 4.4. Deletion of the canonical palmitoylation site abolishes signal transduction in vitro. Alphascreen analysis of (A-B) AKT, (C-D) ERK and (E-F) p38MAPK phosphorylation in HEK cells transiently expressing $R1_{WT}$, $R1_{C54A}$ and $R1_{C124A}$ incubated with 2.5 µg/ml globular adiponectin (gAd) for 15 min (A,C and E) and $R2_{WT}$, $R2_{C11A}$, $R2_{C96A}$ and $R2_{C135A}$ incubated with 2.5 µg/ml gAd for 24 h (B,D and F). ELISA demonstrating AMPK phosphorylation in INS-1 cells transiently expressing (G) $R1_{WT}$, $R1_{C54A}$ and $R1_{C124A}$ treated with 2.5 µg/ml gAd for 15 min and (H) $R2_{WT}$, $R2_{C11A}$, $R2_{C96A}$ and $R2_{C135A}$ incubated with 2.5 µg/ml gAd for 24 h. Parental INS-1 cells were used as control. Results are from 4 independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (I) Western blots (using αHA) and (J) pAKT Alphascreen show levels of HA-tagged $R2_{WT}$ and $R2_{C11A}$ protein (sodium/potassium ATPase (NaK) serves as a loading control) and

adiponectin-stimulated phosphor-AKT following transient transfection of HEK293 cells with increasing concentrations of plasmid (200 ng was used for routine transfection).

Palmitoylation of the canonical cysteine in R1 and R2 is required in vivo.

To investigate the requirement for adiponectin receptor palmitoylation in a more physiological setting we employed *in vivo* electrotransfer (IVE) [212, 215] to characterize the effects of overexpression of WT or canonical palmitoylation defective constructs, namely $R1_{C124A}$ & $R2_{C135A}$ constructs, in the tibialis anterior (TA) muscle of C57BL/6 mice. Receptor constructs were introduced into the right TA whilst the left TA served as control (transduced with empty plasmid). Muscles were harvested after two weeks and analysis of mRNA levels confirmed comparable expression of each of the receptor constructs (Figure 4.5A & B). Phosphorylation of downstream signaling effectors AKT, ERK and AMPK were all increased by approximately 2-fold in muscles expressing the WT receptors (Figure 4.5C-H). This effect was lost in muscles expressing the $R1_{C124A}$ or $R2_{C135A}$ receptors. Overall, these results demonstrate that palmitoylation of the adiponectin receptors at a highly-conserved canonical site is a prerequisite for function *in vitro* and *in vivo*.

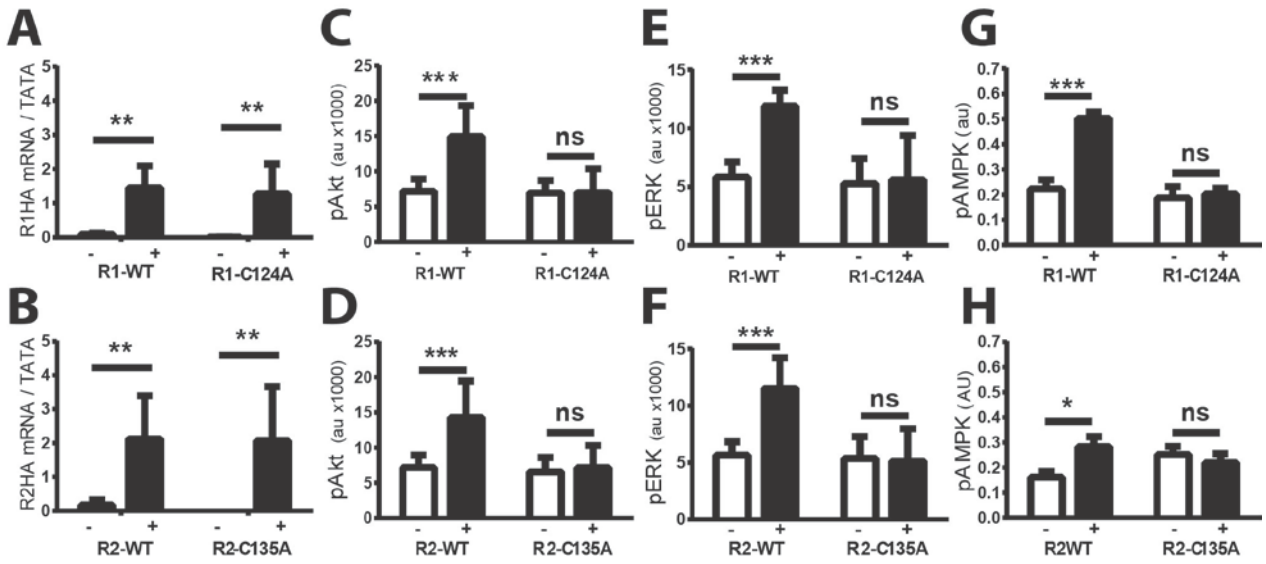

Figure 4.5. Deletion of the canonical palmitoylation site abolishes signal transduction in vivo. Graphs show mRNA expression of (A) $R1_{WT}$ and $R1_{C124A}$ or (B) $R2_{WT}$ and $R2_{C135A}$ in left (-) and right (+) tibialis anterior muscle. Graphs show phosphorylation of (C-D) AKT, (E-F) ERK and (G-H) AMPK in muscles transduced with constructs as indicated. (n=6/group)(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4.6 Discussion

In the current investigation we have provided the first evidence of an important role for PTM of the adiponectin receptors in receptor homeostasis and function. We provided direct biochemical evidence that human R1 and R2 are palmitoylated at multiple sites within the N-terminal, cytoplasmic domains and these events regulate receptor trafficking and stability. In particular, we demonstrated that palmitoylation of a highly conserved canonical cysteine, situated 13 residues prior to the first TMD, was essential for CSE of both receptors and deletion was sufficient to block adiponectin-stimulated signal transduction via R1 and R2 both in vitro and in vivo. These findings identify a new layer of regulation of the adiponectin axis and establish adiponectin receptor palmitoylation as a potential therapeutic target.

The importance of extensive PTM of adiponectin for its efficient production has been well characterized[216] however our appreciation of the role of PTM in the regulation of the adiponectin receptors is in its infancy. The study of protein palmitoylation has advanced rapidly in line with the development of relatively straightforward, non-radioactive approaches to determine palmitoylation status[209] which, aligned with mass-spectrometry approaches, have revealed the extensive nature of protein palmitoylation with a recent estimate suggesting ≥ 10% of proteins are palmitoylated[217]. The adiponectin receptors have not been identified in these large-scale proteomic investigations[217]. This may reflect a limitation of the approaches which typically under-represent the palmitoylome[209]. In the case of transmembrane proteins the direct effects of palmitoylation may include alterations in protein conformation, regulation of protein-protein or protein-membrane interactions that may in turn affect protein localization, stability and or activity[210].

Given the above we set out to determine whether the adiponectin receptors were palmitoylated and, if so, define the molecular details. An in silico search for putative palmitoylation sites[213] revealed two and three putative sites in the N-terminal cytoplasmic domains of R1 and R2 respectively and we subsequently demonstrated that both receptors were palmitoylated using ABE. Palmitoylation status reflects a balance between addition and removal with the former performed by a family of palmitoyl acyltransferases (PATs) whilst the latter is carried out by protein thioesterases[218, 219]. Treatment of cells with 2BP, a widely used pharmacological inhibitor of the acyltransferases[177, 220], abolished palmitoylation of both receptors. Interestingly, whilst the temporal effects of 2BP on receptor palmitoylation were similar for R1 and R2 they differed across cell-types as 1 h was sufficient to inhibit palmitoylation in CHO but not HEK293 cells, where 4 h treatment was required. One possibility is that activity of the thioesterase(s) responsible for receptor

depalmitoylation is higher in the former. Regardless, this observation highlights the potential for cell and tissue specific differences in the dynamics of adiponectin receptor palmitoylation.

A large body of evidence has demonstrated that palmitoylation regulates the trafficking and subcellular localization of a range of proteins[221, 222] including the insulin-responsive glucose transporter GLUT4[223] and associated regulatory proteins[224] as well as numerous GPCRs[177, 220]. GPCRs, such as the protease-activated receptor-2, the D2 dopamine receptor and the α2A and β2-adrenergic receptors, are typically palmitoylated on one or more cysteine residues situated close to the seventh TMD[177, 220, 225]. Moreover, targeted inhibition of this palmitoylation event by mutagenesis of the key cysteine(s) results in impaired receptor trafficking and CSE[177, 220]. Interestingly, we observed similar results when we deleted the canonical palmitoylation site in R1 (C_{124}) and R2 (C_{135}) but not when we deleted the less-conserved R1- or R2-specific sites. We also observed marked staining around the nucleus in cells expressing WT receptor constructs but not constructs lacking the canonical palmitoylation site. Similar palmitoylation-dependent localization of the ER chaperone calnexin to the nuclear envelope has been reported, with further investigation revealing a direct interaction between calnexin and the ribosome-translocon complex of the rough ER[226]. Collectively these observations highlight a role for palmitoylation of the canonical sites in the regulation of subcellular localization and CSE.

The importance of the canonical palmitoylation events was further highlighted by our characterization of the intracellular N-terminal domains of R1 and R2 (that lacked the 7TMDs). The truncated WT constructs exhibited clear morphological and biochemical enrichment with the PM relative to the canonical palmitoylation deficient mutants. Deletion of the canonical palmitoylation site in the truncated R1 construct resulted in a complete loss of palmitoylation, consistent with our observations of full-length R1, and had a greater impact on the distribution and fractionation properties than deletion of the canonical palmitoylation site in the truncated R2 construct, which retained palmitoylation. These findings are consistent with the requirement of a dual-lipid anchor to mediate stable attachment of soluble proteins with the PM[209].

Consistent with our earlier reports[200, 207] we demonstrated that CSE of R2 was relatively modest (20%) compared with that of R1 (60%). Molecular investigations revealed these differences, as well as differences in temporal signaling profiles, were underpinned by the non-conserved, cytoplasmic N-terminal trunks of R1 (1-70) and R2 (1-81)[200, 207]. Thus, it was surprising that the intracellular N-terminal domains characterized here, comprising both the non-conserved and conserved regions (spanning residues 1-127 and 1-138 in R1 and R2) showed similar subcellular distribution profiles.

