Regulation of Human Melanocortin-5 Receptor by Melanocortin-2 Receptor Accessary Proteins and Functional Characterization of hMC5R Mutants

By

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Abstract

Melanocortin-5 receptor (MC5R) is the most recently discovered melanocortin receptor (MCR). It is widely distributed in both central nervous system and peripheral tissues and has unique tissue expression patterns, pharmacological properties, and physiological functions. MC5R influences exocrine gland function and regulates energy metabolism by affecting lipolysis, fatty acid oxidation, and glucose uptake in adipocytes. Preliminary clinical studies also indicated that hMC5R is associated with obesity and type 2 diabetes.

Melanocortin-2 receptor accessory proteins (MRAPs) are important regulators of MCRs, which can regulate their transport, binding, and signal transduction, thereby affecting various physiological processes. There are two members of MRAP family: MRAP1 and MRAP2. Many studies have shown that MRAP1 and MRAP2 primarily interact with MC2R, MC3R, and MC4R. However, the interaction between different isoforms of MRAPs and MC5R is still unclear and needs further study.

To investigate the effects of MRAPs on trafficking, ligand binding and signaling properties of human (h) MC5R, HEK293T cells were transiently co-transfected with plasmids encoding MC5R and different isoforms of MRAPs (hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b, and hMRAP2c). The results showed that hMRAP1a, hMRAP2a and hMRAP2c increased the cell surface expression of hMC5R. All MRAPs have no effect on

affinity to the superpotent analog of α-melanocyte stimulating hormone (α-MSH), NDP-MSH, while hMRAP2c significantly increased maximal binding. All MRAPs had no effect on α-MSH-stimulated cAMP production ($G\alpha_s$-cAMP pathway). Additionally, α-MSH induced ERK1/2 activation of hMC5R. Cells co-transfected with hMC5R and hMRAP1a, hMRAP2a, hMRAP2b, or hMRAP2c had significantly increased pERK1/2 level upon stimulation with α-MSH. In summary, the two MRAP1s and three MRAP2s had differential effects on MC5R trafficking, binding, and signaling. These findings led to a better understanding of the regulation of MC5R by MRAP1s and MRAP2s.

Melanocortin-4 and -5 receptors (MC4R and MC5R) play important roles in regulating energy homeostasis. MRAP2 regulates MC4R and MC5R trafficking, ligand binding, and signaling. Loss of MRAP2 function results in reduced MC4R activity, leading to obesity and metabolic disorders. In this study, we selected five hMRAP2a mutants (G31V, F62C, N77S, K102*, and P195L) and investigated their effects on hMC4R and hMC5R pharmacology. In hMC4R, F62C and P195L reduced cell surface expression and maximal response to α-MSH. Additionally, hMRAP2a and the remaining three mutants (G31V, N77S, and K102*) reduced the maximal response to α-MSH as well as basal activity. For hMC5R, K102* reduced total and cell surface expression. Furthermore, F62C and P195L reduced cell surface expression of hMC5R, whereas N77S increased it. F62C reduced the maximal response to α-MSH, while P195L impaired both the maximal binding and the maximal response to α-MSH. Like MC4R, MC5R exhibits basal cAMP signaling, which was reduced

by hMRAP2a and all five mutants. In conclusion, hMRAP2a and its identified obesity-associated mutants can modulate the pharmacology of hMC4R and hMC5R. Different hMRAP2a mutants exhibited distinct regulatory effects on hMC4R and hMC5R, revealing a complex regulatory mechanism within the melanocortin receptor family.

Recent genomics studies have identified hundreds of naturally occurring *MC5R* mutations. Human MC5R is primally coupled to $G\alpha_s$, resulting in increased intracellular cAMP. Studies have shown that some missense polymorphisms in hMC5R affect its ligand binding activity and downstream functions, particularly those involving conserved amino acids in the transmembrane domain (TMD). Herein, we studied pharmacological properties of 12 hMC5R mutants at highly conserved residues by measuring their expression, ligand binding and signaling. Results showed only Y295H had decreased total expression while I294T had increased cell surface expression. D119Y, P253L, P292S, I294T, and I294M were defective in ligand binding as well as α-MSH-stimulated cAMP production. The remaining seven mutants (P70A, Y141F, R158H, R158L, D291H, Y295C, Y295H) had similar binding affinities for α-MSH as wild-type hMC5R. P70A, Y141F, and R158H had significantly decreased maximal binding and signaling. D291H, Y295H and Y295C were defective in α-MSH-stimulated $G\alpha_s$-cAMP signaling pathway. Y141F and R158H also had decreased α-MSH potency. In summary, we did not identify any mutant with misfolding defect. Five mutants had defects in binding and consequent signaling. Three mutants had binding but no signaling, with a clear defect in G protein binding and/or activation.

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Table of contents

List of Tables

List of Figures

List of Abbreviations

AC: adenylyl cyclase

ACTH: adrenocorticotropic hormone

AgRP: agouti-related peptide

NPY: neuropeptide Y

AMPK: 5'-AMP-activated protein kinase

BSA: bovine serum albumin

cAMP: 3’,5’-cyclic adenosine monophosphate

CNS: central nervous system

EC_{50}: half maximal effective concentration

ECL: extracellular loop

ICL: intracellular loop

TMD: transmembrane domain

ER: endoplasmic reticulum

ERK1/2: extracellular signal-regulated kinases 1/2

GPCR: G protein-coupled receptor

HEK 293T: human embryonic kidney 293T

Bmax: maximal binding

IC_{50}: half maximal inhibitory concentration

MCR: melanocortin receptor

MSH: melanocyte-stimulating hormone

MRAP1: melanocortin-2 receptor accessory protein 1

MRAP2: melanocortin-2 receptor accessory protein 2

NDP-MSH: [Nle4, D-Phe7]-α-MSH

PI3K: phosphatidylinositol 3-kinase

PKA/C: protein kinase A/C

PKB: protein kinase B

POMC: proopiomelanocortin

PVN: paraventricular nucleus

WT: wild type

MC4R: melanocortin-4 receptor

MC5R: melanocortin-5 receptor

NPY: neuropeptide Y

Chapter 1: Literature review

1.1. Introduction

G protein-coupled receptors (GPCRs) are the most diverse families of cell surface receptors, playing critical roles in various physiological processes. They are classified into five groups (GRAFS) based on sequence homology and functional similarities, including: rhodopsin (Class A), secretin and adhesion (Class B), glutamate (Class C) and frizzled/taste2 (Class F) (Fredriksson et al., 2003). GPCRs are characterized by seven transmembrane α-helices, which span the cell membrane. The extracellular N-terminus and the loops connecting the transmembrane domains are involved in ligand binding, while the intracellular C-terminus and loops interact with G proteins and other signaling molecules (Shi and Javitch, 2002). Upon ligand binding, the conformation of GPCR changes, activating the G protein within the cell. These G proteins are heterotrimeric, consisting of α, β, and γ subunits. Activation of GPCR promotes the exchange of GDP for GTP on the Gα subunit, leading to the dissociation of Gα subunit from Gβγ dimer. Both Gα subunit and Gβγ dimer can then interact with various effectors to transmit signals within the cell, thereby producing different physiological responses (Kobilka, 2007).

Melanocortin receptors (MCRs) belong to rhodopsin like family A GPCRs and play

key roles in a variety of physiological processes, including pigmentation (Suzuki et al., 1996), adrenal function (Chida et al., 2007), energy homeostasis (Sutton et al., 2008b; Tao, 2005), exocrine gland function and inflammation (Xu et al., 2020). These receptors can be activated by melanocortin peptides, such as α-melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), and other related peptides derived from the precursor protein proopiomelanocortin (POMC) (Dores et al., 2014). There are five known melanocortin receptors, designated MC1R through MC5R, each with distinct physiological functions and tissue distributions. For example, MC4R is widely expressed in the brain, particularly in hypothalamus, and plays a critical role in energy expenditure, appetite, and body weight (Gantz et al., 1993). Mutations in MC4R are the most common genetic cause of monogenic obesity (Farooqi et al., 2003). MC5R is widely expressed in both central and peripheral tissues, thereby has multiple physiological functions (Ji et al., 2022) . MC5R polymorphisms in humans have been reported to be associated with obesity (Valli-Jaakola et al., 2008). Since MC5R is involved in various physiological processes, studies on skin diseases and metabolism have shown a link between MC5R and dry skin, seborrhea and type 2 diabetes (Xu et al., 2020).

Melanocortin receptor accessory proteins (MRAPs) are accessory proteins that modulate the trafficking, binding and signaling of MCRs, thereby influencing various physiological processes (Chan et al., 2009). There are two members in the MRAP family: MRAP1 and MRAP2. MRAP1 is critical for the trafficking of MC2R from the endoplasmic

reticulum to the cell surface. Without MRAP1, MC2R remains intracellular and cannot effectively bind ACTH (Metherell et al., 2005). There are two alternatively spliced isoforms of human (h) *MRAP1*, *hMRAP1a* and *hMRAP1b*, with similar effects on MC2R trafficking and signaling (Metherell et al., 2005; Roy et al., 2007). MRAP2, a newly described homolog of MRAP1, shares 40% homology with MRAP1 and has a broader expression pattern (Webb and Clark, 2010). It plays a key role in the regulation of energy balance and body weight, particularly through its interaction with MC4R (Bruschetta et al., 2018). There are three alternatively spliced isoforms of human (h) *MRAP2*, *hMRAP2a*, *hMRAP2b*, and *hMRAP2c* (Ji and Tao, 2022). Both hMRAP1 and hMRAP2 modulate all MCRs, however, each with distinct but sometimes overlapping roles in receptor modulation.

This chapter provides an overview of the melanocortin system, including its ligands and metabolism-related receptors, hMC4R and hMC5R. The physiological functions and intracellular signaling pathways of these receptors are discussed. Additionally, the regulation of MC4R and MC5R by MRAP1 or MRAP2 is highlighted.

1.2. Melanocortin system

1.2.1. Endogenous and synthetic ligands

Melanocortin receptors (MCRs) are a family of G protein-coupled receptors (GPCRs)

that can be activated or inhibited by specific endogenous peptides, known as agonists and antagonists, respectively, thereby regulating their activity. There are five endogenous agonists derived from the pro-opiomelanocortin (POMC) precursor protein: α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH). α-MSH is one of the most potent agonists and binds MC1R, MC3R, MC4R, and MC5R, playing a role in regulating pigmentation, appetite, and energy balance (Yang and Harmon, 2017). Endogenous antagonists of MCRs include agouti signaling protein (ASIP) and agouti-related protein (AgRP) (Chai et al., 2005; Cowley et al., 1999). In the skin, ASIP inhibits α-MSH, resulting in the production of yellow or red pigments instead of black or brown, thereby affecting the fur color of mammals (Voisey and van Daal, 2002). AgRP is a potent antagonist of MC3R and MC4R, regulating appetite and energy balance by inhibiting the anorectic effects of α-MSH, thus promoting feeding behavior and weight gain (Fu and van den Pol, 2008).

The development of synthetic agonists and antagonists for melanocortin receptors (MCRs) has been crucial for advancing our understanding of the melanocortin system (Cain et al., 2006). For example, NDP-α-MSH (Nle4, D-Phe7-α-MSH), a synthetic analog of the natural melanocortin peptide α-MSH, is widely used in research to study the physiological roles of MC1R, MC3R, MC4R, and MC5R due to its enhanced stability and potency compared to α-MSH (Frandberg et al., 1994; Heyder et al., 2021). It also aids in understanding the signaling mechanisms activated by melanocortin receptors and their

downstream effects. SHU9119, a synthetic peptide antagonist for MC3R and MC4R, is used in research to study the role of melanocortin receptors in regulating energy balance and feeding behavior (Haskell-Luevano et al., 2000; Sutton et al., 2008a).

1.2.2. Structure, tissue distribution and function of melanocortin-4 receptor

The human *MC4R* was cloned by degenerate PCR in 1993 as an intronless gene with an open reading frame of 999 bp encoding a 332 amino acid protein (Mountjoy et al., 1994). Like other GPCRs, MC4R is characterized by seven transmembrane α-helices connected by alternating extracellular loops (ELs) and intracellular loops (ILs). This configuration allows the receptor to interact with extracellular ligands and intracellular signaling proteins, transmitting signals from the outside to the inside of the cell (Ersoy et al., 2012). α-MSH is the primary agonist that stimulates MC4R activity, while AgRP functions as an endogenous antagonist that inhibits MC4R activity (Bagnol et al., 1999).

MC4R is widely expressed in various tissues, primarily in the central nervous system (CNS), especially the hypothalamus (**Fig. 1.1**). Neurons in the arcuate nucleus of the hypothalamus produce α-MSH and AgRP, which directly interact with MC4R to regulate feeding behavior. POMC-expressing neurons of the hypothalamic arcuate nucleus (ARC) are involved in the control of food intake and metabolic processes. The fed state is signaled by the abundance of circulating hormones such as leptin and insulin in the hypothalamus,

promoting the processing of POMC to the mature hormone α-MSH. Activation of MC4R by α-MSH leads to reduced food intake (Baldini and Phelan, 2019). In contrast, under the "starvation state," the activity of AgRP- and neuropeptide Y (NPY)-expressing neurons increases due to decreased circulating leptin and insulin levels and the secretion of the appetite-promoting hormone ghrelin. Projections from AgRP/NPY neurons converge with projections from POMC neurons to MC4R neurons in the paraventricular nucleus (PVN) to promote food intake (Baldini and Phelan, 2019).

MC4R is also present in other brain regions, such as the thalamus, cortex, brainstem, hippocampus, hypothalamus, and spinal cord (Mountjoy et al., 1994). Although MC4R is mainly involved in central regulation, it is also expressed in some peripheral tissues, including the heart, kidney, muscle, lung, and testes (Mountjoy et al., 2003). MC4R can influence glucose metabolism independently of its effects on food intake by improving insulin sensitivity and promoting glucose uptake in peripheral tissues, contributing to overall metabolic health. For example, in adipose tissue, MC4R affects lipolysis and adipocyte function (Møller et al., 2015). In muscle tissue, it is involved in regulating glucose uptake and energy expenditure (Podyma et al., 2018).