One possible explanation is that these contrasting observations may reflect the effects of intramolecular interactions between the intracellular N-terminal domains and the 7TMDs. Indeed, the recently solved crystal structures of R1 and R2 support such a model[71]. Although the crystals lacked the entire non-conserved N-terminal trunks and a portion of the conserved domains (spanning residues 1-88 and 1-99) the remaining intracellular N-terminal regions were closely juxtaposed to a large internal cavity postulated to be involved in receptor activity[71]. Moreover, these intracellular N-terminal regions contain a short intracellular helix (spanning residues 121-129 and 132-140)[71] that includes the canonical palmitoylation site and an ER exit motif, $F(x)_3F(x)_3F$, required for efficient CSE of both adiponectin receptors[167, 207]. This complex arrangement is also found in some GPCRs, where the helix is referred to as helix 8, and steroid hormones[220, 227]. Localization of the helix is often regulated by palmitoylation. Hence it is tempting to speculate palmitoylation, particularly at the canonical site, may modulate interactions between the intracellular-N-terminal domains and the 7TMD to alter accessibility of motifs in R1 or R2 and or interactions affecting the position of the intracellular N-terminal domain. Whilst such modulation may also be expected to affect activity, we feel that palmitoylation at the non-conserved site(s) is unlikely to affect receptor activity per se, given the comparable, albeit right-shifted, adiponectin-stimulated AKT phosphorylation dose-response of $R2_{C11A}$ compared with $R2_{WT}$. It is also noteworthy that the helix containing the canonical palmitoylation site also constitutes an ER exit motif, $F(x)_3F(x)_3F$, required for efficient CSE of both adiponectin receptors[167, 207]. Palmitoylation of a cysteine located within an $F(x)_6LL$ ER exit motif (FxxCxxxLL) within the human estrogen receptor α (ERα) promotes membrane localization and signaling and, as for other proteins, facilitates the association with caveolin-1[227]. Emerging evidence supports an important role for an interaction between R1 and caveolins 1 and 3[228-230]. Thus, canonical palmitoylation of the adiponectin receptors is likely to have multilayered effects that require further, comprehensive investigations to elaborate the molecular details.

Deletion of the canonical palmitoylation event abolished CSE of both receptors and, consistent with this, adiponectin-mediated phosphorylation of downstream signaling effectors was similarly lost in cell-based assays. We attempted to extend these observations and demonstrate the physiological significance of adiponectin receptor palmitoylation by performing ABE on a range of tissue samples however we were unable to detect palmitoylation of the endogenous receptors. This likely reflects a combination of the low level of expression of the receptors and intrinsic limitations of the approach[209]. Thus, we employed an alternate approach to address the physiological significance by characterizing the effect of overexpression of WT and canonical palmitoylation defective receptor

constructs in mouse skeletal muscle[212, 215]. Overexpression of the WT receptors for two weeks resulted in a constitutive increase in phosphorylation of AMPK, AKT and ERK. This effect was not observed in muscles overexpressing the mutant receptors. These observations strongly support the notion that canonical palmitoylation of the receptors is a strict requirement for physiological function.

Deletion of the canonical palmitoylation event did not affect protein receptor levels. In contrast, deletion of either of the R2-specific cysteines resulted in reduced protein levels indicating a strict requirement for palmitoylation of both cysteines to maintain R2 stability. A role for palmitoylation in maintenance of protein stability is well established, with numerous studies demonstrating that inhibition of palmitoylation results in increased ubiquitin-mediated degradation[209, 210, 220]. Further studies are required to determine whether such mechanisms explain the observed reduction in $R2_{C11A}$ and $R2_{C96A}$ protein levels. As mentioned above, the ability of these mutant receptors to transduce the adiponectin-signal across the membrane appears largely intact. Whilst this suggests a relatively limited impact on receptor function per se, at physiological levels of expression any reduction in receptor levels may be expected to contribute to decreased adiponectin sensitivity. Indeed, our titration experiments highlight the high levels of overexpression achieved in transient transfection systems employed both here and elsewhere[71] provide a level of redundancy that make it likely that subtle, but potentially important, differences in receptor function may be overlooked.

A role for dysregulation of palmitoylation contributing to human diseases is well established[217]. More specifically, obesity has been shown to alter the palmitoylation status of key metabolic proteins such as GLUT4[224] whilst altered palmitoylation has been implicated in endothelial dysfunction[231], lipid-induced ER stress and β-cell death [232], and modulation of TNFα activity [233]. Having established that adiponectin receptor function is dependent on palmitoylation it remains to be determined whether altered palmitoylation of R1 and R2 may compromise the adiponectin axis and play a role in human disease. Elucidation of the molecular details governing adiponectin receptor palmitoylation may identify new strategies to ameliorate adiponectin resistance and improve cardiometabolic outcomes.

Acknowledgements

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Chapter Five:

“Muscle-specific overexpression of AdipoR1 or AdipoR2 gives rise to common and discrete local effects whilst AdipoR2 promotes additional systemic effects.”

5.1 introduction to this chapter

This chapter was submitted to *Scientific Reports* Journal as an original investigation.

In the current report we have compared the effects of overexpression of AdipoR1 or AdipoR2 in skeletal muscle (tibaialis anterior muscle – TAM) of lean or obese mice.

We report the following key observations:

- In lean mice, TAM-specific overexpression of AdipoR1 or AdipoR2 increased phosphorylation of downstream signalling effectors (AMPK, AKT and ERK) and expression of the insulin responsive glucose transporter glut4.
- Only TAM-specific overexpression of AdipoR2 increased pparα and a target gene acox1.
- These local effects were decreased in obese mice, despite no reduction in circulating adiponectin levels, consistent with the existence of adiponectin resistance.
- TAM-specific overexpression of AdipoR2 increased expression of adipoQ in TAM and this was unaffected by obesity.
- TAM-specific overexpression of AdipoR2 in obese mice promoted systemic effects including decreased weight gain, reduced epididymal fat mass and inflammation, increased epididymal adipoQ expression and increased circulating adiponectin.

Collectively, these results demonstrate that AdipoR1 and AdipoR2 exhibit overlapping and distinct effects in skeletal muscle consistent with enhanced adiponectin sensitivity but these appear insufficient to ameliorate established obesity-induced adiponectin resistance. They also provide evidence of unexpected systemic effects of muscle-specific overexpression of AdipoR2 in obese mice.

Muscle-specific overexpression of AdipoR1 or AdipoR2 gives rise to common and discrete local effects whilst AdipoR2 promotes additional systemic effects.

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5.2 Abstract

Hypoadiponectinemia and adiponectin resistance are implicated in the aetiology of obesity-related cardiometabolic disorders, hence represent a potential therapeutic axis. Here we characterised the effects of *in vivo* electrotransfer-mediated overexpression of the adiponectin receptors, AdipoR1 or AdipoR2, into tibialis anterior muscle (TAM) of lean or obese mice. In lean mice, TAM-specific overexpression of AdipoR1 (^{TAM}R1) or AdipoR2 (^{TAM}R2) increased phosphorylation of AMPK, AKT and ERK and expression of the insulin responsive glucose transporter *glut4*. In contrast, only ^{TAM}R2 increased *pparα* and a target gene *acox1*. These effects were decreased in obese mice despite no reduction in circulating adiponectin levels. ^{TAM}R2 also increased expression of *adipoQ* in TAM of lean and obese mice. Furthermore, in obese mice ^{TAM}R2 promoted systemic effects including; decreased weight gain; reduced epididymal fat mass and inflammation; increased epididymal *adipoQ* expression; increased circulating adiponectin. Collectively, these results demonstrate that AdipoR1 and AdipoR2 exhibit overlapping and distinct effects in skeletal muscle consistent with enhanced adiponectin sensitivity but these appear insufficient to ameliorate established obesity-induced adiponectin resistance. We also identify systemic effects upon ^{TAM}R2 in obese mice and postulate these are mediated by altered myokine production. Further studies are warranted to investigate this possibility which may reveal novel therapeutic approaches.

5.3 Introduction

Adiponectin is a key adipokine that displays a variety of beneficial effects to reduce diabetes, atherosclerosis and cardiometabolic disease[205]. Adiponectin regulates carbohydrate and lipid metabolism, reducing hepatic glucose production and enhancing fatty acid oxidation in skeletal muscle[205]. In contrast to most other adipokines, circulating adiponectin levels are typically reduced in obesity, type 2 diabetes and associated conditions[205]. Moreover, mice lacking adiponectin or humans with polymorphisms that compromise adiponectin production develop metabolic dysfunction and or type 2 diabetes[12]. Hence, therapeutic strategies to reverse hypoadiponectinaemia are attractive. Increasing evidence indicates that adiponectin resistance also contributes to the development of metabolic and cardiovascular diseases[42, 43, 45, 46, 208, 234-237]. While the molecular mechanisms that give rise to adiponectin resistance are poorly defined strategies to overcome adiponectin resistance are also of therapeutic potential.

The beneficial effects of adiponectin are mediated primarily via the action of two atypical seven-transmembrane domain (7TMD) receptors, AdipoR1 and AdipoR2[48]. These receptors are structurally and functionally distinct from other 7TMD receptors, having intracellular N-termini and extracellular C-termini, and couple adiponectin to a range of downstream effectors including AMPK, PPARα, AKT and ERK by mechanisms that are incompletely understood[48, 71, 182, 238]. Molecular and cellular studies have revealed differences between AdipoR1 and AdipoR2 that include different binding properties[48], cell surface expression[168, 200, 206] and temporal signaling profiles[152, 207]. Furthermore, investigations in mice have demonstrated contrasting expression profiles, with AdipoR1 expressed relatively ubiquitously compared with AdipoR2[48], and activation of alternate signaling pathways[90]. For example, studies in knockout mice indicate that in liver activation of AMPK is mediated primarily by AdipoR1 whilst PPARα appears to be downstream of AdipoR2[90].

In the current study we aimed to extend our cell-based investigations demonstrating differences between AdipoR1 and AdipoR2[200, 207] to compare the effects of overexpression of either AdipoR1 or AdipoR2 in skeletal muscle in lean and obese mice. Overexpression was achieved by *in vivo* electrotransfer (IVE) of the tibialis anterior muscle (TAM) that allowed characterization of local, TAM-specific changes in phosphorylation of downstream signaling effectors and expression of genes involved in glucose and lipid metabolism as well as determination of somewhat unexpected systemic effects in response to overexpression of AdipoR2.

5.4 Materials and methods

5.4.1 Animals

All experimental procedures were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee or the University of Queensland Animal Ethics Unit. Male WT C57Bl/6 mice (AMREP AS, Melbourne, VIC, Australia or University of Queensland Biological Resource, Brisbane, QLD, Australia) were used for all experiments and commenced when mice were 8 weeks of age. Animals were maintained at 22.0±0.5°C under a 12-h day, 12-h night cycle and fed standard chow diet containing 5% of total energy from fat. At 8 weeks of age, animals were divided into two groups. Half kept on standard chow diet and the rest were fed high fat diet containing 43% of total calories from fat (lard) (Specialty Feeds, Glen Forrest, WA, Australia) for 8 weeks before in vivo electrotransfer.

5.4.2 Reagents and antibodies

Reagents were from Sigma–Aldrich (Castle Hill, Australia) unless otherwise stated. Primary antibodies against HA and Sodium Potassium ATPase were from Covance (Washington, USA) and Abcam (Melbourne, Australia) respectively. AdipoR2 antibody has been described previously[70]. Secondary antibodies were from Life Technology (Invitrogen).