1.2.3. Multiple signaling pathways of MC4R

The main endogenous agonists of MC4R are α-MSH and ACTH. Upon binding to its agonists, MC4R undergoes conformational changes and promotes the exchange of GDP for GTP on the $G\alpha_s$ subunit, resulting in the dissociation of the $G\alpha_s$ subunit from the Gβγ dimer. The $G\alpha_s$-GTP complex activates adenylate cyclase (AC), which catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP). Increased cAMP levels act as a second messenger, activating PKA, which then phosphorylates target proteins, leading to changes in cellular function and gene expression (Fatima et al., 2022; Gantz et al., 1993).

In vitro experiments have shown that MC4R can functionally couple to other major G proteins (Gαi and Gαq/11) and may recruit other downstream effectors through β-arrestin (Breit et al., 2011; Gillyard et al., 2019) . MC4R couples to Gαq, which activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to produce two second messengers: inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 binds receptors in the endoplasmic reticulum (ER), resulting in the release of stored calcium ions into the cytoplasm (Newman et al., 2006; Sharma et al., 2020). After MC4R binds to the Gi protein, it inhibits adenylate cyclase (AC) activity and reduces cAMP levels (Büch et al., 2009; Clément et al., 2018). Recently, Inoue and colleagues have confirmed that MC4R can also bind to G12/13 (Inoue et al., 2019).

MC4R also activates extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, but the underlying mechanisms vary by cell type. In CHO cells expressing hMC4R, NDP-MSH-

induced ERK1/2 activation is mediated by phosphatidylinositol 3-kinase (PI3K) instead of PKA (Vongs et al., 2004). In GT1-7 cells, α-MSH-induced ERK1/2 activation through MC4R is exclusively PKA-dependent (Damm et al., 2012). Another study reported that ERK1/2 activation through MC4R is mediated by Gi proteins in HEK293 cells, while in GT1-1 cells, it is mediated by calcium and PKC (Chai et al., 2006). Additionally, gain-of-function *MC4R* variants exhibit signaling biased toward β-arrestin recruitment and enhanced ERK1/2 signaling (Lotta et al., 2019). MC4R is also involved in other pathways, including 5'-AMP-activated protein kinase (AMPK), c-Jun N-terminal kinase (JNK), protein kinase B (PKB or AKT), and the potassium channel Kir7.1 (Anderson et al., 2019; Chai et al., 2009; Ghamari-Langroudi et al., 2015; Minokoshi et al., 2004; Yang and Tao, 2016).

1.2.4. *MC4R* mutations in obesity pathogenesis

MC4R is a pivotal regulator of energy homeostasis, influencing both appetite and energy expenditure. Mutations of *MC4R* are found in approximately 2-6% of individuals with severe early-onset obesity, making it the most common monogenic form of obesity (Farooqi et al., 2003). Loss-of-function mutations in *MC4R* affect receptor expression, ligand binding, or signal transduction, causing hyperphagia due to decreased anorexigenic signals and reduced energy expenditure, leading to obesity. Conversely, gain-of-function mutations enhance *MC4R* activity either by improving ligand binding or through constitutive activation,

increasing energy expenditure and thermogenesis, thereby promoting weight loss (Fatima et al., 2022; Tao and Segaloff, 2005).

Previous studies have proposed a scheme for classifying natural mutations in *MC4R* gene associated with obesity (Tao and Segaloff, 2003). Class I mutants have no receptor protein due to defective synthesis and/or accelerated degradation. Class II mutants are retained within the cell. Class III mutants show relatively normal expression on cell surface but have impaired ligand binding, as evidenced by reduced maximal binding and/or binding affinity, and therefore impaired ligand-stimulated signaling. Class IV mutants are relatively normal on the cell surface and bind to ligands but have impaired agonist-stimulated signaling. Class V mutations have no known defects.

To date, more than 170 naturally occurring mutations of human *MC4R* have been identified in gnomAD v2.1.1 database (https://gnomad.broadinstitute.org/) based on Ref. (Lek et al., 2016; Liu et al., 2022) (**Fig. 1.2**). These mutations include at least 79 Class II mutations, accounting for nearly 45% of all naturally occurring human *MC4R* mutations discovered. The severity of the observed dysfunction varies, with some mutants showing complete loss of function, some severely impaired function, and some mildly impaired or normal function.

1.2.5. Structure, tissue distribution and function of melanocortin-5 receptor

The final MCR to be characterized is MC5R, a 325 amino acid protein encoded by the *MC5R* gene located on chromosome 18p11.2 (Chhajlani et al., 1993; Logan et al., 2003). It also has a common structural framework characterized by seven transmembrane α-helices connected by three ECLs and three ICLs. Additionally, like other members of MCR family, MC5R has several characteristic motifs that are critical for its structure and function. The highly conserved DRY motif is located at the end of TM3 and the beginning of ICL2, stabilizing the active state of the receptor and promoting G protein coupling (Hawtin, 2005; Yang and Tao, 2020). The NPxxY motif, located near the end of TM7 and the beginning of the C-terminal tail, is essential for receptor activation, G protein coupling, and interaction with other intracellular signaling molecules (Kim et al., 2004; Liu et al., 2015).

MC5R is expressed in both central and peripheral tissues (**Fig. 1.1**), giving it a variety of physiological functions. In the brain, MC5R is involved in fetal brain development, cognitive function, and stress response (Ji et al., 2022). In peripheral tissues, MC5R regulates exocrine gland secretion, such as sebum production by sebaceous glands and potentially sweat production by sweat glands which plays a key role in maintaining healthy skin and hair (Springer and Gatesy, 2018; Zhang et al., 2006). Additionally, MC5R is involved in regulating immune cell activity and inflammatory responses, highlighting its importance in immune regulation. MC5R also regulates key metabolic processes in adipocytes, including lipolysis, re-esterification, fatty acid oxidation, and glucose uptake (Ji et al., 2022). These functions are essential for maintaining energy balance and metabolic health. Therefore,

understanding the structure and function of MC5R can help develop targeted therapies for diseases such as acne, seborrheic dermatitis, and other skin conditions. Furthermore, the role of MC5R in energy metabolism makes it a potential target for the treatment of metabolic diseases.

1.2.6. Multiple signaling pathways of MC5R

Like MC4R, MC5R is activated by melanocortin peptides such as α-MSH and ACTH. Upon ligand binding, MC5R undergoes a conformational change that activates associated heterotrimeric G proteins, primarily $G\alpha_s$ protein. This activation initiates a cascade of intracellular signaling events. The $G\alpha_s$-GTP complex activates AC, catalyzing the conversion of ATP to cAMP, thereby triggering the cAMP-PKA signaling pathway. In turn, the cAMP-PKA pathway triggers downstream signaling cascades, including the lipolytic pathway (involving inhibition of acetyl-CoA carboxylase and activation of hormone-sensitive lipase (HSL), perilipin, and adipose triglyceride lipase (ATGL)) and the cAMP response element binding protein (CREB) pathway, which plays a key role in balancing pro- and anti-inflammatory responses (Rodrigues et al., 2013).

Recently, MC5R activates ERK1/2 through a PI3K-dependent signaling mechanism, and then inhibit fatty acid re-esterification, mediate cell proliferation or differentiation, and induce the expression of early genes involved in immune responses, such as c-fos (Rodrigues

et al., 2015). Furthermore, in 3T3-L1 adipocytes, MC5R inhibits the secretion of leptin, indicating that MC5R may indirectly regulate food intake and energy expenditure via the leptin-melanocortin pathway (Jun et al., 2010). In both Ba/F3 pro-B-lymphocyte cells and human IM-9 lymphoblastoid cells expressing *MC5R*, α-MSH binding stimulates Janus kinase 2 (JAK2) and signal transducers and activator of transcription (STAT1) tyrosine phosphorylation (Buggy, 1998).

1.2.7. *MC5R* mutations in obesity pathogenesis

MC5R is a functional candidate gene for human obesity and livestock obesity. Although MC5R mutations in Quebec families and Finns are significantly associated with obesity phenotypes, current studies on these mutations are insufficient to prove a causal relationship between them and human obesity. Recent extensive genomic studies have discovered many additional *MC5R* mutations (**Fig. 1.3**), such as F209L, A81A, D108D, S125S, T248T and L283L, which are associated with obesity, type 2 diabetes (T2DM) as well as schizophrenia and bipolar disorder, but without effects on skin condition like acne vulgaris (Chagnon et al., 1997; Hebebrand et al., 2018; Valli-Jaakola et al., 2008). In fact, the gnomAD v2.1.1 database (https://gnomad.broadinstitute.org/) reports more than 130 mutations in *MC5R*, which have been poorly studied to date.

There are many functional studies of naturally occurring mutations in *MC3R* and *MC4R*, and potential strategies to correct these mutations have been identified, especially pharmacological partners that correct misfolded mutant receptors (Granell et al., 2010; Maya-Nunez et al., 2012; Tao, 2020). However, whether *MC5R* mutations lead to receptor defects and the exact nature of these molecular defects remain to be investigated. Correlating molecular defects with phenotype is necessary to convincingly demonstrate the clinical significance of these mutations in human disease.

1.3. Melanocortin-2 receptor accessory proteins

1.3.1. Discovery and structure of MRAPs

Over the past decade, several families of GPCR accessory proteins have been identified as regulators of receptor trafficking, ligand binding, and signal transduction, increasing the control and complexity of GPCR functional expression and signaling (Tao, 2020). Like other known GPCR accessory proteins, melanocortin 2 receptor (MC2R) accessory proteins (MRAPs) are small proteins with a hydrophobic transmembrane domain. There are two members in the human MRAP family, MRAP1 and MRAP2 (Chan et al., 2009). MRAP1 was originally discovered as an essential partner for MC2R, facilitating the trafficking of MC2R from the endoplasmic reticulum to the cell membrane, and was required for ACTH binding and the stimulation of cyclic AMP (cAMP) (Novoselova et al., 2013). *MRAP2,* identified through whole human genome sequence analysis, shares approximately

40% sequence homology with *MRAP1* (Ji and Tao, 2022). Unlike MRAP1, MRAP2 only facilitates MC2R trafficking and does not affect ACTH signaling. Recent studies have shown that MRAP1 and MRAP2 can regulate the surface expression and signaling of all melanocortin receptors (MC1-5R), whereas MRAP2 has lower receptor fidelity and affects the signaling of the orexin receptor, ghrelin receptor, and growth hormone secretagogue receptor 1a (GHSR1a) (Berruien and Smith, 2020; Rouault et al., 2017a; Rouault et al., 2020).

MRAPs are single transmembrane proteins with an unusual dual topology. *MRAP1* is encoded by a single gene composed of six exons. Alternative splicing of the *MRAP1* gene produces two isoforms: the 172-amino-acid MRAP1a and the 102-amino-acid MRAP1b. These isoforms share the identical N-terminal and transmembrane domain but differ at the C-terminus. Two predicted splice variants of *MRAP2* are encoded after transcription and alternative splicing: MRAP2a with 205 amino acids and MRAP2b with 154 amino acids. Like MRAP1, the carboxy-terminal region differs considerably among *MRAP2* variants, whereas the amino-terminal and transmembrane domains are conserved (Ji and Tao, 2022).

Several critical and functionally distinct regions of MRAPs were identified by site-directed and saturation mutagenesis. The conserved motif LKANKHS/LKAHKYS, preceding the transmembrane region, is required for dual topology and has been observed in both MRAP1 and MRAP2, allowing for the formation of homodimers (MRAP/MRAP; MRAP2/MRAP2) or heterodimers (MRAP/MRAP2; MRAP2/MRAP) (Sebag and Hinkle,

2007). Furthermore, the YEYY motif is evident in MRAP1 and MRAP2 from almost all vertebrates examined and is crucial for their association with Gαs, as well as for mediating the downstream signal leading to the activation of PKA and lipolysis. However, the LDYL motif, required for ACTH binding to MC2R, is absent in MRAP2, which is a critical difference between MRAP1 and MRAP2 (Dores, 2016). Co-precipitation studies with MC2R suggest that the identical transmembrane domain is necessary for the formation of a functional MRAP-MC2R complex (Chan et al., 2009). However, the exact residues required for MRAPs to interact with GPCRs have not been fully characterized.

1.3.2. Tissue distribution and function of MRAPs

MRAPs are widely distributed in the central nervous system and peripheral tissues **(Fig. 1.1)**, including the adrenal glands, digestive tract, testes, ovaries, and adipose tissue, suggesting they have multiple functions. In humans, MRAP1 is expressed at the highest levels in adipose tissue and the adrenal cortex, with lower expression levels in the skin and male reproductive organs (Novoselova et al., 2018). In 3T3-L1 adipocytes, knockdown or overexpression of *MRAP1* reduced or increased ACTH-induced lipolysis, respectively. Furthermore, transgenic mice (aP2-MRAP) overexpressing MRAP specifically in adipose tissue exhibited increased lipolysis in response to ACTH compared with wild-type mice. When fed a high-fat diet (HFD), these transgenic mice had significantly reduced fat and weight gain and improved glucose and insulin tolerance, indicating the essential role of

MRAP1 in regulating ACTH-induced lipolysis and whole-body energy balance. Effective ACTH signaling is vital for glucocorticoid production, mutations in MRAP1 result in the loss of a functional ACTH receptor (MC2R), causing familial glucocorticoid deficiency type 2 (Metherell et al., 2005).

MRAP2, a newly identified paralog of MRAP, is known to modulate the activity of MC3R and MC4R, both of which are critical for the regulation of energy homeostasis. By modulating MC4R activity, MRAP2 influences the anorexigenic (appetite-suppressing) effects mediated by melanocortin peptides like α-MSH (Berruien and Smith, 2020; Soletto et al., 2019). Recent genetic studies have found that reduced MC4R activity caused by loss of function of MRAP2 may contribute to obesity. Interestingly, some *MRAP2* genetic variants associated with severe obesity have a slightly different phenotype than *MC4R* genetic variants (da Fonseca et al., 2021). Both brain-targeted *Mrap2*$^{-/-}$ or global *Mrap2*$^{-/-}$ mice exhibit morbid obesity, and Mrap2-deficient mice do not show reduced energy consumption or hyperphagia (Asai et al., 2013).

Additionally, MRAP2 can regulate energy homeostasis by modulating the activity of prokineticin receptor (PKR1). PKR1, a $G\alpha_s$ and $G\alpha_q$/11 coupled receptor expressed in multiple tissues, reduces food intake and body weight in a mouse model of human obesity. In contrast to the activity of MC4R, MRAP2 inhibits PKR1 trafficking and signaling *in vitro*. Furthermore, the anorexigenic effect of the PKR1 agonist prokineticin 2 is enhanced in

MRAP2 knockout mice compared with wild-type (Rouault et al., 2017a). Recently, MRAP2 was found to be localized in uterine glandular epithelial cells and was significantly upregulated in women with unexplained infertility, suggesting that MRAPs have a potential impact on reproductive regulation (D'Aurora et al., 2019). These studies illustrate the promiscuity of MRAP2 and underscore its importance in various physiological processes, including energy homeostasis, appetite regulation, and metabolic balance.

1.3.3. Pharmacological effect of MRAPs on melanocortin receptors

MC2R was initially difficult to express on the cell surface until an adrenal cell line was utilized and found that MRAP1 acts as an essential accessory protein for MC2R. It is required for ACTH binding and affects cAMP production in response to $G\alpha_s$ activation. The interaction of MRAP and MC2R also influences receptor internalization and recycling (Roy et al., 2007).

MRAPs are intriguing and versatile regulatory proteins that can also modulate the trafficking, ligand specificity, and signaling of other MCRs. For MC4R, MRAP1a decrease cell surface expression of MC4R and inhibits α-MSH- and NDP-MSH-induced or has no effect on α-MSH- and ACTH-stimulated hMC4R signaling (Chan et al., 2009; Kay et al., 2013; Liang et al., 2018). MRAP2 converts MC4R into an ACTH receptor by increasing its

sensitivity to ACTH stimulation (Soletto et al., 2019; Zhang et al., 2017). For MC3R, MRAP2 decreases cell surface expression of hMC3R, but increases α-MSH-stimulated and decreases NDP-MSH-induced cAMP production of hMC3R (Chan et al., 2009; Kay et al., 2013; Liang et al., 2018).

Compared to MC3R and MC4R, the effects of MRAPs on MC5R are less well studied. It has been reported that co-expression with MRAP1 retains MC5R intracellularly. MRAP1 can disrupt the dimerization and surface localization of MC5R (Chan et al., 2009). cAMP assay studies have found that MRAP1 acts as a negative regulator of MC5R in response to NDP-MSH, due to the disruption of MC5R dimerization (Sebag and Hinkle, 2009a). However, it does not affect α-MSH and ACTH-induced hMC5R signaling. Like MRAP1, MRAP2 has also been found to have a significant inhibitory effect on MC5R.

Additionally, MRAPs play essential roles in the regulation of MCRs across various species, highlighting their evolutionary conservation and functional importance. Studies in zebrafish, amphibians, and birds provide valuable insights into the evolutionary adaptation of MRAP functions and their roles in stress response, development, and energy regulation (Tai et al., 2021; Wang et al., 2019; Wang et al., 2022; Zhang et al., 2017; Zhu et al., 2019b).

1.3.4. *MRAP2* mutations in obesity pathogenesis

Like *MC3R* and *MC4R* KO mice, *MRAP2* KO mice exhibit an obese phenotype. Analysis of obese patient populations has revealed multiple loss-of-function mutations in the *MRAP2* gene that cause monogenic hyperphagic obesity with concomitant hyperglycemia and hypertension (Asai et al., 2013; Baron et al., 2019). To date, large-scale sequencing has revealed around 30 rare heterozygous *MRAP2a* variants that are associated with obesity in adults and children (**Fig. 1.4**)(Asai et al., 2013; Baron et al., 2019; Schonnop et al., 2016). Study found that rare alleles in the *MRAP2* p. Gln174Arg variant reduced MC4R signaling in HEK293 cells, suggesting that *MRAP2* mutations can affect MC4R function (Schonnop et al., 2016). Furthermore, in two independently derived *Mrap2*-deficient mouse strains ($Mrap2^{tm1a/tm1a}$), cholesterol metabolism was altered, and transcriptome analysis revealed a significant reduction in the expression of *Sim1*, *Trh*, *Oxt*, and *Crh* in the PVN of hypothalamus. This supports the idea that MRAP2 is involved in the MC4R signaling pathway *in vivo* (Novoselova et al., 2016).

However, the data further highlights the phenotypic differences between *Mrap2*-deficient and *Mc4r*-deficient mice. Although both strains developed severe early-onset obesity, $Mc4r^{-/-}$ mice showed pronounced hyperphagia and reduced energy expenditure, whereas *Mrap2*-deficient mice did not (Balthasar et al., 2005; Huszar et al., 1997; Novoselova et al., 2016). This suggests that there may be MC4R-independent mechanisms and possibly MCR-independent pathways in the pathogenesis of MRAP2-associated obesity. Therefore, understanding the multiple regulatory mechanisms of MCRs by MRAP2 could

provide valuable insights into its role in health and disease. This knowledge may open new avenues for therapeutic interventions targeting obesity and related metabolic disorders.

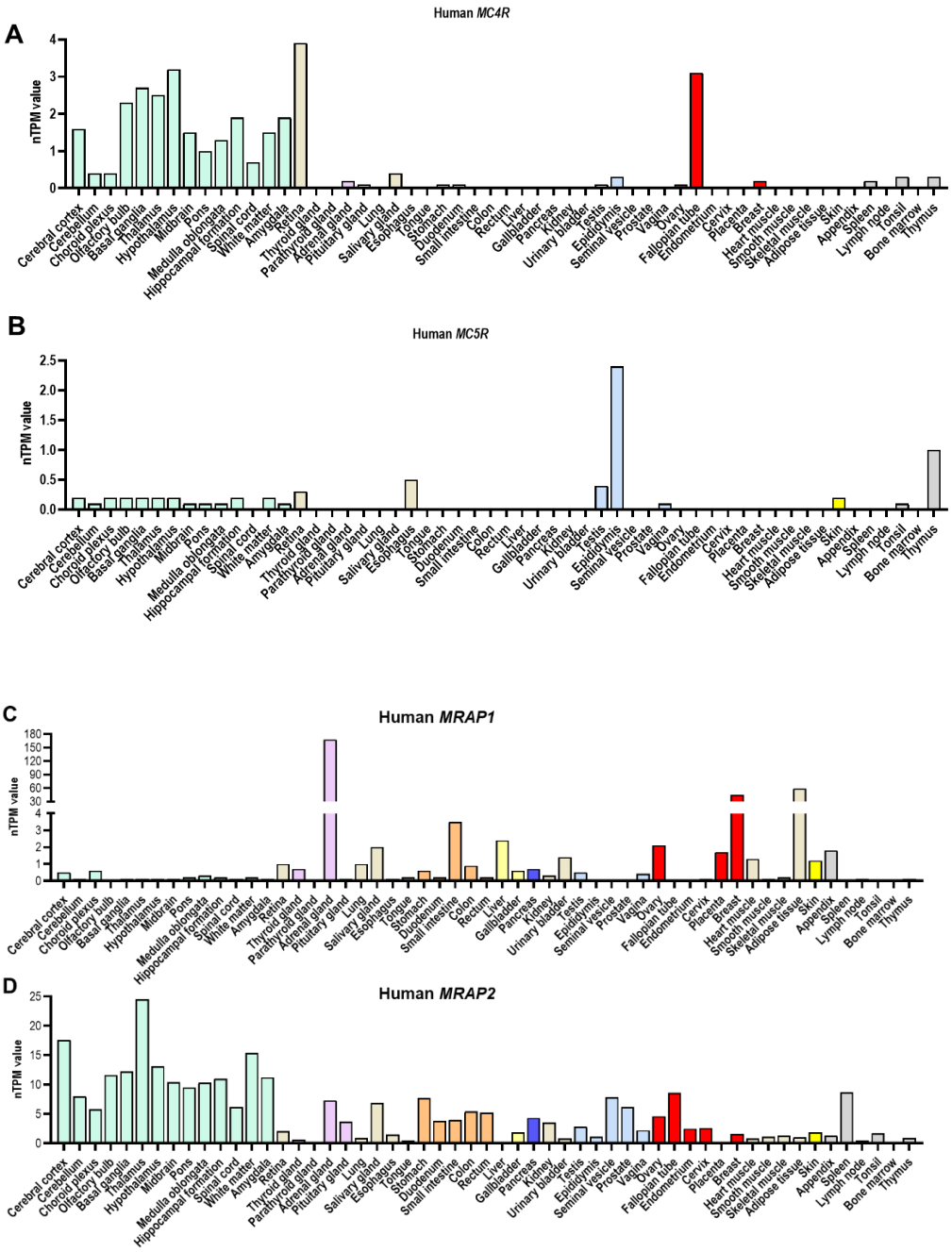


Figure 1.1 Gene expression. *MC4R* (A), *MC5R* (B), *MRAP1* (C), and *MRAP2* (D). https://www.proteinatlas.org/ (Uhlen et al., 2015). nTPM indicates normalized protein-coding transcripts per million. Color-coding is based on tissue groups with functional features in common.

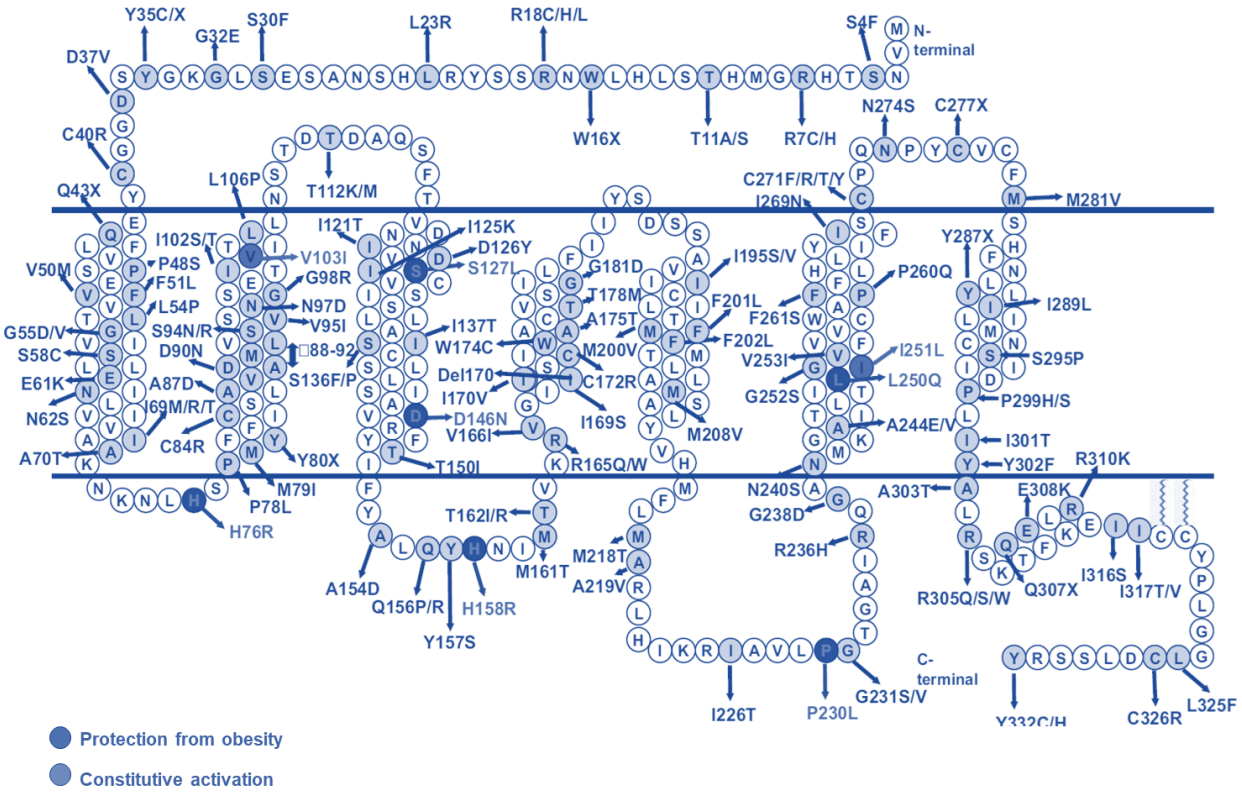


Figure 1.2 Naturally occurring mutations in human *MC4R*. The data was registered in gnomAD v2.1.1 database (https://gnomad.broadinstitute.org/) in 2010 based on Ref. (Lek et al., 2016; Liu et al., 2022). The circles filled with color were missense and nonsense mutations/polymorphisms. Frameshift mutations were not included in the figure.