5.4.3 Molecular biology

Original constructs encoding C-terminally epitope-tagged (HA) human AdipoR1 and AdipoR2 were as described[70]. Plasmid DNA was prepared using a Plasmid Purification Gigaprep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. The DNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (Biolab, Scoresby, VIC, Australia) and the DNA dissolved in saline (154 mmol/l NaCl) to a final concentration of 4 μg/μl.

5.4.4 In vivo Electrotransfer

IVE was performed as described[212]. Briefly, mice were anaesthetised with isoflurane, their hind limbs were shaved and TA muscles were injected with 30 μl of 0.5 U/μl hyaluronidase. Two hours later mice were again anaesthetised with isoflurane and 100 μg empty vector, GFP, HA-tagged AdipoR1 or HA-tagged AdipoR2 (in 25 μl saline) was injected into the right TA muscle. The left TA muscle was injected with 25 μl empty vector. Stainless steel electrodes attached to an ECM-830 electroporator (BTX, Holliston, MA) were placed on the muscle and square-wave electrical pulses

(200 V/cm) were applied eight times with an electrical pulse generator at a rate of 1 pulse/s, with each pulse being of 20 ms duration. Two weeks later muscles and other tissues were dissected, snap frozen in liquid nitrogen and stored at -80°C.

5.4.5 Real-time PCR

Gene expression levels were quantified using real-time PCR assay. Mice tibialis anterior muscles were powdered under liquid nitrogen and homogenized using Fast prep-24 (MP Biomedicals, NSW, Australia) in Trizol (Invitrogen, Australia). Epididymal and subcutaneous adipose tissue and liver were homogenized using Fast prep-24 (MP Biomedicals, NSW, Australia) in Trizol (Invitrogen, Australia). Total RNA was then extracted as per the manufacturer's instructions and resuspended in nuclease-free water. Genomic DNA contamination of RNA preparations was eliminated by digestion with DNase I amplification grade (Invitrogen, Australia). cDNA was synthesized from 1 mg total RNA using cDNA synthesis kit (Bioline, NSW, Australia) and RT-PCR was performed using the SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real-time PCR system (Ambion Life Technologies). Results are quoted to the mRNA level compared to TATA box, the expression of which was unchanged by the treatments.

5.4.6 Western blot

Mice tibialis anterior muscles were powdered under liquid nitrogen and homogenized using Fast prep-24 (MP Biomedicals, NSW, Australia) in lysis buffer. Lysates were depleted of nuclei via centrifugation at 800 g for 10 min. The supernatant was then centrifuged at 257,000 g for 1 h, and the pellet (HSP containing membrane) was resuspended in lysis buffer and protein concentration was determined by bicinchononic acid (BCA) assay (Pierce) using BSA as the standard. 60μg protein was then loaded and resolved by SDS-PAGE on polyacrylamide gels, transferred to membranes and blocked with 5% BSA. The immunoreactive proteins were detected with enhanced chemiluminescence after incubation with appropriate primary and secondary antibodies.

5.4.7 Total adiponectin ELISA

Total circulating adiponectin was measured using adiponectin ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction.

5.4.8 AMPK phosphorylation ELISA

TA muscles were powdered under liquid nitrogen and homogenized using Fast prep-24 (MP Biomedicals, NSW, Australia) in lysis buffer. Phosphorylation of AMPK in TA muscle was measured using PathScan Phospho-AMPKα (Thr172) Sandwich ELISA (Cell-Signaling technology, Danvers, Massachusetts) according to manufacturer's instruction.

5.4.9 Akt and ERK phosphorylation assays

Alphascreen analysis was performed as previously described[207]. TA muscles were powdered under liquid nitrogen and homogenized using Fast prep-24 (MP Biomedicals, NSW, Australia) in Alphascreen lysis buffer. Phosphorylation of Akt and ERK was measured using AlphaScreen SureFire kits essentially as described (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Plates were read using a POLARstar Omega plate reader.

5.4.10 Statistical analysis

Data are presented as mean ± SEM. Significance was determined using paired t test to compare control and test TAM and two-way ANOVA followed by Tukey's multiple comparison test with statistical significance defined as $p < 0.05$.

5.5 Results

5.5.1 Overexpression of AdipoR2 in TAM of obese mice has unexpected systemic effects.

Using *in vitro*, cell-based systems we previously demonstrated that acute treatment with globular adiponectin resulted in different temporal signaling profiles in cells overexpressing AdipoR1 or AdipoR2, with the former promoting relatively acute activation (peaking at 15 min) and the latter more chronic activation (peaking after 24 h) respectively[207]. In the present study we have employed *in vivo* electrotransfer (IVE) to extend these observations and determine the effects of short-term (14 day) overexpression of AdipoR1 and AdipoR2 in mouse tibialis anterior muscle (TAM) of lean (chow fed) and obese mice fed a high fat diet (HFD) for 8 weeks.

IVE is a powerful experimental approach that allows manipulation of the gene of interest in the test leg and comparison with the control leg in the same animal[212, 215]. Here, we first used IVE to introduce a plasmid encoding GFP into the right TAM (test) and empty plasmid into the left TAM (control) of 16 week old lean or obese C57BL/6 mice. After 14 days mice were sacrificed and GFP expression was examined visually and by qRT-PCR. Visual inspection revealed robust GFP

expression throughout the entire target muscle and none in neighbouring muscles (Figure 5.1A). GFP expression in the test TAM, but not the control TAM, was also confirmed by qRT-PCR (Figure 5.1 B).

Having confirmed the efficacy and specificity of the IVE protocol we then went on to perform experiments to characterise the effects of TAM-specific overexpression of AdipoR1 (^{TAM}R1) or AdipoR2 (^{TAM}R2) in lean (average body weight 29.85 g) and obese mice (average body weight 40.75 g). Following IVE of AdipoR1 or AdipoR2 into the test TAM and empty plasmid into the control TAM mice were maintained on chow or HFD for a further 14 days then sacrificed for analysis. To our surprise in obese mice ^{TAM}R2 resulted in significantly reduced weight gain and elevated circulating adiponectin levels compared with ^{TAM}R1 obese mice (weight gain: -0.6±0.3 vs 1.3±0.3 g, p=0.001; adiponectin: 3.0±0.3 vs 1.8±0.2 µg/ml, p<0.01; n=6/group).

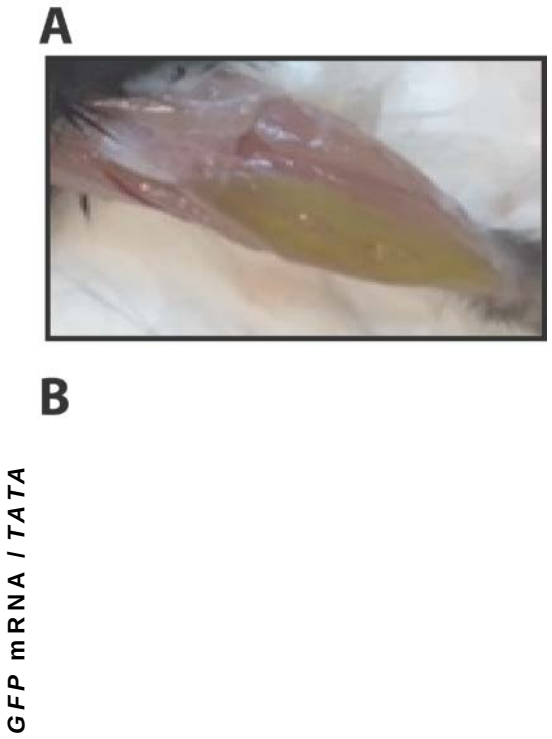

Figure 5.1. IVE-mediated expression of GFP. (A) GFP protein expression in TAM at the time of tissue collection **(B)** *GFP* expression in test TAM (T) compared to control TAM (C) in lean and obese mice; *p < 0.05; n=3 in each group.

5.5.2 HFD induced obesity does not alter AdipoR1 or AdipoR2 expression in TAM.

The effects on weight gain and circulating adiponectin levels were not anticipated and prompted us to redesign the study to include lean and obese control (sham) groups that were transduced with empty plasmid in both the left and right TAMs in parallel to the AdipoR1 or AdipoR2 transduced mice to allow direct comparison across groups. The extent of ^{TAM}R1 or ^{TAM}R2 was determined 14 days post IVE. Expression of both endogenous and exogenous genes was determined using primers specific for either mouse or human receptors respectively. The expression of endogenous receptors was unaffected by either IVE or diet (Figure 5.2A & B), with *adipoR1* expressed at levels 10-fold higher than *adipoR2*. Human *AdipoR1* and *AdipoR2* were only detected in the test TAM and were expressed at similar levels in lean and obese mice (Figure 5.2C & D). Western blot analysis was performed to characterise overexpression at the protein level. Western blot using HA-antibody confirmed expression of the exogenous HA-tagged proteins in only the test TAM and demonstrated that AdipoR1 and AdipoR2 proteins were expressed at similar levels in lean and obese mice (Figure 5.2E & F). Western blot with a validated AdipoR2 antibody (that recognises both human and mouse AdipoR2[70]) revealed a 2-fold increase in total AdipoR2 in TAM from the test leg compared to the control leg. Unfortunately we were unable to perform a similar analysis of AdipoR1 protein due to the lack of a suitable, validated antibody that recognised both human and mouse AdipoR1 efficiently. Nevertheless, these results demonstrated the success and efficiency of the IVE approach and also showed that expression of the endogenous or exogenous receptors was not affected by HFD-induced obesity.

5.5.3 ^{TAM}R1 or ^{TAM}R2 promotes activation of proximal signaling events and these effects are reduced in HFD-induced obesity.

We next sought to examine the local effects of ^{TAM}R1 or ^{TAM}R2 at the level of proximal phosphorylation events using ELISA or Alphascreen technology. Phosphorylation of AMPK was increased around 2-fold in response to overexpression of either receptor in TAM of lean mice (Figure 5.3A). Overexpression of either receptor also significantly increased AMPK phosphorylation in obese mice however the magnitude of this effect was less than that observed in lean mice. Similar results were seen for AKT phosphorylation. ^{TAM}R1 or ^{TAM}R2 increased AKT phosphorylation in TAM by around 50%, whilst the magnitude of this effect was reduced by around 20% in obese mice (Figure 5.3B). In lean mice, phosphorylation of ERK was increased 3-fold by ^{TAM}R1 and 2-fold by ^{TAM}R2 (Figure 5.3C). The magnitude of these effects was reduced by 55%

and 45% in obese mice such that overexpression of AdipoR1 or AdipoR2 no-longer resulted in a significant increase in test verses control TAM (Figure 5.3C). These results show that, at least under these experimental conditions, overexpression of AdipoR1 or AdipoR2 have similar effects on proximal signaling effectors and also suggest the development of HFD-obesity induced adiponectin resistance.

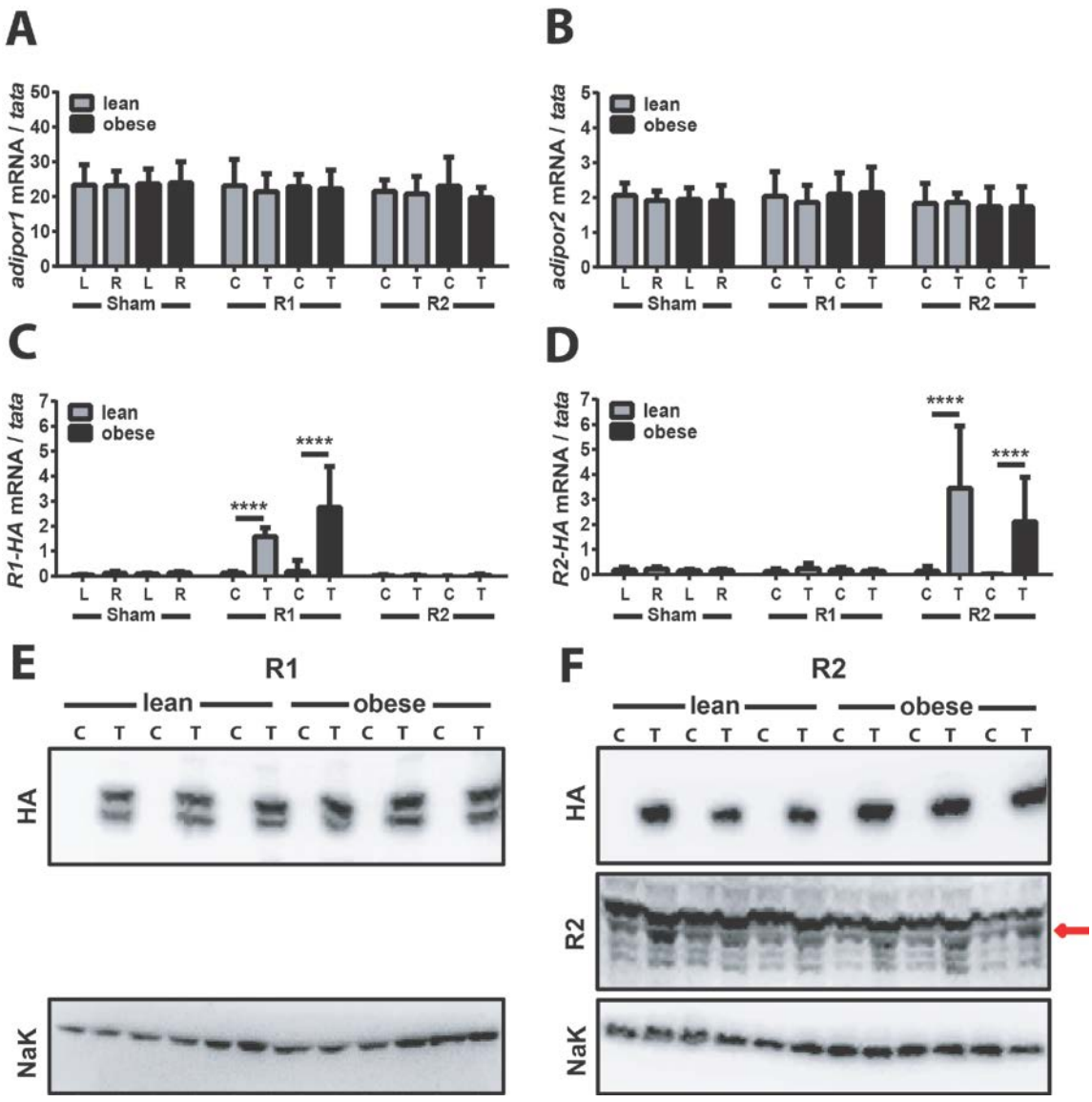

Figure 5.2. IVE-mediated Overexpression of AdipoR1 and AdipoR2. qRT-PCR analysis of **(A)** endogenous a*dipoR1*, **(B)** endogenous *adipoR2*, **(C)** *R1-HA* and **(D)** *R2-HA* expression in right vs left TAM of control (sham) and test (T) vs control (C) TAM of ^{TAM}R1 (R1) and ^{TAM}R2 (R2) mice. Data are expressed as mean±SEM; ****p < 0.0001; n=6 in each group. **(E)** Representative western blot analysis of HSP (membrane fractions) of TAM lysates derived from ^{TAM}R1 mice probed with HA antibody (top panel) and NaK ATPase antibody (lower panel). **(F)** Immunoblotting of HSP (membrane fractions) of TAM lysates

derived from ^{TAM}R2 mice probed with HA antibody (top panel), in-house R2 antibody (middle panel) and NaK ATPase antibody (lower panel); n=3 in each group of lean and obese mice.

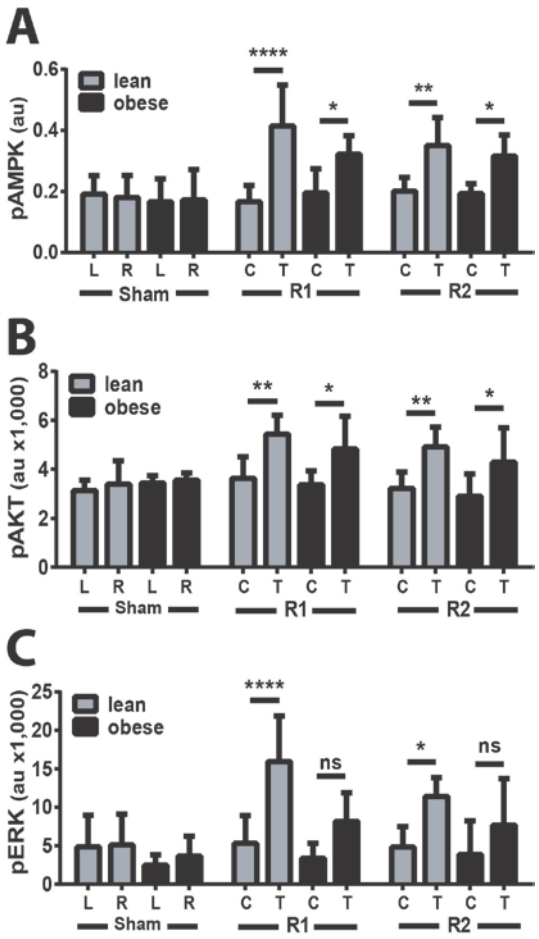

Figure 5.3. ^{TAM}R1 and ^{TAM}R2 activate proximal signalling pathways. (A) ELISA demonstrating AMPK phosphorylation of TAM lysates of control (sham), ^{TAM}R1 (R1) and ^{TAM}R2 (R2) mice. Alphascreen analysis of **(B)** Akt and **(C)** ERK phosphorylation in TAM lysates of sham, R1 and R2 groups. Data are expressed as mean±SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; n=6 in each group; significant difference of test TAM (T) compared to control TAM (C) in lean and obese mice.

5.5.4 ^{TAM}R2, but not ^{TAM}R1, promotes increased expression of genes involved in lipid metabolism and these effects are reduced in HFD-induced obesity.

We then investigated the effects of overexpression of the receptors on expression of key genes involved in glucose and lipid metabolism. Overexpression of AdipoR1 or AdipoR2 resulted in a 2-fold increase in expression of the insulin-responsive glucose transporter *glut4* in lean mice but this effect was not observed in obese mice (Figure 5.4A). In contrast to the effects described above that were common to both AdipoR1 and AdipoR2, only the latter effected changes in genes involved in lipid metabolism, *pparα* and *acox1*. Indeed, in lean but not obese ^{TAM}R2 mice *pparα* and a downstream target gene *acox1* (encoding acyl-CoA oxidase) were both increased around 2-fold (Figure 5.4B & C). These results demonstrate different effects on local gene expression following ^{TAM}R1 or ^{TAM}R2 and also provide further evidence of adiponectin resistance in the face of HFD-induced obesity.

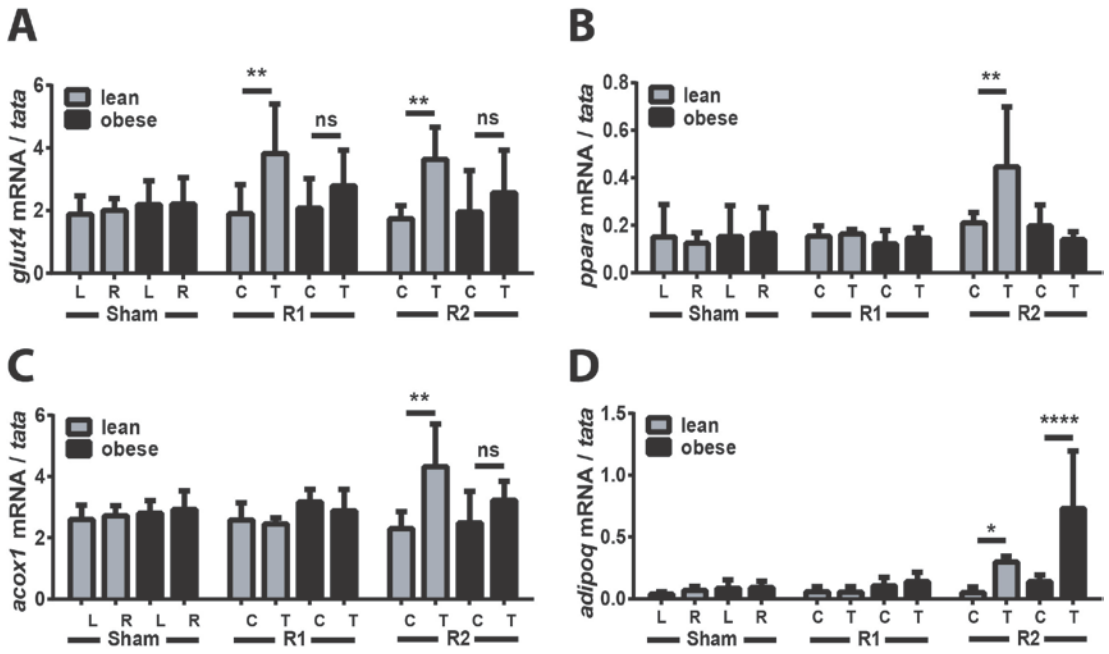

Figure 5.4. ^{TAM}R1 and ^{TAM}R2 increase expression of glut4 but only the latter increase the expression of adipoQ and genes involved in lipid metabolism. qRT-PCR analysis of **(A)** *glut4*, **(B)** *pparα*, **(C)** *acox1* **(D)** *adipoQ* expression in control (sham), ^{TAM}R1 (R1) and ^{TAM}R2 (R2) mice. Data are expressed as mean±SEM; *p < 0.05, **p < 0.01, ****p < 0.0001; n=6 in each group; significant difference of test TAM (T) compared to control TAM (C) in lean and obese mice.

5.5.5 ^{TAM}R2, but not ^{TAM}R1, promotes increased expression of adipoQ in TAM from lean and obese mice.