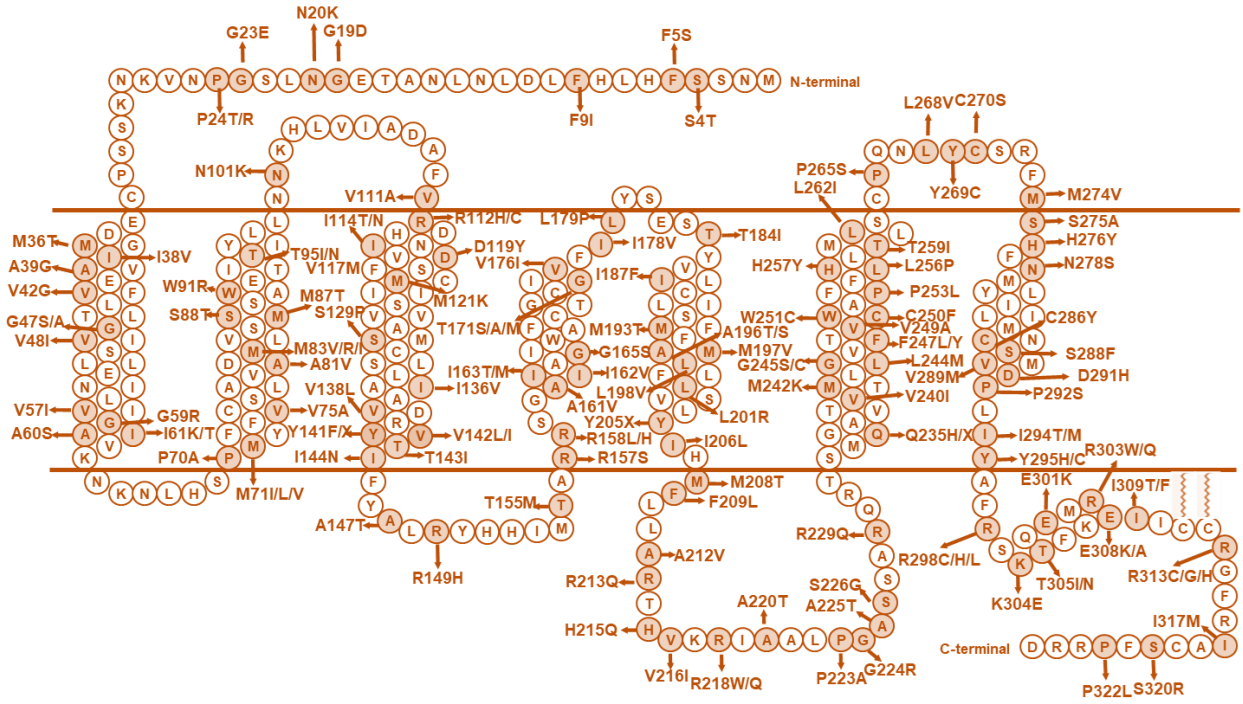


Figure 1.3 Naturally occurring mutations in human *MC5R*. The data were obtained in 2020 from gnomAD v2.1.1 database (https://gnomad.broadinstitute.org/). The circles filled with color were missense and nonsense mutations/polymorphisms. Frameshift mutations were not included in the figure.

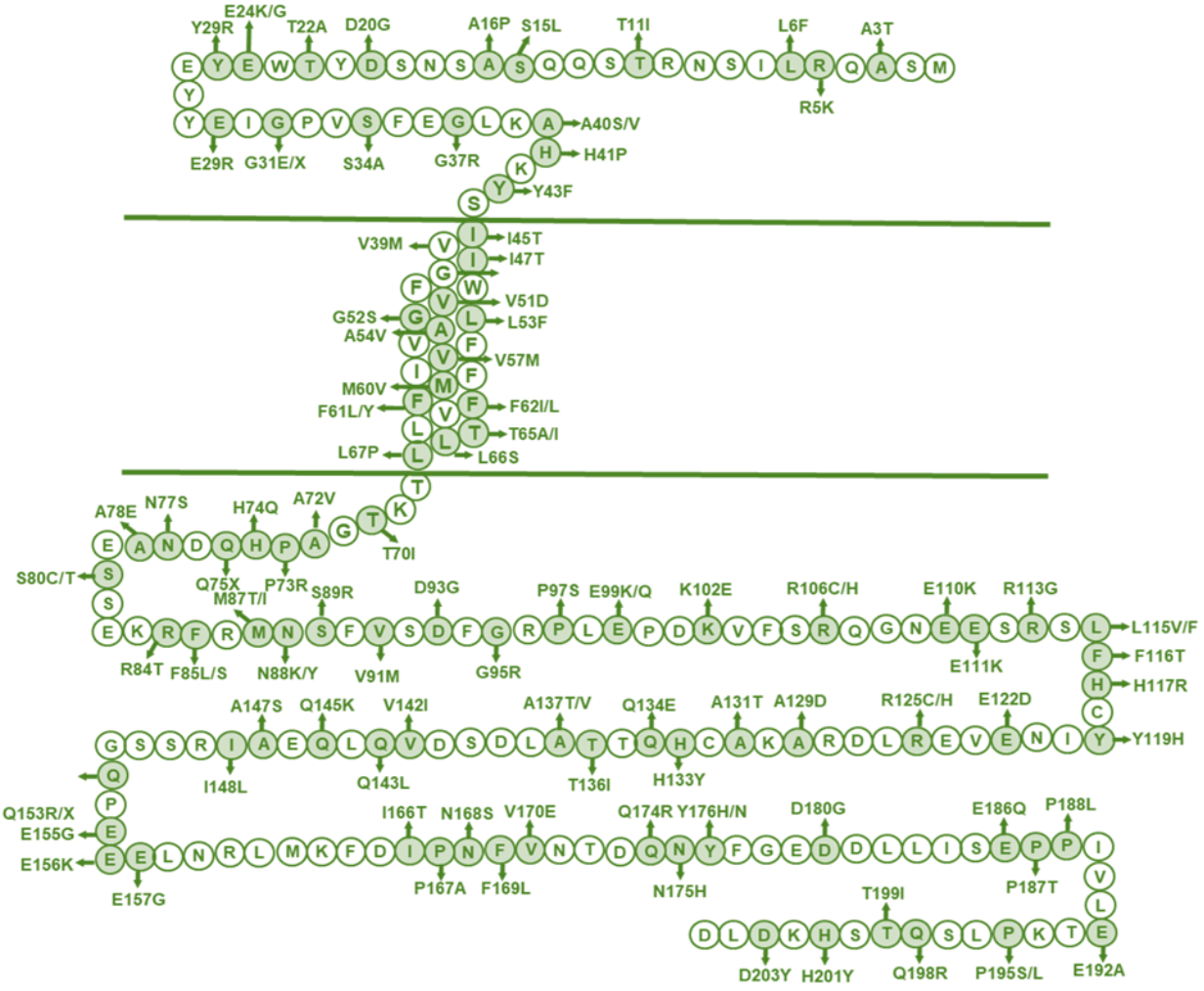


Figure 1.4 Naturally occurring mutations in human *MRAP2*. The data were obtained in 2021 from gnomAD v2.1.1 database (https://gnomad.broadinstitute.org/). The circles filled with color were missense and nonsense mutations/polymorphisms. Frameshift mutations were not included in the figure.

Chapter 2: Regulation of human melanocortin-5 receptor by isoforms of melanocortin-2 receptor accessory proteins

2.1. Introduction

Melanocortins are a group of structurally related peptides derived from proopiomelanocortin (POMC). They are named for their melanotropic and corticotropic activities, including adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), β-MSH, and γ-MSH (Chakraborty et al., 1996). The effects of melanocortins are mediated through the activation of melanocortin receptors (MCRs), specifically from melanocortin-1 receptor (MC1R) to MC5R. Each receptor has distinct tissue expression patterns and unique profiles for the relative potency of different melanocortins, resulting in diverse physiological functions (MacNeil et al., 2002). MC1R is involved in skin pigmentation in mammalian melanocytes (Almeida Scalvino et al., 2018; Voisey and van Daal, 2002). MC2R regulates steroidogenesis in adrenocortical cells and mediates the action of ACTH (Penhoat et al., 2000). MC3R and MC4R regulate energy homeostasis in the central nervous system (CNS) (Cone, 1999). MC5R, the last cloned member of MCR family, is widely expressed in peripheral tissues, such as skin, skeletal muscle, adipocytes, adrenal and exocrine glands, and various types of immune cells (Chowdhary et al., 1995; Ji et al., 2022). It is involved in exocrine and endocrine gland secretion (van der Kraan et al., 1998), energy metabolism (Chagnon et al., 1997; Møller et

al., 2015), thermoregulation (Chen et al., 1997), inflammation (Webering et al., 2019), and immune response (Ng et al., 2021; Taylor et al., 2006). MC5R is also found in the brain, where it plays a role in stress response (Liao et al., 2019), cognitive function (Zhou et al., 2021), and fetal brain development (Simamura et al., 2011).

MC5R belongs to rhodopsin-like Family A G protein-coupled receptor (GPCR), which consists of an extracellular amino terminus, an intracellular carboxylic terminus, and seven transmembrane domains connected by intracellular and extracellular loops. Upon binding to its agonists, MC5R couples to G proteins and transduce extracellular signals into intracellular space through various intracellular pathways, thereby exerting physiological functions (Cone, 2006; Tao, 2008, 2017). Generally, MC5R displays the highest binding affinity to α-MSH (MacNeil et al., 2002). Studies have shown that peripheral α-MSH promotes glucose uptake in skeletal muscle through MC5R pathway, suggesting its potential association with obesity and diabetes mellitus (Chagnon et al., 1997; Enriori et al., 2016; Valli-Jaakola et al., 2008). After MC5R binds to its agonist α-MSH, the active $G\alpha_s$ protein triggers cAMP signaling pathway, which plays a role in inflammation (Hoogduijn et al., 2002). Additionally, activated $G\alpha_i$ induces the activation of ERK1/2 through PI3K-dependent signaling pathway, thereby affecting fatty acid re-esterification, lipolysis, and immune response (Rodrigues et al., 2012; Rodrigues et al., 2009). Some synthetic agonists have been developed to understand the physiological roles played by MCRs. These include [Nle^4-D-Phe^7]-α-MSH (super-potent analog of α-MSH), melanotan II (MTII) and SHU9119, which

exhibit higher potency for MC5R than endogenous agonists but do not exhibit good selectivity for MC5R (Grieco et al., 2003; Xu et al., 2020).

Several families of GPCR accessory proteins have been identified as regulators of receptor trafficking, ligand binding, and signal transduction over the past decade, increasing the control and complexity of GPCR functional expression and signaling (Brady and Limbird, 2002). Like all known GPCR accessory proteins, melanocortin 2 receptor (MC2R) accessory proteins (MRAPs) are single hydrophobic transmembrane proteins with an unusual dual topology (Sebag and Hinkle, 2009b). MRAP (also called MRAP1) was initially discovered as an essential partner of MC2R, assisting its trafficking from endoplasmic reticulum (ER) to cell surface. Further studies showed that MRAP1 is required for ACTH binding and affects cAMP production in response to $G\alpha_s$ activation (Hinkle and Sebag, 2009; Roy et al., 2012). MRAP2, the newly discovered paralog of MRAP1 through whole human genome sequence analysis, shares approximately 40% sequence homology with MRAP1 (Chan et al., 2009; Webb and Clark, 2010). Recent studies have shown that both MRAP1 and MRAP2 can interact with all MCRs and play different functional roles among them (Berruien and Smith, 2020; Cerdá-Reverter et al., 2013; Rouault et al., 2017b). However, MRAP2 has lower receptor fidelity and can also modulate the trafficking, ligand specificity and signaling of GPCRs outside of MCRs, such as orexin receptor, ghrelin receptor and growth hormone secretagogue receptor 1a (GHSR1a) (Rouault et al., 2017a; Rouault et al., 2020; Srisai et al., 2017; Yin et al., 2020). MRAP2 regulates energy homeostasis by modulating the activity of

prokineticin receptor (PKR1), a $G\alpha_s$ and $G\alpha_q$/11 coupled receptor expressed in multiple tissues. In contrast to its activity on MC4R, MRAP2 inhibits PKR1 trafficking and signaling *in vitro*, suggesting that MRAP2 can either potentiate or inhibit GPCRs depending on the target (Berruien and Smith, 2020; Rouault et al., 2017a).

Alternative splicing generates two human MRAP1 isoforms, 172-amino-acid hMRAP1a and 102-amino-acid hMRAP1b. Additionally, it produces three predicted isoforms of hMRAP2, 205-amino-acid hMRAP2a, 154-amino-acid hMRAP2b, and 126-amino-acid hMRAP2c (Ji and Tao, 2022). Although many studies have focused on the pharmacological effects of MRAPs on MCRs, the potential effects of all five isoforms of hMRAPs on hMC5R have not been systematically studied. Therefore, in this study, c-myc-tagged hMC5R and Flag-tagged hMRAPs were synthesized and co-transfected into human embryonic kidney (HEK) 293T cells. Pharmacological properties were then investigated by measuring cell surface expression, ligand binding, and signaling (cAMP production and ERK1/2 phosphorylation).

2.2. Materials and methods

2.2.1. Reagents and supplies

The ligands used included NDP-MSH, obtained from Peptides International (now

Vivitide, Louisville, KY, USA), and α-MSH, purchased from Pi Proteomics (Huntsville, AL, USA). [^{125}I]-NDP-MSH and [^{125}I]-cAMP were iodinated using chloramine T method (Steiner et al., 1969). N-terminal c-myc-tagged hMC5R and N-terminal Flag-tagged hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b, and hMRAP2c were subcloned into pcDNA3.1(-) vector and commercially synthesized by Synbio Technologies (Monmouth Junction, NJ, USA) to generate plasmids for cell transfection. Paraformaldehyde (ACROS, 96%, NJ, USA) was used to prepare 4% PAF for flow cytometry. Falcon® 5 mL round bottom polystyrene test tube, with cell strainer snap cap (Franklin Lakes, NJ, USA) were used to collect dissociated samples for flow cytometry.

2.2.2. Cell culture and DNA transfection

HEK293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine essence (Avantor, Allentown, PA, USA), 10mM HEPES, 0.25 mg/mL amphotericin B, 50 mg/mL gentamicin, 100 IU/mL penicillin, and 100 mg/mL of streptomycin. The cells were incubated at 37 °C in a 5% CO_2 atmosphere (Tao and Segaloff, 2003). For experiments, cells were plated into gelatin-coated 24-well or 6-well plates. When the cells reached 50-70% confluency, they were co-transfected with 0.25 mg/mL hMC5R with or without hMRAPs (hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b, or hMRAP2c) at a ratio of 1:5 using calcium phosphate precipitation

method (Chen and Okayama, 1987). Total DNA was normalized using empty vector pcDNA3.1.