Next, we examined expression of *adipoQ* (encoding adiponectin) in test and control TAM. As expected, expression of *adipoQ* was relatively low in control muscle from lean mice (1000-fold less than in epididymal fat). Obesity increased *adipoQ* expression 2.2-fold (n=18/group; p=0.001), consistent with previous reports[239], whilst ^{TAM}R2 increased *adipoQ* levels 6-7 fold in TAM from both lean and obese mice respectively (Figure 5.4D).

5.5.6 ^{TAM}R2 in obese mice reduces HFD-induced weight gain, adipose tissue mass and inflammation, and increases circulating adiponectin levels.

Consistent with the findings in our pilot study (see above) we again observed a significant decrease in weight gain in ^{TAM}R2 obese mice compared with obese control mice (transduced with empty plasmid in both legs) or ^{TAM}R1 obese mice (Figure 5.5A). The latter also promoted a modest but significant reduction in weight gain compared with the obese control group (Figure 5.5A). To address this further we measured epididymal and subcutaneous fat pad weights. Consistent with the reduced weight gain, fat pad weights were also significantly reduced in the ^{TAM}R2 obese mice (Figure 5.5B & C). In light of these surprising observations we performed qRT-PCR on the epididymal and subcutaneous fat pads (and liver) using primers specific for human *AdipoR2* to rule out the possibility that these effects may reflect leaky expression of AdipoR2 in tissues other than the test TAM. In all cases we were unable to detect human *AdipoR2* expression (data not shown) leaving us to conclude that these effects are most likely mediated indirectly via the increased expression of AdipoR2 in TAM. Having established this, we performed further characterisation of the epididymal fat pads aiming to define the impact on the inflammatory signature. As expected, HFD-induced obesity resulted in a significant increase in expression of inflammatory markers including the pro-inflammatory cytokine TNFα[240], the chemokine monocyte chemoattractant protein (MCP)-1[241], and the monocyte/macrophage markers CD68, CD11b and F4/80 [242] (Figure 5.6A-E). ^{TAM}R1 or ^{TAM}R2 had no effect on inflammatory gene expression in lean mice. However, in ^{TAM}R2 obese mice there was a significant reduction in the expression of all inflammatory markers, such that expression levels were comparable to those in lean mice (Figure 5.6A-E). Moreover, ^{TAM}R1 obese mice presented an intermediate profile with significant reductions in *mcp1*, *cd11b*, *cd68* and *f4/80* compared with obese sham mice (Figure 5.6A-E). We also determined the effects of diet and gene transduction on *adipoQ* expression. All groups showed similar expression except for the ^{TAM}R2 obese mice, where *adipoQ* expression was significantly elevated (Figure

5.6F). To investigate this further we measured circulating adiponectin levels. Consistent with the gene expression, and our pilot study, circulating adiponectin levels were significantly increased in ^{TAM}R2 obese mice (Figure 5.6G). We performed a similar analysis of the subcutaneous adipose tissue which revealed a more modest inflammatory response in the face of HFD-induced obesity and no effect of ^{TAM}R1 or ^{TAM}R2 (Figure 5.7A-E). Surprisingly, *adipoQ* expression was significantly increased by obesity in this depot (Figure 5.7F). Collectively, these results suggest that TAM-specific overexpression of AdipoR2, and to a lesser extent AdipoR1, results in reduced HFD-induced weight gain concomitant with amelioration of HFD-induced adipose inflammation in epididymal fat pads, increased adiponectin expression and increased circulating adiponectin.

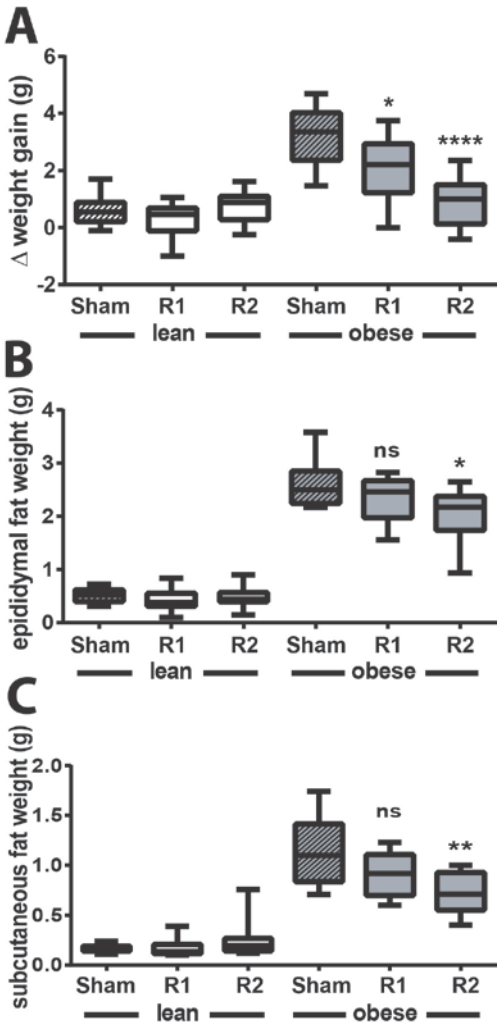

Figure 5.5. ^{TAM}R2 reduces weight gain and fat pad weight. **(A)** Average weight gain over two weeks following IVE. **(B)** Epididymal fat and **(C)** subcutaneous fat pad weight. Data are expressed as mean±SEM; *p < 0.05, **p < 0.01, ****p < 0.0001; n=6 in each group; significant difference compared to control (sham) group of same diet.

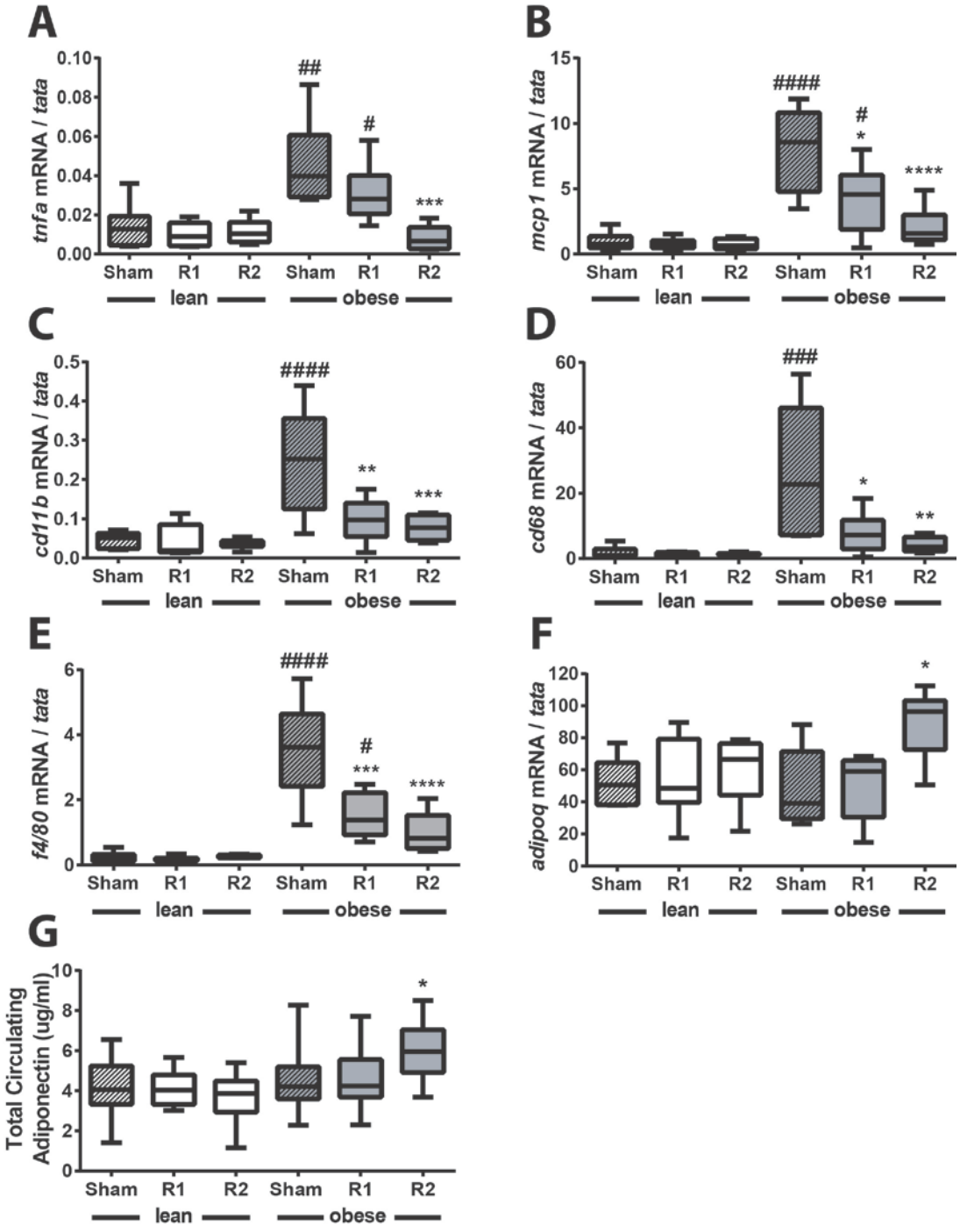

Figure 5.6. ^{TAM}R2 improves HFD induced inflammation in epididymal fat pad of obese mice and increases circulating adiponectin levels. qRT-PCR analysis of **(A)** *tnfα* **(B)** *mcp1* **(C)** *cd11b* **(D)** *cd68* and **(E)** *f4/80* and **(F)** a*dipoQ* expression in epididymal fat pad. **(G)** ELISA analysis of serum total circulating adiponectin. Values are presented as mean±SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; significant difference compared to control (sham) of same diet. #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001; significant comparison of lean vs obese mice in same group; n=6 in each group.

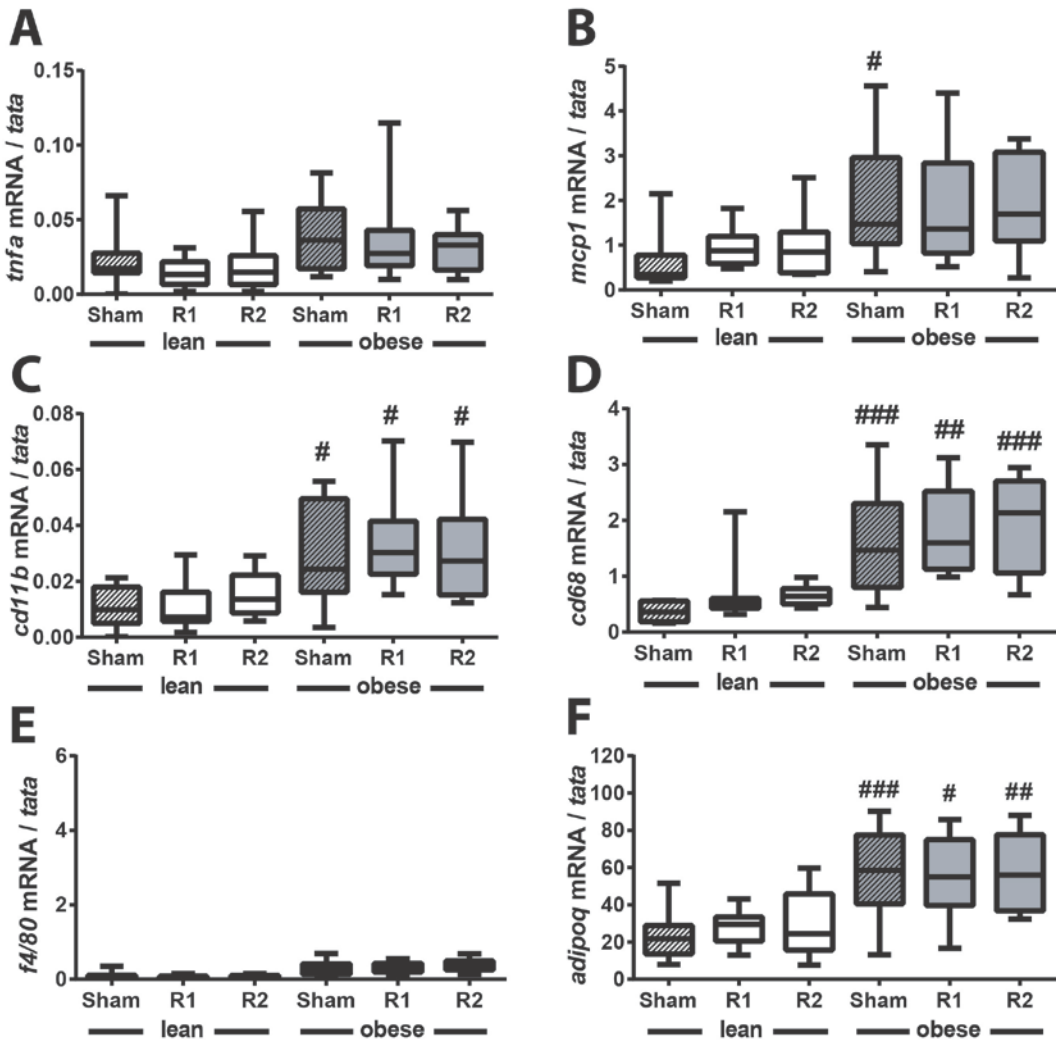

Figure 5.7. ^{TAM}R1 or ^{TAM}R2 do not affect diet induced inflammation in subcutaneous fat pad. qRT-PCR analysis of **(A)** *tnfa* **(B)** *mcp1* **(C)** *cd11b* **(D)** *cd68* and **(E)** *f4/80* and **(F)** *adipoQ* expression in subcutaneous fat pad. Data are presented as mean±SEM; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001; significant comparison of lean vs obese mice in same group; n=6 in each group.

5.6 Discussion

In the current study we aimed to extend molecular and cellular studies by comparing the effects of overexpression of AdipoR1 or AdipoR2 in skeletal muscle in lean and obese mice. We employed IVE to mediate overexpression of AdipoR1 or AdipoR2 in TAM of lean or HFD-induced obese mice. In lean mice TAM-specific overexpression of either receptor resulted in increased phosphorylation of downstream effectors and elevated expression of the insulin responsive glucose transporter *glut4*. In contrast, only overexpression of AdipoR2 resulted in increased expression of *pparα* and the target gene *acox1*. In obese mice the magnitude of all of these effects was reduced even though receptor expression and circulating adiponectin levels were maintained or increased. Surprisingly, ^{TAM}R2 in obese mice resulted in a significant decrease in weight gain, adipose tissue mass and inflammation and a significant increase in circulating adiponectin levels. Collectively, these results identify overlapping effects of AdipoR1 and AdipoR2 as well as additional, distinct effects of the latter that provide a foundation for further investigations aimed at reducing obesity-related complications.

Investigations at the molecular and cellular level have provided clear evidence that AdipoR1 and AdipoR2 display different properties in terms of adiponectin binding[48], cell surface expression, oligomerization and signaling[152, 193, 200, 207]. Consistent with this scenario, whole animal studies, predominantly involving characterization of AdipoR1 or AdipoR2 knockout mice, have demonstrated different signaling outputs, such as coupling of hepatic AMPK and PPARα activity to AdipoR1 and AdipoR2 respectively[90], and phenotypic consequences following deletion of either receptor[90, 183, 184, 243]. Whilst informative, there are caveats to this loss of function approach given oligomerization of AdipoR1 and AdipoR2[48, 57, 152] has been shown to alter properties of the receptor complex and downstream signaling outputs[152, 200]. For example, our observations that under physiological conditions (no serum withdrawal) cell surface expression of AdipoR2 is limited unless it is co-expressed with AdipoR1[200] has clear implications when considering the impact of AdipoR1 deletion, which is also likely to compromise AdipoR2 function. Furthermore, adiponectin receptor interacting proteins such as ERp46 have been shown to modulate receptor oligomerization, cell surface expression and downstream signaling[70]. Thus, a gain of function approach may be expected to provide important complementary information. Typically such studies have tended to focus on the effects of overexpression of either AdipoR1 or AdipoR2. For example, overexpression of AdipoR1 in rat skeletal muscle was reported to improve insulin sensitivity[215] whilst overexpression of AdipoR2 in liver increased PPARα and protected against progression of

NASH[192]. To the best of our knowledge there are no examples where such studies have been compared the effects of overexpression of AdipoR1 or AdipoR2 *in vivo*.

Skeletal muscle is a recognized target of adiponectin action, with adiponectin increasing fatty acid oxidation and glucose uptake and enhancing insulin sensitivity by activation of pathways involving AMPK and PPARα[205, 239, 244]. In addition, numerous studies have provided evidence of adiponectin resistance in skeletal muscle from rodents[40, 45, 234, 235] and humans[42, 43, 236]. Whilst muscle-specific deletion of AdipoR1 has established a key role for AdipoR1[120] this does not preclude a role for AdipoR2 in mediating the beneficial effects of adiponectin in skeletal muscle or the potential of AdipoR2-based therapies. Thus, in the current study we used IVE to compare the effects of overexpression of AdipoR1 or AdipoR2 in TAM of lean or HFD-induced obese mice.

Neither IVE nor HFD-induced obesity affected expression of endogenous receptors at the mRNA level and endogenous *adipoR1* was expressed around 10-fold higher than *adipoR2,* consistent with a previous report[48]. Measurement of both gene and protein (HA) indicated that the exogenous human receptors were expressed at similar levels, with IVE increasing total AdipoR2 levels in TAM by around 100%. Unfortunately we were unable to determine the impact of AdipoR1 overexpression on total levels due to the lack of a suitable antibody. Nevertheless, similar levels of overexpression of AdipoR1 and AdipoR2 in TAM of lean mice resulted in comparable elevation of phosphorylation events including AMPK, AKT and ERK as well as increased *glut4* expression suggesting that, at least under these conditions, they mediate similar effects consistent with enhanced adiponectin and insulin sensitivity. Furthermore, these beneficial effects were reduced in the context of HFD-induced obesity by 20-60%. Given circulating adiponectin levels were not decreased these findings are consistent with the development of adiponectin resistance, at a level distal to receptor expression.

Only overexpression of AdipoR2 resulted in increased expression of *pparα* and *acox1*. This is consistent with the findings of impaired hepatic PPARα activity in AdipoR2 knockout mice [90] but contrasts with observations in endothelial cells, where overexpression of either AdipoR1 or AdipoR2 was sufficient to mediate PPARα activation[245]. Once again, these effects were blunted in obesity providing further evidence of adiponectin resistance.

Emerging evidence suggests adiponectin is produced by skeletal muscle and that this is increased in response to obesity or inflammation[27, 239, 246]. Consistent with these observations, we observed a 2-fold increase in *adipoQ* levels in TAM from obese mice. Intriguingly, overexpression of AdipoR2 promoted increased expression of *adipoQ* in both lean and obese mice. The molecular basis for this

effect is unclear, particularly given that all other local effects of ^{TAM}R2 were diminished in obese mice. Further investigations are warranted to elaborate the underlying mechanisms, which may reveal novel strategies to induce adiponectin expression more globally.

Perhaps the most surprising observations in this study relate to the effects of ^{TAM}R2 reducing weight gain in obese mice. Indeed, weight gain in HFD-fed obese mice transduced with AdipoR2 was indistinguishable from that in the lean, chow-fed mice. This effect was also reflected by a modest but significant reduction in epididymal and subcutaneous fat pad weights, compared with those from obese sham or ^{TAM}R1 mice, and a striking resolution of adipose tissue inflammation in the epididymal fat pad. Moreover, *adipoQ* expression was significantly elevated in the epididymal fat pad from the obese ^{TAM}R2 mice as were circulating levels of adiponectin. It is noteworthy that these observations (reduced weight gain and elevated adiponectin) are consistent across two independent studies, performed in two distinct research facilities, using different sets of reagents with different mouse cohorts. Whilst unexpected, evidence from the literature supports the notion that overexpression of either AdipoR1 or AdipoR2 may prevent weight gain. Hydrodynamic delivery of AdipoR2 to the liver resulted in reduced diet-induced weight gain and adipose tissue mass[247] whilst global or macrophage-specific overexpression of AdipoR1 were also sufficient to reduce diet-induced weight gain[248, 249]. Whilst it remains possible that the IVE approach employed in the current study may have resulted in transduction of cells other than the TAM we were unable to detect evidence of such leaky expression in epididymal or subcutaneous fat pads or in liver. Thus, we propose that overexpression of AdipoR2 in skeletal muscle results in altered expression of a circulating factor, possibly a myokine in a manner similar to that detailed for *adipoQ*, and that this underpins the reduced weight gain and associated improvements. Clearly, further studies are required to investigate this intriguing hypothesis.

In summary, we have demonstrated that overexpression of AdipoR1 or AdipoR2 in mouse skeletal muscle promote similar effects at the level of proximal signaling events and *glut4* expression whilst only AdipoR2 promotes activation of the PPARα axis. All of these effects were blunted in the face of obesity, consistent with the development of adiponectin resistance at the level of skeletal muscle. Finally, muscle-specific overexpression of AdipoR2 gave rise to several unexpected local and systemic effects that included increased expression of *adipoQ* in muscle and epididymal adipose as well as increased circulating levels of adiponectin, and reduced HFD-induced weight gain, adipose tissue mass and inflammation. However, these effects appeared unable to ameliorate muscle adiponectin resistance. Future studies, investigating the effects of more global muscle-specific

overexpression of AdipoR2 may provide further insights into the underlying mechanisms which may provide novel strategies to reverse hypoadiponectinemia and or adiponectin resistance.