2.2.3. Flow cytometry assay

Flow cytometry is a high-throughput single-cell analytical method that has evolved from identifying cell surface proteins to characterizing intracellular proteins. To investigate the effect of hMRAP1s or hMRAP2s on total (including cell surface and intracellular proteins) and cell surface expression of hMC5R, flow cytometry (Accuri Cytometers, Ann Arbor, MI, USA) was used as described previously (Wang et al., 2008). Forty-eight hours after co-transfection, cells for quantification of total expression were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. After incubating with blocking solution (5% BSA) for 1 h, both permeabilized and non-permeabilized cells (for quantification of cell surface expression) were incubated with mouse anti-myc 9E10 monoclonal antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) diluted 1:40, followed by Alexa Fluro @ 488 goat anti-mouse antibody (Invitrogen, Grand Island, NY, USA) diluted 1:2,000 for 1 h respectively. The C6 Accuri Cytometer (Accuri Cytometers, Ann Arbor, MI, USA) was used to collect fluorescence signals. Empty vector (pcDNA3.1) fluorescence was used for correction of background staining. The expression of hMC5R was calculated as the percentage of cells transfected with hMC5R in absence of hMRAPs (set as 100%).

2.2.4. Radioligand ligand binding assays

To incontainsthe regulation of hMRAP1s or hMRAP2s on binding properties of hMC5R, forty-eight hours after co-transfection, cells were incubated at 37 °C with warm DMEM containing 1 mg/mL bovine serum albumin (DMEM/BSA) and a fixed amount of radioactive [^{125}I]-NDP-MSH (~100,000 cpm) with or without different concentrations of unlabeled NDP-MSH (ranging from 10^{-10} to 10^{-5} M) for 1 hour. After incubation, cells were washed with cold Hank's balanced salt solution (Sigma-Aldrich) containing 1 mg/ml BSA and then solubilized with 0.5 N NaOH solution. Cell lysates were collected with swabs and counted in a gamma counter. The concentration of ligand that results in 50% displacement of radiolabeled ligand (IC_{50}, an indicator of binding affinity) and maximal binding capacity (B_{max}) were calculated.

2.2.5. Ligand-stimulated cAMP assays

To investigate the regulation of hMRAP1s or hMRAP2s on cAMP signaling properties of hMC5R, forty-eight hours after co-transfection, cells were pre-incubated with warm DMEM/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 minutes and then treated with or without different concentrations of α-MSH (ranging from 10^{-10} to 10^{-4} M) for another hour. Intracellular cAMP was extracted

using 0.5 N perchloric acid containing 180 mg/mL theophylline and neutralized with 0.72 M KOH/0.6 M $KHCO_3$. The cAMP levels were determined by radioimmunoassay (RIA) as described previously (Yang et al., 2019). The maximal response (R_{max}) and concentration of agonist that results in 50% maximal response (EC_{50}, an indicator of agonist potency) were calculated.

2.2.6. ERK1/2 phosphorylation assay

To investigate the regulation of hMRAP1s or hMRAP2s on phosphorylated ERK1/2 (pERK1/2) activity of hMC5R, twenty-four hours after co-transfection, cells were starved overnight in DMEM/BSA and then treated with either buffer alone or 1 μM α-MSH for 5 min the next day. Cells were washed with cold OG and then solubilized in lysis buffer. Total protein concentrations were determined by Bradford protein assay. Protein samples (32 μg) were separated by 10% SDS-PAGE and transferred onto pre-wetted PVDF membrane using wet transfer conditions. The membrane was blocked in nonfat dry milk and then immunoblotted with primary antibodies for pERK1/2 and β-tubulin: rabbit anti-pERK1/2 antibody (1:1800~1:2000, Cell Signaling, Billerica, MA, USA) and mouse anti-β-tubulin antibody (1:20,000, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA) overnight, respectively. After washing by TBST, blots were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies: anti-rabbit (1:1800 ~ 1:2000, Cell Signaling, Billerica, MA, USA) and anti-mouse (1:20,000, Jackson

ImmunoResearch Laboratories, West Grove, PA) antibodies at room temperature for 2h. Specific bands were detected with enhanced chemiluminescence reagent (Thermo Scientific, Rockford, IL, USA) and quantified using ImageJ 1.44 software (National Institute of Health, Bethesda, MD) after densitometric scanning of films. ERK1/2 phosphorylation was normalized to protein loading by expressing data as a ratio of pERK1/2 to β-tubulin.

2.2.7. Statistical analysis

All data were represented as mean ± S.E.M. The parameters and significance of differences were calculated GraphPad Prism 8.3 software (GraphPad, San Diego, CA, USA). The significance of differences in flow cytometry, ligand binding and cAMP signaling parameters were determined by one-way ANOVA (determine differences between the means of three or more independent groups), followed by Dunnett's post hoc test, with $p < 0.05$ set as the threshold for significant. The significance of difference in p-ERK1/2 levels between cells transfected with hMC5R alone and cells co-transfected with hMC5R and MRAP was analyzed using unpaired Student's t-test.

2.3. Results

2.3.1. Regulation of hMC5R expression by hMRAPs

Flow cytometry was used to determine the regulation of hMRAPs on cell surface and total expression of hMC5R (Fig. 2.1). Total expression of hMC5R increased by 35.40% and decreased by 42.06% when co-transfected with hMRAP1a or hMRAP1b, respectively, but the difference was not significant (Fig. 2.1A). However, hMRAP1a hMRAP2a and hMRAP2c significantly increased cell surface expression of hMC5R (2.94-, 2.08- and 2.00-fold of control, respectively, $p < 0.05$) (Fig. 2.1B). The other two hMRAPs had no significant effect on cell surface expression of hMC5R (Fig. 2.1B).

2.3.2. Ligand binding properties of hMC5R regulated by hMRAPs

Different concentrations of unlabeled synthetic agonist, NDP-MSH, were used to compete with a fixed amount of labeled ^{125}I-NDP-MSH. Results showed that unlabeled NDP-MSH displaced radiolabeled NDP-MSH bound to hMC5R with an IC_{50} of 14.56 nM in control group (co-transfected hMC5R with empty vector) (Fig. 2.2 and Table 2.1). The experimental group (co-transfected hMC5R with hMRAPs) had similar binding affinities (IC_{50s}) for NDP-MSH as control (Fig. 2.2 and Table 2.1). In addition, only hMRAP2c significantly increased B_{max} of hMC5R (1.79-fold of control, $p < 0.05$), while others had no significant effect on B_{max}s of hMC5R (Fig. 2.2 and Table 2.1).

2.3.3. α-MSH-stimulated cAMP signaling properties of hMC5R regulated by hMRAPs

MC5R displays the highest binding affinity to its endogenous ligand, α-MSH, and then stimulates intracellular signaling pathways. Therefore, we studied signaling properties of hMC5R using α-MSH as the ligand. Our results showed that hMC5R exhibited a dose-dependent increase in cAMP generation upon α-MSH stimulation in all groups, however, none of hMRAPs affected the potencies (EC_{50}s) and maximal response (R_{max}) of hMC5R (Fig. 2.3 and Table 2.2).

2.3.4. Effects of hMRAPs on hMC5R-mediated ERK1/2 activation

To evaluate the efficacy of α-MSH on MAPK signaling, we first analyzed α-MSH-induced ERK1/2 phosphorylation in HEK293T cells transiently transfected with hMC5R alone. We found that α-MSH behaved as an agonist, inducing a significantly activation of ERK1/2 with a 1.56-fold maximal increase at 5 minutes upon stimulation with 1 μM α-MSH ($p < 0.05$). No significant change of pERK1/2 level was observed upon α-MSH treatment in cells transfected with empty vector (Fig. 2.4A).

To evaluate the effect of MRAP on hMC5R-mediated MAPK signaling, we compared ERK1/2 phosphorylation in cells transfected with hMC5R alone and cells co-transfected with hMC5R and MRAP, both in the absence (basal activity) and presence of ligand. Our results showed that hMRAP1a and hMRAP2c significantly decreased basal p-ERK1/2 levels of hMC5R (Fig. 2.4B & C) . Significantly increased pERK1/2 levels were observed in cells co-

transfected with hMC5R and hMRAP1a, hMRAP2a, hMRAP2b, or hMRAP2c upon stimulation with α-MSH, with increases of approximately 1.82-, 1.69-, 1.50-, and 1.96-fold, respectively ($p < 0.05$) (Fig. 2.4B & D). However, no significantly ERK1/2 activation was observed in cells co-transfected with hMC5R and hMRAP1b (Fig. 2.4B & D). Furthermore, hMRAP2c significantly increased hMC5R-mediated ERK1/2 activation compared to cells transfected with MC5R alone upon stimulation with 1 μM α-MSH ($p < 0.05$) (Fig. 2.4E).

2.4. Discussion

Precursor messenger RNA (pre-mRNA) splicing is key to post-transcriptional regulation of gene expression and can significantly expand the functional proteome of eukaryotes with a limited number of genes (Black, 2003). Typically, alternative splicing processes pre-mRNA into multiple mRNA isoforms that differ in the precise combination of exon sequences, resulting in different protein isoforms with varying peptide sequences and distinct chemical and biological activities (Grabowski and Black, 2001). To date, two human (h) *MRAP1* splice variants (*MRAP1a* and *MRAP1b*) and three *MRAP2* splice variants (*MRAP2a*, *MRAP2b* and *MRAP2c*) have been identified. Furthermore, isoforms of hMRAPs have been found to have different effects on the trafficking, binding, and signaling of hMC2R, hMC3R and hMC4R (Berruien and Smith, 2020; Chan et al., 2009; Ji and Tao, 2022; Kay et al., 2013; Roy et al., 2007). However, the regulation of hMC5R by hMRAP proteins has been poorly studied, especially hMRAP2c, the newly identified isforms of hMRAP2. To further

explore the functional diversity of hMRAP protein isoforms, the potential impact of two hMRAP1s and three hMRAP2s on hMC5R pharmacology was investigated in this study.

Unlike hMC2R, which requires hMRAP1a to reach the cell surface, hMRAP1a was found to have the opposite effect on hMC5R, greatly reducing cell surface expression of hMC5R in CHO cells in a concentration-dependent manner (Sebag and Hinkle, 2009a). Chan et al. also found that addition of hMRAP1a and hMRAP2a, either alone or in combination, significantly inhibited hMC5R trafficking to plasma membrane in CHO cells (Sebag and Hinkle, 2007). However, our results showed that hMRAP1a and hMRAP2a significantly increased cell surface expression of hMC5R in HEK293T cells. This contradictory phenomenon also appeared in experiments with hMC4R, where hMRAP1a and hMRAP2a significantly increased cell surface expression of hMC4R in HEK293 cells but attenuated it in CHO cells (Chan et al., 2009; Ji and Tao, 2022). These findings suggest that the trafficking of MCRs mediated by hMRAPs may be cell-line dependent. Since research on the regulation of MCRs by hMRAPs is limited, more studies are needed to confirm this.

Subsequent studies showed that MRAPs can also regulate MC5R trafficking and pharmacology in many species. In zebrafish, there are two *mc5r* genes, *mc5ra* and *mc5rb*, with phylogenetic analysis showing that zebrafish MC5Ra (zMC5Ra) is closer to hMC5R. There are two isoforms of MRAP2 in zebrafish, zMRAP2a and zMRAP2b (Agulleiro et al., 2010). The interactions between zMC5Rs and zMRAP2s were determined by co-

immunoprecipitation, zMRAP2a decreased the surface expression of both zMC5Ra and zMC5Rb, whereas zMRAP2b had no significant effect (Zhu et al., 2019a). In elephant shark(es), esMrap1 and esMrap2 had no effect on esMc5r trafficking in transfected CHO cells (Barney et al., 2019), similar to findings in whale shark (Hoglin et al., 2020). In addition, MRAP2 displayed a significant modulatory effect on cell surface expression of MC5R in mouse (Agulleiro et al., 2010), ricefield eel (Liu et al., 2021) and rainbow trout (Dores et al., 2020). These results suggest that the effects of MRAPs on MC5R trafficking may be species dependent.

As a typical GPCR, hMC5R can trigger $G\alpha_s$-cAMP signaling pathway and ERK1/2 signaling pathway. However, few studies have investigated the modulation of hMC5R signaling by hMRAPs. Previous study has shown that both hMRAP1a and hMRAP2a decrease NDP-MSH-stimulated cAMP production of hMC5R (Sebag and Hinkle, 2007, 2009a). Our study is the first to explore the potential modulation of hMC5R by hMRAPs using α-MSH. Results showed that hMRAP1s and hMRAP2s had no effect on α-MSH-stimulated $G\alpha_s$-cAMP pathway. Interestingly, another study has found that MRAP2 dose-dependently inhibits the efficacy of MC5R with α-MSH in mouse. Furthermore, in zebrafish, zMRAP2a inhibits the efficacy of both zMC5Ra and zMC5Rb with α-MSH, while zMRAP2b inhibits zMC5Ra but increases efficacy of zMC5Rb with α-MSH (Zhu et al., 2019a). In gar, MRAP1 increases efficacy with NDP-MSH, while MRAP2 increases efficacy with ACTH (Wolverton et al., 2019). Ricefield eel MC5R (maMC5R) and MRAP2X1

(maMRAP2X1) exhibit similar structural characteristics to MC5Rs and MRAP2s from other species, whereas maMRAP2X2 does not possess regions conserved in other species. Both maMRAP2X1 and maMRAP2X2 inhibit efficacy of maMC5R with α-MSH and ACTH (Liu et al., 2021). In whale shark, both MRAP1 and MRAP2 increase sensitivity to ACTH but not des-acetyl-α-MSH, while only MRAP1 increase sensitivity to ACTH in elephant shark (Barney et al., 2019; Hoglin et al., 2020). These results suggest that the effect of MRAPs on MC5R ligand efficiency may be species- or ligand-dependent.

In addition to cAMP activation, α-MSH-stimulated MC5R also induces ERK1/2 phosphorylation in a dose-dependent manner, with the maximum level obtained 5 min after stimulation (Rodrigues et al., 2009). Our study also found that α-MSH exhibited agonist activity on the MAPK pathway of hMC5R. Interestingly, α-MSH significantly increased ERK1/2 phosphorylation levels in cells co-transfected with hMC5R and hMRAP1a, hMRAP2a, hMRAP2b, or hMRAP2c, while hMRAP1b did not stimulate MC5R-mediated ERK1/2 activation. Additionally, hMRAP2c significantly increased MC5R-mediated ERK1/2 activation upon binding to α-MSH. Previous studies have shown that in HEK293 cells, upon α-MSH activation, MC5R activates ERK1/2 using a specific transduction pathway that is independent of adenylyl cyclase, PKA, PKC and Akt/PKB pathways but involves PI3K (Rodrigues et al., 2012). This is consistent with our finding that hMRAPs had no effect on α-MSH-stimulated $G\alpha_s$-cAMP pathway of hMC5R but affected MC5R-mediated activation of ERK1/2. This suggests that different transduction pathways are

differentially affected when the receptor binds to different hMRAP isoforms generated by alternative splicing. Since studies on the regulation of hMC5R signaling by hMRAPs are limited, further research is needed to explore this.

2.5. Conclusion

In summary, hMRAP1b and two newly identified hMRAP2 isoforms, hMRAP2b and hMRAP2c, played potential roles in regulating MC5R pharmacology. Additionally, different MRAPs had varying effects on hMC5R trafficking, binding, $G\alpha_s$-cAMP signaling, and ERK1/2 activation. Only hMRAP1a and hMRAP2a significantly increased trafficking of hMC5R from ER to cell membrane. All MRAPs had no effect on hMC5R affinity for NDP-MSH or on α-MSH-stimulated $G\alpha_s$-cAMP signaling. However, hMRAPs had different effects on MC5R-mediated ERK1/2 activation. These findings suggest the complexity of hMRAPs in modulating hMC5R and provide a new opportunity for regulating hMC5R signaling.

Table 2.1 Effects of MRAPs on ligand binding properties of hMC5R to NDP-MSH

	Bmax (%)	NDP-MSH binding
		IC_{50} (nM)
hMC5R	**100**	**14.56 ± 1.77**
hMC5R+hMRAP1a	**146.1 ± 18.96**	**18.03 ± 2.54**
hMC5R+hMRAP1b	**41.34 ± 8.32**	**12.22 ± 2.76**
hMC5R+hMRAP2a	**152.20 ± 27.17**	**17.45 ± 2.57**
hMC5R+hMRAP2b	**66.76 ± 12.82**	**11.80 ± 1.82**
hMC5R+hMRAP2c	**179.90 ± 27.03***	**12.18 ± 2.21**

Results are presented as the mean ± SEM (n = 4 - 6).

* Significant difference from the parameter of hMC5R, $P < 0.05$.

Table 2.2 Effects of MRAPs on α-MSH-stimulated cAMP signaling properties of hMC5R

	α-MSH	
	EC_{50} (nM)	**R_{max} (%)**
hMC5R	**32.76 ± 6.69**	**100**
hMC5R+hMRAP1a	**38.37 ± 5.54**	**105.80 ± 17.98**
hMC5R+hMRAP1b	**41.56 ± 5.32**	**80.42 ± 20.29**
hMC5R+hMRAP2a	**46.75 ± 6.99**	**90.25 ± 10.65**
hMC5R+hMRAP2b	**29.00 ± 4.06**	**107.20 ± 17.58**
hMC5R+hMRAP2c	**40.50 ± 4.49**	**93.59 ± 10.47**

Results are presented as the mean ± SEM (n = 4 - 6).

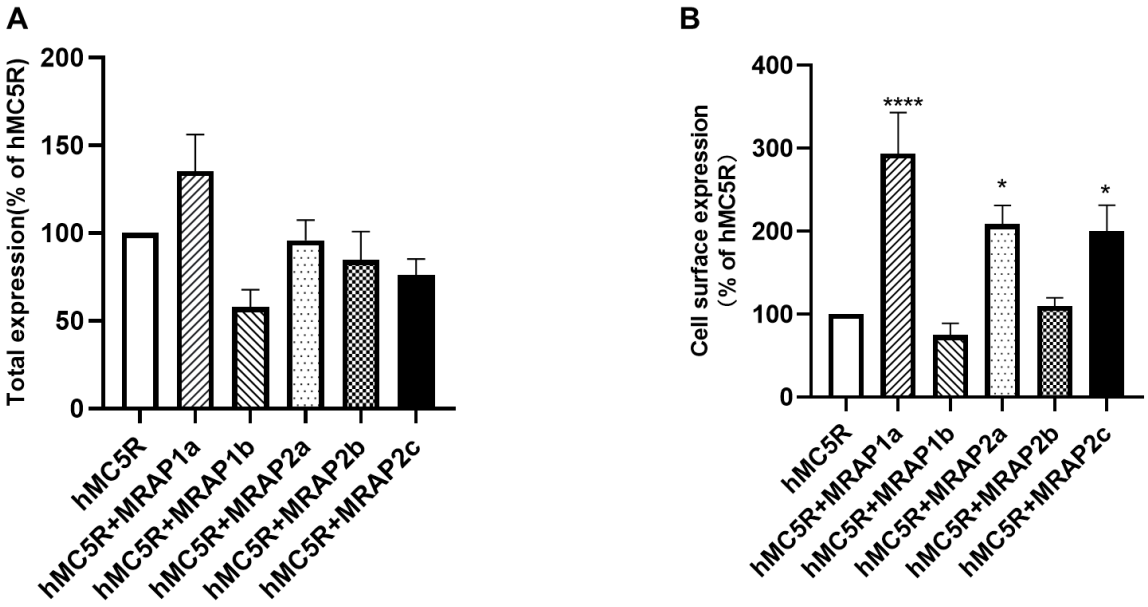


Figure 2.1 Regulation of hMC5R expression by MRAPs. The total (A) and cell surface (B) expression of hMC5R were measured by flow cytometry. HEK293T cells were co-transfected with hMC5R and hMRAPs (1:5). The empty vector pcDNA3.1 fluorescence was used for background staining. The results are calculated as % of 1:0 group. Each data point represented as the mean ± SEM (n = 4). Different letters indicate significant difference ($P < 0.05$) (One-way ANOVA followed by Dunnett's test). * Significant difference from the parameter of hMC5R, $P < 0.05$.

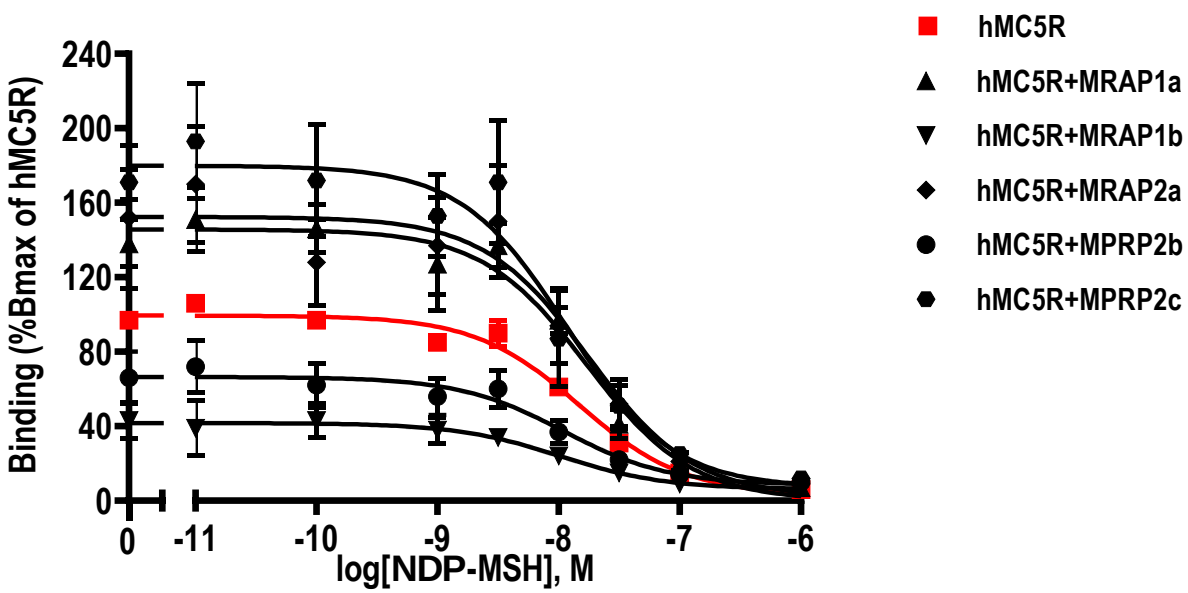


Figure 2.2 Effects of MRAPs on ligand binding properties of hMC5R to NDP-MSH. HEK293T cells were transiently transfected with hMC5R with or without hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b or hMRAP2c plasmids (1:5), and the binding properties were measured 48 h later by displacing the binding of ^{125}I-NDP-MSH using different concentrations of unlabeled NDP-MSH. Data are expressed as % of hMC5R binding ± range from duplicate measurements within one experiment. The curves are representative of at least four independent experiments.

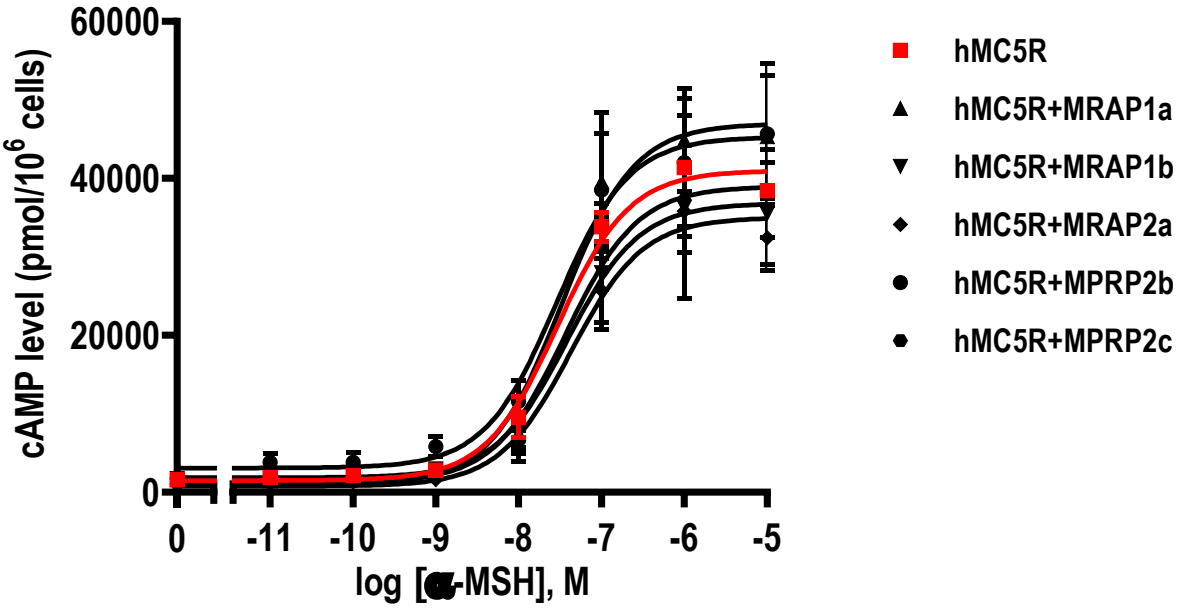
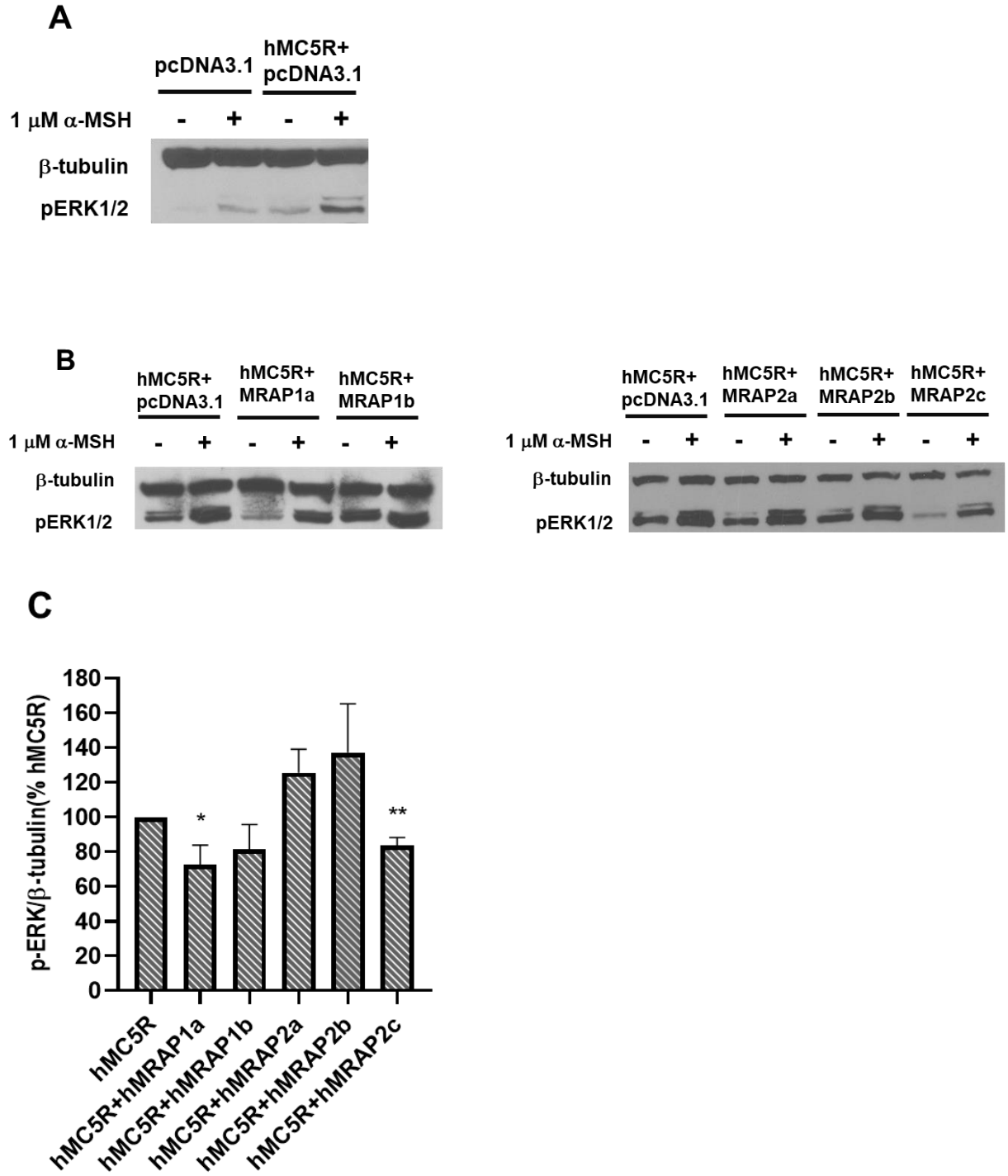
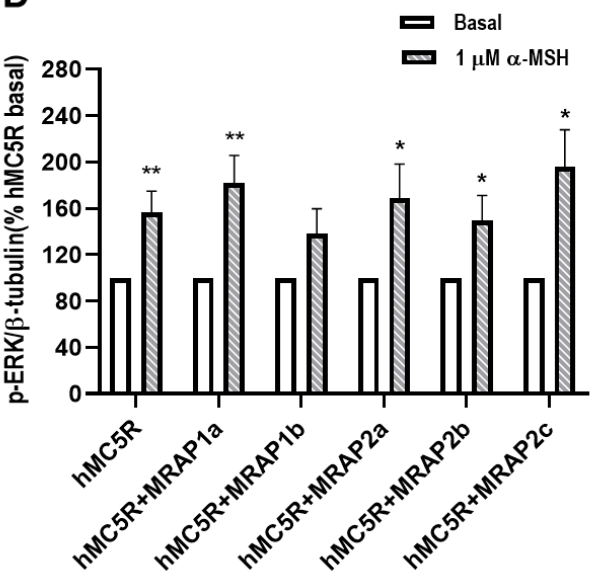