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Chapter Six:

General Discussion

6.1 Overview

The global increase in prevalence of cardiovascular and metabolic diseases and comorbidities has had profound implications on public health systems. Thus, there is a need for the development of more effective therapeutics. Adiponectin and its receptors are considered promising therapeutic targets for cardiometabolic disorders[12]. Population-based studies had documented that circulating adiponectin levels correlate inversely with metabolic complications such as hypertension, insulin resistance and the incidence of some cancers[10, 30, 250-252]. Pre-clinical studies, principally in mouse models, had demonstrated that adiponectin administration[40, 41], and more importantly increased HMW adiponectin[253], was able to alleviate most of these associated complications. Since the discovery of AdipoR1 and AdipoR2 in 2003[48], they were demonstrated as main receptors for adiponectin and mediating its beneficial effects. AdipoR1 and AdipoR2 are predicted to be seven transmembrane domain proteins and, importantly, are shown to have opposite topology to GPCRs and function through atypical pathways[48]. In addition to adiponectin expression, accumulating evidence demonstrates adiponectin resistance at the level of the adiponectin receptors in cardiometabolic disorders. For instance, pre-clinical studies demonstrated that adiponectin failed to stimulate glucose uptake and fatty acid oxidation in insulin sensitive organs of obese, diabetic and hypertensive rats despite increased or unchanged AdipoR1 and AdipoR2 mRNA expression[43, 46]. Thus understanding the processes that promote receptor function will provide opportunities to define mechanisms that give rise to adiponectin dysfunction.

This thesis investigated the molecular characterisation governing the subcellular localisation and signal transduction of AdipoR1 and AdipoR2. Key findings are outlined below:

1- Under steady-state conditions (no serum starvation) AdipoR1 exhibits robust (50%) cell-surface expression, whereas AdipoR2 is predominantly restricted to the ER and it is undetectable at the cell-surface [200].
2- The non-conserved, intracellular, N-terminal region of AdipoR2 ($AdipoR2_{1-81}$) restricted cell-surface expression, whilst the same region in AdipoR1 ($AdipoR1_{1-70}$) promoted cell-surface expression[200].
3- Co-expression of AdipoR1 with AdipoR2 leads to the formation of hetero-dimers and promotes the cell-surface expression of AdipoR2[200].
4- 16 h of serum starvation promoted cell-surface expression of AdipoR2 (to 20%) but had no discernible effect on the cell-surface expression of AdipoR1[207].

5- Two conserved motifs proximal to the first transmembrane domain, an ER exit motif (FxxxFxxxF) and Di-Leucine motif (DxxxLL), are required for efficient cell-surface expression of AdipoR1 and AdipoR2[207].
6- Overexpression of AdipoR1 resulted in acute adiponectin-stimulated activation of downstream signalling networks (Akt, ERK & P38MAPK) whereas overexpression of AdipoR2 promoted more chronic activation (peaking at 15 min and 24 h respectively)[207].
7- Difference in the temporal signalling profiles of AdipoR1 and AdipoR2 is also underpinned by the non-conserved N-terminal domain ($AdipoR1_{1\text{-}70}$ and $AdipoR2_{1\text{-}81}$)[207].
8- AdipoR1 and AdipoR2 are both palmitoylated at multiple sites in the N-terminal domain (AdipoR1($C_{54\ \&\ 124}$) and AdipoR2($C_{11,\ 96\ \&\ 135}$).
9- Palmitoylation of the conserved cysteine in the juxta-membrane region of AdipoR1(C_{124}) and AdipoR2(C_{135}) is required for efficient cell-surface expression and signal transduction of AdipoR1 and AdipoR2 (both *in vitro* and *in vivo*).
10- Palmitoylation of non-conserved cysteines in AdipoR2 (C_{11} and C_{96}) contribute to the maintenance of AdipoR2 stability.
11- Muscle-specific overexpression of AdipoR1 or AdipoR2 *in vivo* enhances downstream signalling (AMPK, Akt & ERK) and regulated glucose uptake, but activation of the PPARα axis and fatty acid oxidation is specific to AdipoR2.
12- Diet-induced obesity in mice results in adiponectin resistance.
13- Over-expression of AdipoR2 in TA muscle prevents HFD induced weight gain and regulates adiponectin secretion.
14- Overexpression of AdipoR2 in TA muscle of obese mice resulted in marked systemic effects including reduced fat mass and markers of adipose tissue inflammation.

6.2 General discussion and future directions

6.2.1 Characterisation of the non-conserved N-terminal domains (NC-NTD)

During the course of this thesis we demonstrated that despite highly conserved sequences, there are fundamental differences between AdipoR1 and AdipoR2.

In the early chapters of this thesis, we established major differences in the cell-surface expression (CSE) of the adiponectin receptors. Examining the subcellular localisation of the endogenous receptors revealed that AdipoR1 is predominantly located in the PM; however, AdipoR2 is mainly localised in the ER. Using cell biology approaches we further investigated the subcellular localisation of adiponectin receptors and we demonstrated that CSE of AdipoR2 was undetectable

by semi-quantitative approaches such as microscopy and plate-based assay. Due to the highest sensitivity, flow cytometry was then used to accurately investigate the number of cells expressing receptors at the cells surface. Results revealed that under steady state only 10-15% of the cells transiently transfected with AdipoR2 are expressing it at the cell-surface, whilst 50-60% CSE was detected for AdipoR1[200, 207]. By generating chimeric and truncated receptors we demonstrated that the difference in the subcellular localisation of AdipoR1 and AdipoR2 is underpinned by the NC-NTD[200] (figure 6.1). Our results showed that CSE of AdipoR2 is restricted by amino acids 1–81 whilst same region in AdipoR1 (amino acids 1-70) promoted cell-surface expression. These observations are consistent with those from the original study by Kadowaki and colleagues, who characterised a truncated form of AdipoR2 that lacked the N-terminal region and was localised at the cell-surface[48]. In chapter 4 of this thesis however, we showed enriched localisation of the cytoplasmic domain of both AdipoR1 and AdipoR2 under the PM. These constructs contain NC-NTD and conserved N-terminal domains (C-NTD) (figure 6.1) without the transmembrane region. However, our previous results suggested that the NC-NTD of AdipoR2 inhibits CSE. One explanation for this difference is that the AdipoR2 NC-NTD may interact with one or more of the intracellular loops to inhibit CSE. Therefore, future studies are required to investigate the exact role of NC-NTD in AdipoR2. So far different groups investigated adiponectin receptors properties. Some groups reported that AdipoR2 is not expressing at the cell-surface and therefore AdipoR1 was the main focus of most of the studies[168, 169]. In contrast, a group demonstrated that both receptors were expressed at the cell-surface to same extent[152]. In this study, recombinant AdipoR1 and AdipoR2 with a series of phrGFP, ECFP, Venus-YFP, and DsRed tags on the cytoplasmic N-terminal region of the receptors were used and co-localisation of the receptors with a membrane marker was shown[152]. One explanation for cell-surface localisation of AdipoR2 in this study is the addition of a large tag (> 200 amino acids) on the N-terminus which may prevent the NC-NTD acting as a brake, possibly via interference of the proposed interaction of the NC-NTD with intracellular loops (see above) or other interacting proteins.

In accordance with previous investigations on different CSE of AdipoR1 and AdipoR2, we identified that the temporal signal profile of AdipoR1 and AdipoR2 also differs [207]. We showed that AdipoR1 increases the adiponectin stimulated downstream signalling pathways acutely (15 min), whilst, AdipoR2 acts chronically (24 h). This was also shown to be underpinned by the NC-NTD of the receptors. Interestingly, although CSE of AdipoR2 is relatively limited, its ability to activate downstream effectors is comparable with AdipoR1 and CSE is necessary for AdipoR2 downstream

signalling. This could be explained by AdipoR2 redundancy after overexpression as it was demonstrated in chapter 4, figure 4.4.

During the course of this thesis the crystal structure of adiponectin receptors was reported by Kadowaki and colleagues[71]. In this study a truncated form of AdipoR1 and AdipoR2 was used and it was suggested that the deletion of the non-conserved and part of the conserved N-terminal domain of the receptors does not affect their function and signalling[71]. However, our investigations provided the evidence that the NC-NTD underpinned the different cell-surface expression[200] and temporal signalling profile[207] of AdipoR1 and AdipoR2. Previous studies from the host lab also demonstrated that ERP46, the first AdipoR1-specific interacting protein, interacts with the NC-NTD of AdipoR1 and modulates adiponectin signalling. It is also well established that the C-NTD of AdipoR1 and AdipoR2 interacts with APPL1, the best characterised interacting protein for AdipoR1 and AdipoR2[72, 100], and other interacting proteins such as RACK and protein kinase CK2[73, 74]. All these interacting proteins have been shown to play important roles in mediating adiponectin signalling raising some concerns about the validity of the recent findings from the Kadowaki group[71].

6.2.2 Dimerisation effect on receptor CSE

Further characterisation of adiponectin receptors during this thesis demonstrated that co-expression of AdipoR1 with AdipoR2 increases the cell-surface expression of AdipoR2[200]. It is well established that AdipoR1 and AdipoR2 form homo and hetero dimers[48, 57, 169]. Previously it was reported that a motif consisting of two conserved glycine (GxxxG) in the fifth transmembrane domain is responsible for dimerisation of AdipoR1[151] (figure 6.1). This motif provides a flat interaction surface for receptor dimerisation, as described for several transmembrane proteins including some GPCRs[254, 255]. Later in 2012, it was demonstrated that dimerisation of both AdipoR1 and AdipoR2 occurs mainly in the plasma membrane and less in the ER suggesting that dimerisation of the receptors may regulate CSE of the receptors[152]. This is the case for some of the GPCRs where dimerisation is essential for their efficient CSE[147, 162]. It was demonstrated by both groups that adiponectin treatment reduces dimerisation of the receptors[151, 152] suggesting that dimerisation facilitates the CSE and that these complexes dissociate in response to ligand binding. Our observations suggest that in organs such as the liver where AdipoR1 and AdipoR2 expression at least at the mRNA level [89] is comparable, it seems likely that the major species is AdipoR1/R2 heterodimer. Given that CSE expression of AdipoR1 and AdipoR2 is required to activate downstream signalling and considering limited CSE of AdipoR2 homodimer, this question may rise

whether AdipoR2 homodimer plays a major role serving as classic receptor. However, future studies are required to characterise the role of AdipoR2 homo dimerisation.