Figure 2.3 Effects of MRAPs on α-MSH-stimulated cAMP signaling properties of hMC5R. HEK293T cells were transiently transfected with hMC5R with or without hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b or hMRAP2c plasmids (1:5). Intracellular cAMP levels were measured by RIA after stimulation with different concentrations of α-MSH. The curves are representative of at least four independent experiments. All experiments were performed at least three times independently.


A
pcDNA3.1
hMC5R+ pcDNA3.1
1 μM α-MSH
- + - +
β-tubulin
pERK1/2
B
hMC5R+ pcDNA3.1
hMC5R+ MRAP1a
hMC5R+ MRAP1b
1 μM α-MSH
- + - + - +
β-tubulin
pERK1/2
hMC5R+ pcDNA3.1
hMC5R+ MRAP2a
hMC5R+ MRAP2b
hMC5R+ MRAP2c
1 μM α-MSH
- + - + - + - +
β-tubulin
pERK1/2
C
p-ERK/β-tubulin(% hMC5R)
180
160
140
120
100
80
60
40
20
0
*
**
hMC5R
hMC5R+hMRAP1a
hMC5R+hMRAP1b
hMC5R+hMRAP2a
hMC5R+hMRAP2b
hMC5R+hMRAP2c

D


Basal
1 μM α-MSH
p-ERK/β-tubulin(% hMC5R basal)
280
240
200
160
120
80
40
0
**
**
*
*
*
hMC5R
hMC5R+MRAP1a
hMC5R+MRAP1b
hMC5R+MRAP2a
hMC5R+MRAP2b
hMC5R+MRAP2c

E

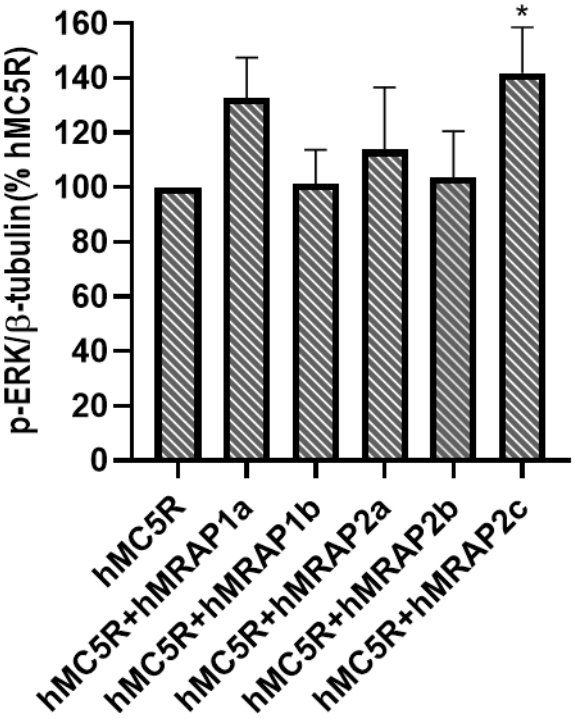

p-ERK/β-tubulin(% hMC5R)
160
140
120
100
80
60
40
20
0
*
hMC5R
hMC5R+hMRAP1a
hMC5R+hMRAP1b
hMC5R+hMRAP2a
hMC5R+hMRAP2b
hMC5R+hMRAP2c

Figure 2.4 Effects of MRAPs on MC5R-mediated ERK1/2 activation. ERK1/2 activation experiments were performed as described in Materials and methods, 24 hours later cells were starved overnight and stimulated with/without 1 μM α-MSH for 5min. HEK293T cells were transiently transfected with emptor vector or hMC5R (A), or co-transfected hMC5R with or without hMRAP1a, hMRAP1b, hMRAP2a, hMRAP2b or hMRAP2c plasmids (1:5) (B). (C) Basal pERK1/2 levels, results are expressed as percentage of basal pERK1/2 activity of cells transfected with hMC5R alone. (D) ERK1/2 phosphorylation levels upon stimulation with 1 μM α-MSH. Results are expressed as percentage of the value obtained in non-stimulated cells of each co-transfected group and represent the mean ± SEM of three independent experiments. (E) α-MSH-induced ERK1/2 activation. Results are expressed as percentage of α-MSH-stimulated ERK1/2 phosphorylation level in cells transfected hMC5R alone and represent the mean ± SEM of three independent experiments. The statistical significance is indicated as follows: *: significantly different from pERK activity, $p < 0.05$; **: significantly different from pERK activity, $p < 0.001$.

Chapter 3: Pharmacological effect of human melanocortin-2 receptor accessory protein 2 variants on human melanocortin-4 and -5 receptor

3.1. Introduction

The melanocortin receptor (MCR) family consists of five G protein-coupled receptors (GPCRs) designated MC1R through MC5R. These receptors are activated by melanocortin peptides, including α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH), which are derived from the precursor protein proopiomelanocortin (POMC) (Dores et al., 2014). Each receptor has specific ligand affinities and signals through G proteins, primarily activating adenylate cyclase to increase cyclic AMP (cAMP) levels, which activates protein kinase A (PKA) and other downstream effectors (Cabrera-Vera et al., 2003; Yang and Harmon, 2017). Different MCRs have distinct tissue distributions and physiological roles. For example, centrally expressed MC3R and MC4R play critical roles in energy homeostasis (Tao, 2005). MC5R, widely expressed in both central and peripheral tissues, participates in stress response (Liao et al., 2019), exocrine gland function (Chen et al., 1997) and immune response (Rodrigues et al., 2009), etc. Additionally, MC5R primarily regulates energy metabolism via adipocyte lipolysis and re-esterification, fatty acid oxidation, and glucose uptake (Chagnon et al., 1997; Cho et al., 2005; Møller et al., 2015; Valli-Jaakola et al., 2008).

Melanocortin-2 receptor accessory proteins (MRAPs) are small proteins with a hydrophobic transmembrane domain, which can regulate receptor trafficking, ligand binding and signaling transduction of MCRs (Chan et al., 2009). There are two members in the human MRAP family: MRAP1 and MRAP2. MRAP1 is predominantly expressed in adrenal glands and is essential for the trafficking of MC2R from endoplasmic reticulum to cell surface, binding of ACTH to MC2R and the subsequent activation of the receptor (Roy et al., 2007). MRAP2, identified through whole human genome sequence analysis, shares approximately 40% sequence homology with MRAP1 (Berruien and Smith, 2020). Although both MRAP1 and MRAP2 regulate the activity of all MCRs, MRAP2 can either enhance or inhibit signaling of these receptors depending on the context, thereby regulating more general physiological processes, particularly energy balance and body weight by affecting signaling at MC4R (Chan et al., 2009; Kay et al., 2013; Liang et al., 2018; Soletto et al., 2019).

Obesity is a complex and multifactorial disease characterized by excessive accumulation of body fat, posing a significant risk to health (Marti et al., 2004). It is associated with multiple comorbidities, including cardiovascular disease, type 2 diabetes, and certain cancers. Energy imbalance, adipose tissue dysfunction, and central regulation of appetite and energy expenditure are the main causes of obesity (Haslam and James, 2005). Genetic mutations in MC3R and MC4R can lead to obesity and associated metabolic disorders (Hani et al., 2001; Li et al., 2000; Pritchard et al., 2002). Although MC5R has been relatively poorly studied, it has been shown to play a key role in regulating energy

homeostasis. Single nucleotide polymorphisms of MC5R have been found to be associated with type 2 diabetes and obesity in Finnish people (Valli-Jaakola et al., 2008). In addition, recent studies have found that MRAP2 is also involved in regulating energy homeostasis, particularly through modulation of MC4R activity. Reduced MC4R activity caused by the loss of function of MRAP2 contributes to obesity and metabolic disorders (Baron et al., 2019; Liang et al., 2018). Interestingly, some MRAP2 genetic variants associated with severe obesity exhibit a slightly different phenotype compared to MC4R genetic variants. The different functional domains in MRAP2 suggest that these variants could influence the interactions between MRAP2 and MC4R (Berruien and Smith, 2020). Therefore, understanding the mechanisms and effects of these variants provides valuable insights into disease pathogenesis and offers potential pathways for therapeutic intervention.

Large-scale sequencing revealed 23 rare heterozygous MRAP2a variants that are associated with obesity in adults and children. Functional evaluation of each variant revealed that loss-of-function MRAP2a variants are pathogenic for monogenic hyperphagic obesity and hyperglycemia (Baron et al., 2019). The pleiotropic metabolic effects of MRAP2a mutations may result from the failure of different MRAP2a-regulated GPCRs in various tissues, including pancreatic islets. Given the role of MC4R and MC5R in energy regulation, as well as the widespread distribution and physiological regulatory functions of MC5R, we selected 5 hMRAP2a mutants (G31V, F62C, N77S, K102* and P195L) and co-transfected them with hMC4R or hMC5R into HEK293T cells to identify pharmacological effects of

these MRAP2a variants on hMC4R and hMC5R. This may provide new insights into therapeutic strategies for treating obesity and related metabolic diseases by targeting MRAP2 or its interaction with melanocortin receptors.

3.2. Materials and Methods

3.2.1. Reagents and supplies

The ligands used included NDP-MSH, obtained from Peptides International (now Vivitide, Louisville, KY, USA), and α-MSH, purchased from Pi Proteomics (Huntsville, AL, USA). [^{125}I]-NDP-MSH and [^{125}I]-cAMP were iodinated using chloramine T method (Steiner et al., 1969). N-terminal c-myc-tagged hMC4R, hMC5R and N-terminal Flag-tagged hMRAP2a, hMRAP2a mutants (G31V, F62C, N77S, k102*, P195L) were subcloned into pcDNA3.1(-) vector and commercially synthesized by Synbio Technologies (Monmouth Junction, NJ, USA) to generate plasmids for cell transfection. Paraformaldehyde 16% w/v solution (EMS, Hatfield, PA, USA) was used to prepare 4% PAF for flow cytometry. Flowmi™ Cell Strainers (SP BEL-ART, Wayne, NJ, USA) were used for filtering small volume samples prior to flow analysis.

3.2.2. Cell culture and DNA transfection

HEK293T cells were obtained from American Type Culture Collection (ACTT,

Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 2% fetal bovine serum (ATCC, Manassas, VA, USA) and 12.5% newborn calf serum (R&D Systems, Flowery Branch, GA, USA), 10mM HEPES, 0.25 μg/mL amphotericin B, 50 μg/mL gentamicin, 100 IU/mL penicillin, and 100 μg/mL of streptomycin. The cells were incubated at 37 °C in a 5% CO_2 atmosphere (Tao and Segaloff, 2003). For experiments, cells were plated into gelatin-coated 6-well plates. When the cells reached 50-70% confluency, they were co-transfected with 0.25 μg/μL hMC4R or hMC5R with or without hMRAP2a and its mutants (G31V, F62C, N77S, k102*, P195L) at a ratio of 1:5 using calcium phosphate precipitation method (Chen and Okayama, 1987). Total DNA was normalized using empty vector pcDNA3.1.

3.2.3. Flow cytometry assay

Flow cytometry is a high-throughput single-cell analytical method that has evolved from identifying cell surface proteins to characterizing intracellular proteins. To investigate the effect of hMRAP2a mutants on total (including cell surface and intracellular proteins) and cell surface expression of hMC4R or hMC5R, flow cytometry (Accuri Cytometers, Ann Arbor, MI, USA) was used as described previously (Yang and Tao, 2012). HEK293T cells were co-transfected with hMC4R or hMC5R (N-terminal c-myc tag) with or without hMRAP2a and its mutants (G31V, F62C, N77S, k102*, P195L) at a ratio of 1:5. The C6 Accuri Cytometer (Accuri Cytometers, Ann Arbor, MI, USA) was used to collect

fluorescence signals. Fluorescence from cells transfected with empty vector (pcDNA3.1) was used for correction of background staining. The expression of hMC4R or hMC5R was calculated as the percentage of cell transfected with hMC4R or hMC5R alone set as 100%.