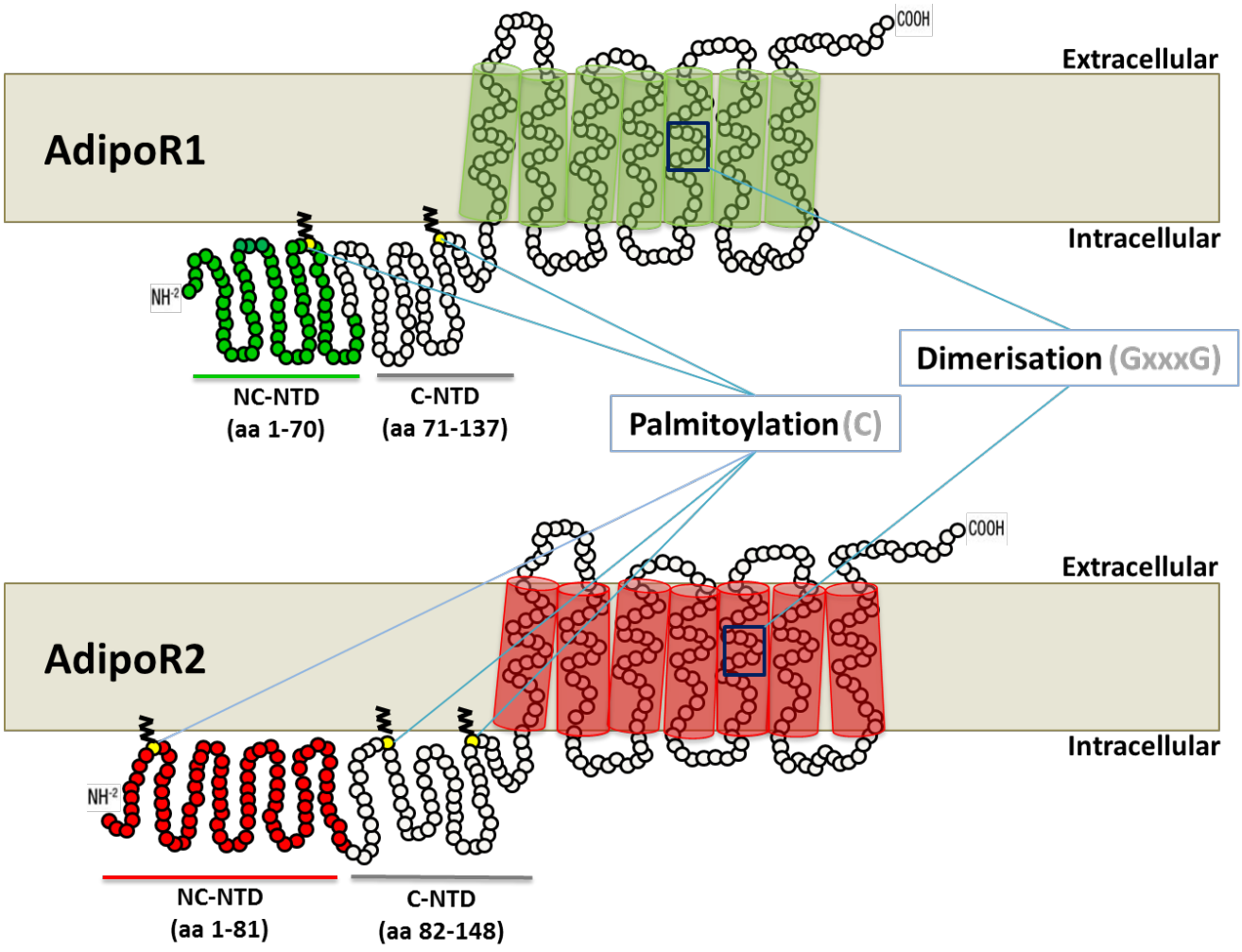

Figure 6.1. Schematic representation of AdipoR1 and AdipoR2 structure, palmitoylation and dimerisation sites. Non-conserved N-terminal domain (NC-NTD) is shown in red for AdipoR2 (inhibit CSE) and green for AdipoR1 (promote CSE). Conserved N-terminal domain (C-NTD) of AdipoR1 and AdipoR2 is highlighted with grey line. Palmitoylation sites for AdipoR1 (C54 & C124) and AdipoR2 (C11, C96 &C124) are shown in yellow. Dimerisation site in the fifth transmembrane domain is highlighted by dark blue boxes.

6.2.3 Characterisation of the C-NTD

Beside the NC-NTD which causes the differences, the C-NTD however, drives important parallel facets of AdipoR1 and AdipoR2. In 2012, two motifs known as ER exit motif (FxxxFxxxF) and Di-Leucine motif (DxxxLL) was identified as modulators of AdipoR1 anterograde trafficking[167]. Given that these motifs are identical between AdipoR1 and AdipoR2, we demonstrated that they are required for CSE of both AdipoR1 and AdipoR2 and subsequently essential for their downstream signalling[207].

6.2.4 Identification and characterisation of receptor palmitoylation

A major finding of this thesis was investigating the palmitoylation of AdipoR1 and AdipoR2. The important role of post-translational modification for the stability[176, 256], localisation[177, 220, 257, 258] and function[233, 259] of GPCRs is well recognised. However, not many studies have focused on characterising the properties that govern adiponectin receptor function other than a recent study demonstrating the phosphorylation of AdipoR1 in post-myocardial infarction heart failure in pre-clinical models[208]. Our investigations provided the first direct biochemical and molecular evidence showing that AdipoR1 and AdipoR2 are palmitoylated. We identified that a conserved cysteine in the juxtamembrane region of AdipoR1 and AdipoR2, which is a classical palmitoylation site in many of the 7-transmembrane GPCRs[177, 220, 225], is subjected to palmitoylation. Further investigations revealed that palmitoylation of the mentioned 'canonical' cysteines are essential for efficient cell-surface expression and therefore are required for downstream signalling of AdipoR1 and AdipoR2. We also identified two additional cysteines in the cytoplasmic domain of AdipoR2 which are subjected to palmitoylation. These cysteines are not conserved between AdipoR1 and AdipoR2 but conserved within species. We demonstrated that palmitoylation of these cysteines is required for the stability of AdipoR2. Interestingly, we did not see such effect in AdipoR1 suggesting that only AdipoR2 stability is palmitoylation dependent.

As mentioned before, adiponectin resistance has been reported in cardiometabolic disorders such as obesity and type-2 diabetes. In these studies, despite reduced signalling, increased or unchanged mRNA expression of AdipoR1 and AdipoR2 was demonstrated [43, 46]. The protein expression of the receptors was not reported in these studies. However, reported reduced signalling could be, at least partly, explained by reduced protein expression and/or impaired CSE which was reported by another group in overweight patients with coronary heart disease[34]. On the other hand, reduced palmitoylation of other proteins such as LIM domain only 4 (LMO4), an inhibitor of Protein tyrosine phosphatase 1B (PTP1B) activity, has been reported in metabolic stresses such as

obesity[260]. To this end, we attempted to examine the level of AdipoR1 and AdipoR2 palmitoylation in the muscle and liver of lean and obese pre-clinical mouse models to further investigate the physiological effects of palmitoylation. Unfortunately, we were unable to detect palmitoylation of the endogenous receptors. This probably reflects limitations of the acyl-biotinyl exchange assay, in terms of sensitivity, combined with the low levels of expression of the adiponectin receptors and high number of other palmitoylated proteins. Notwithstanding, to extend our cell-based observations to a more physiological setting we used *in vivo* electrotransfer (IVE) to overexpress the receptors in mouse skeletal muscle. Our studies revealed that disruption of palmitoylation of the canonical cysteines impairs the ability of AdipoR1 and AdipoR2 to activate downstream signalling pathways.

6.2.5 IVE-mediated overexpression of AdipoR1 and AdipoR2 in skeletal muscle

Another purpose of IVE study was to examine the effects of overexpression of wild-type AdipoR1 and AdipoR2 in mouse tibialis anterior muscle (TAM). Adenovirus-mediated overexpression of AdipoR1 and AdipoR2 in mouse liver was reported to activate AMPK phosphorylation and PPARα pathway respectively[90]. The IVE system on the other hand, gives the opportunity to examine the local effect of receptors overexpression compared to an internal control (test leg vs. control leg in same animal). TAM specific overexpression of AdipoR1 in rats was also reported to amplify local insulin sensitivity[215]. However, the effect of AdipoR2 overexpression in muscle which is not a primary organ for AdipoR2 was not examined before. Our study revealed that TAM-specific overexpression of either AdipoR1 or AdipoR2 may be enough to activate downstream signalling pathways including phosphorylation of AMPK, Akt and ERK. Overexpression of both AdipoR1 and AdipoR2 also increased Glut4 expression, which would be consistent with increased insulin or exercise stimulated glucose uptake in muscle. However, activation of fatty acid oxidation pathways is specific to AdipoR2. Given the limited CSE of AdipoR2 it seems unlikely that overexpression of AdipoR2 alone is responsible for such effects. A plausible explanation is that exogenous AdipoR2 forms heterodimers with endogenous AdipoR1 and that these mediate many of the downstream effects. This may suggest that both AdipoR1 homodimers and AdipoR1/R2 heterodimers stimulate downstream signalling pathways, whilst only AdipoR1/R2 heterodimers stimulate fatty acid oxidation pathways. As mentioned before, due to its limited CSE, the role and function of AdipoR2 homodimer is questionable. Further studies are required to identify the presence and function of homo/hetero dimers after AdipoR1 and AdipoR2 IVE. The most striking finding in this study was the systemic effects we observed after overexpressing AdipoR2 in the skeletal muscle of obese mice. We observed increased circulating adiponectin and decreased body weight gain following

overexpression of AdipoR2. Interestingly these effects were unchanged in the absence of canonical cysteine palmitoylation suggesting that CSE of AdipoR2 is not required to stimulate these effects. Whilst unexpected, it has been previously reported that overexpression of either AdipoR1 or AdipoR2 may prevent weight gain. Hydrodynamic delivery of AdipoR2 to the liver resulted in reduced diet-induced weight gain and adipose tissue mass [247] whilst global or macrophage-specific overexpression of AdipoR1 were also sufficient to reduce diet-induced weight gain [248, 249]. Although, it remains possible that the IVE approach may have resulted in transduction of cells other than the TAM we were unable to detect evidence of such exogenous AdipoR2 expression in iother organs such as epididymal or subcutaneous fat pads or in liver. Therefore, we propose that overexpression of AdipoR2 in skeletal muscle results in altered expression of a circulating factor, possibly a myokine in a manner similar to that detailed for *adipoQ*, and that this underpins the reduced weight gain and associated improvements. Clearly, further investigations are required to identify the mechanism by which TAM-specific overexpression of AdipoR2 regulates adiponectin secretion and improves diet-induced inflammation.

6.3 Conclusions

Adiponectin and its receptors are recognised attractive potential targets for treatment of metabolic complications. Nevertheless, therapeutic strategies are constrained by a rudimentary understanding of the adiponectin receptors and therefore greater understandings of how the AdipoR1 and AdipoR2 mediate adiponectin's beneficial effects are essential. In this project we demonstrated (i) fundamental differences between AdipoR1 and AdipoR2, highlighting the importance of the cytoplasmic domains, (ii) post-translational regulation (palmitoylation) of the receptors and its significance for cell-surface expression, signal transduction and stability and (iii) overlapping and discrete local and systemic effects of AdipoR1 and AdipoR2. This work provides a foundation for future studies that promise to extend our knowledge of adiponectin receptors biology. Such studies are likely to contribute to the development of much needed therapeutics for cardiometabolic disorders.

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