3.2.4. Radioligand ligand Binding assays

To investigate the regulation of hMRAP2a mutants on binding properties of hMC4R or hMC5R, competitive binding assay was used as previously described. HEK293T cells were co-transfected with hMC4R or hMC5R (N-terminal c-myc tag) with or without hMRAP2a and its mutants (G31V, F62C, N77S, k102*, P195L) at a ratio of 1:5. Two ligands, α-MSH (from 10^{-10} to 10^{-5} M) were used for hMC4R and NDP-MSH (from 10^{-10} to 10^{-5} M) were used for hMC5R in this study. Radioactive [^{125}I]-NDP-MSH (~100,000 cpm) was used as the competitor. The concentration of ligand that results in 50% displacement of radiolabeled ligand (IC_{50}, an indicator of binding affinity) and maximal binding capacity (B_{max}) were calculated.

3.2.5. Ligand-stimulated cAMP assays

To investigate the regulation of hMRAP2a mutants on cAMP signaling properties of hMC5R, radioimmunoassay (RIA) was used to measure cAMP production as described previously (Yang et al., 2019). HEK293T cells were co-transfected with hMC4R or hMC5R

(N-terminal c-myc tag) with or without hMRAP2a and its mutants (G31V, F62C, N77S, k102*, P195L) at a ratio of 1:5. Ligand, α-MSH (from 10^{-11} to 10^{-5} M) were used for hMC4R and α-MSH (from 10^{-10} to 10^{-4} M) were used for hMC5R in this study. The maximal response (R_{max}) and concentration of agonist that results in 50% maximal response (EC_{50}, an indicator of agonist potency) were calculated.

3.2.6. Statistical analysis

All data were represented as mean ± S.E.M. The parameters and significance of differences were calculated GraphPad Prism 8.3 software (GraphPad, San Diego, CA, USA). The significance of differences in flow cytometry, ligand binding and cAMP signaling parameters were determined by one-way ANOVA, followed by Dunnett's post hoc test, with $p < 0.05$ set as the threshold for significant.

3.3. Results

3.3.1. Regulation of hMC4R expression by MRAP2a mutants

Flow cytometry was used to detect the regulatory effects of hMRAP2a mutants (G31V, F62C, N77S, K102* and P195L) on total and cell surface expression of hMC4R or hMC5R (**Fig. 3.1**). The results showed that hMRAP2a and all hMRAP2a mutants had no effect on

total expression of hMC4R (**Fig. 3.1A**). However, F62C and P195L significantly reduced cell surface expression of hMC4R by 67.57% and 64.05%, respectively, compared to cells transfected only with hMC4R ($p < 0.05$) (**Fig. 3.1B**).

3.3.2. Ligand binding and α-MSH-stimulated cAMP signaling properties of hMC4R regulated by MRAP2a mutants

For competitive binding assay, different concentrations of unlabeled α-MSH were used to compete with a fixed amount of labeled ^{125}I-NDP-MSH. Results showed that in control group (co-transfected hMC4R and empty vector), unlabeled α-MSH replaced radiolabeled NDP-MSH in binding to hMC4R with an IC_{50} of 83.10 nM (**Fig. 3.2 and Table 3.1**). All experimental group (co-transfected hMC4R with hMRAP2a/hMRAP2a mutants) had similar binding affinities (IC_{50s}) to α-MSH and B_{max} of hMC4R compared to the control group, indicating that hMRAP2a mutants have no effect on the binding of hMC4R and α-MSH (**Fig. 3.2 and Table 1**).

The hMC4R exhibits basal (constitutive) activity, meaning it can signal in the absence of an external ligand. We found that hMRAP2a, hMRAP2a mutants G31V, N77S, and K102* significantly decreased basal activity of hMC4R by 55.47%, 62.96%, 62.67% and 43.80%, respectively ($p < 0.05$) (**Fig. 3.3 and Table 2**). MC4R displays the highest binding affinity to α-MSH, which then stimulates intracellular signaling pathways. cAMP assay results

showed that both hMRAP2a and hMRAP2a mutants (G31V, F62C, N77S, k102*, P195L) significantly decreased R_{max} values of hMC4R in response to α-MSH, while having no effect on EC50 values (potencies) of α-MSH for hMC4R (**Fig. 3.3 and Table 2**).

3.3.3. Regulation of hMC5R expression by MRAP2a mutants

The hMRAP2a mutant K102* significantly reduced the total expression of hMC5R by 60.48% compared to cells transfected with hMC5R alone, while the other mutants had no effect on the total expression of hMC5R (**Fig. 3.4A**). Compared to cells transfected with hMC5R alone, the hMRAP2a mutants F62C, K102* and P195L significantly reduced the cell surface expression of hMC5R by 45.36%, 55.43%, and 61.20%, respectively, ($p < 0.05$) (**Fig. 3.4B**). However, N77S mutant significantly increased cell surface expression of hMC5R by 50.40 % ($p < 0.05$) (**Fig. 3.4B**).

3.3.4. Ligand binding and α-MSH-stimulated cAMP signaling properties of hMC5R regulated by MRAP2a mutants

NDP-MSH is a synthetic analog of the natural melanocortin peptide α-MSH. They have similar receptor binding profiles, but NDP-MSH typically has a higher binding affinity. Due to its enhanced properties, NDP-MSH is frequently used in pharmacological studies. For competitive binding assay of hMC5R, we used different concentrations of unlabeled NDP-

MSH to compete with a fixed amount of labeled ^{125}I-NDP-MSH. Results showed that in control group (co-transfected hMC5R and empty vector), unlabeled NDP-MSH replaced radiolabeled one in binding to hMC5R with an IC_{50} of 42.71 nM (**Fig. 3.5 and Table 3**). No significant effect was observed for hMRAP2a and hMRAP2a mutants on NDP-MSH affinities (IC_{50s}) at hMC5R. However, only P195L mutant significantly decreased the B_{max} of hMC5R, reduced it by 66.20% ($p < 0.05$) (**Fig. 3.5 and Table 3**).

There is currently a lack of understanding regarding the basal activity of hMC5R. Our original data showed that cAMP production of hMC5R without external ligand binding was higher than the basal activity of hMC4R (data not shown here). This suggests the possibility of basal activity in hMC5R. In addition, we also found that both hMRAP2a and hMRAP2a mutants significantly decreased the basal activities of hMC5R (**Fig. 3.6 and Table 4**).

For α-MSH-stimulated cAMP signaling, both hMRAP2a and hMRAP2a mutants had no effects on α-MSH potencies (EC_{50s}) of hMC5R (**Fig. 3.6 and Table 4**). However, F62C and P195 mutant significantly decreased the R_{max} of hMC5R in response to α-MSH, reduced it by 39.66 % and 46.42%, respectively ($p < 0.05$) (**Fig. 3.6 and Table 4**).

3.4. Discussion

MC3R, MC4R, and MC5R are key members of MCR family and play important roles

in regulating energy homeostasis, appetite, and metabolism. Dysregulation or mutations of these receptors are associated with obesity and related metabolic disorders (Girardet and Butler, 2014; Ji et al., 2022; Lee et al., 2002). Recent studies on the function of MRAP2 have found that it directly interacts with MC4R and enhances the production of second messenger cAMP mediated by MC4R (Asai et al., 2013; Bruschetta et al., 2018). Additionally, MRAP2 has been implicated in energy control in rodents, particularly through its interaction with MC4R (Baron et al., 2019). However, MRAP2 knockout (KO) mice exhibit different metabolic phenotypes compared to mice lacking MC4R (Asai et al., 2013). These differences include variations in bone mass, bone density, eating behavior, and fat-to-lean mass ratio. Baron et al. sequenced the exonic region of MRAP2 in a cohort of 9,418 subjects and found 23 rare heterozygous variants that independently increased the risk of obesity (Baron et al., 2019). Based on this, we first identified the regulation of hMC4R by five hMRAP2a mutants (G31V, F62C, N77S, K102* and P195L) that may be associated with obesity.

F62C and P195L significantly reduced the cell surface expression of hMC4R while having no effect on its total expression. Therefore, two mutants have little effect on the synthesis of hMC4R but prevent its normal trafficking to the cell surface, thereby retaining most of it inside the cell. Meanwhile, F62C and P195L did not affect ligand binding but reduced the maximal response of hMC4R to its ligand, altering the activation of the receptor by endogenous agonist α-MSH. In the first study to describe inactivating mutations in MC4R associated with childhood obesity, mutations that caused the receptor to become trapped

inside the cell were identified as the most common mechanism (Lubrano-Berthelier et al., 2003b; Tao and Segaloff, 2003; Yeo et al., 2003). In contrast, G31V, N77S, and K102* significantly reduced the maximal response of hMC4R to α-MSH but did not affect its expression and binding. This is like Class IV mutants of certain GPCRs that are produced and trafficked to cell surface, bind their ligands normally, but are defective in signaling. Defects in receptor activation can be caused by the receptor's inability to undergo conformational changes to produce an active state, its inability to couple to G proteins, or its failure to activate G proteins (catalyzing the exchange of GDP for GTP) even when coupled. Additionally, defects in receptor regulation can also render the receptor inactive (Tao, 2006). For example, R147H mutation in human vasopressin type 2 receptor (V2R) exhibits unchanged binding affinity but fails to stimulate the $G\alpha_s$/adenylate cyclase system (Rosenthal et al., 1993). In rhodopsin, spectrally normal autosomal dominant retinitis pigmentosa (ADRP) mutants (R135L and R135W) failed to activate guanine nucleotide exchange by 75ransducing, resulting in defects in the signal transduction pathway (Min et al., 1993).

Previous study has found that, compared to wild-type (WT) MRAP2a, the five MRAP2a variants G31V, F62C, N77S, K102*, and P195L significantly reduced MC4R downstream cAMP-PKA signaling in response to α-MSH (Baron et al., 2019). Our experimental results showed that although the MRAP2a mutants caused the loss of hMC4R signaling function, there was no significant difference compared to WT MRAP2a. However, there are few studies in this area, and it is not yet known whether this difference is caused by

variations in cell type, transfection method, or signal measurement technology.

Given the widespread distribution of MC5R, its role in metabolic regulation, and its potential association with obesity, we also explored the pharmacological effects of these hMRAP2a mutants on hMC5R. The study found that hMRAP2a mutants K102* significantly decreased both total and cell surface expression of hMC5R, indicating defective receptor biosynthesis. It may be caused by synthesis/processing defects and/or accelerated degradation. However, no reduction in ligand binding or impairment of α-MSH-stimulated cAMP production was found. F62C and P195L affected normal trafficking of hMC5R to cell surface, similar to their effect on hMC4R. Additionally, F62C only reduced the maximal response of hMC5R to α-MSH and had no effect on the binding properties, while P195L impaired both binding and signal transduction of hMC5R. The observed decreases in ligand binding or signaling of hMC5R by F62C and P195 mutants are associated with decreased cell surface expression due to intracellular retention of receptor. Unlike hMC4R, G31V and N77S had no effect on the binding and signaling of hMC5R. This can provide insights into the diverse regulatory mechanisms within the melanocortin receptor family and their implications for obesity and metabolic diseases.

Basal (constitutive) activity is a characteristic of many GPCRs, including MCRs, which refers to their ability to signal in the absence of an external ligand (Arvanitakis et al., 1998; Srinivasan et al., 2004). This activity can be modulated by various factors, including

mutations, accessory proteins (such as MRAPs), and other interacting molecules (Tao, 2014; Wang et al., 2019). For example, naturally occurring and laboratory-generated mutations that impaired basal activity of hMC4R can lead to metabolic disorders, including obesity (Lubrano-Berthelier et al., 2003a). The Mrap2- and Agrp-suppressed basal activity of Mc4r plays an important role in promoting the growth of zebrafish and culter (Sebag et al., 2013; Tao et al., 2020). In our study, MRAP2a and its mutants G31V, N77S, and K102* significantly reduced the basal cAMP signaling of both hMC4R and hMC5R. F62C and P195L only decreased the basal cAMP signaling of hMC5R. The reduction of MC4R basal activity by MRAP2 has also been reported in other species, indicating that MC4R might be involved in energy balance regulation by interacting with MRAP2. However, further studies are needed to investigate the basal cAMP signaling of hMC5R and its interaction with MRAP2.

3.5. Conclusion

F62C and P195L impaired the trafficking of both hMC4R and hMC5R, reducing ligand binding or α-MSH-induced signaling. When cells were co-transfected with hMC4R and G31V, N77S, and K102*, hMC4R was normally produced and trafficked to the cell surface and could bind to ligands normally, but signaling was defective. However, these three mutants had no effect on hMC5R binding and signaling. N77S significantly increased the cell surface expression of hMC5R, while K102* significantly reduced its cell surface

expression. Additionally, there is basal cAMP signaling in hMC5R compared to hMC4R. Except for F62C and P195L, which only reduce the constitutive activity of hMC5R, the other three mutations reduce the constitutive activity of both hMC4R and hMC5R. Our results suggest that different MRAP2a mutants have diverse regulatory effects on different MCRs, which may provide insights into the varied regulatory mechanisms within MCRs and their impact on obesity and metabolic diseases.

Table 3.1 Effects of hMRAP2a mutants on ligand binding and α-MSH-stimulated cAMP signaling properties of hMC4R

	Binding		α-MSH stimulated cAMP		
	IC_{50} (nM)	B_{max} (% hMC4R)	EC_{50} (nM)	R_{max} (% hMC4R)	Basal (%)
hMC4R	**83.10 ± 11.27**	**100**	**3.99± 1.08**	**100**	**100**
hMC4R+WT MRAP2a	**169.20 ± 10.76**	**123.80 ± 8.83**	**2.72 ± 0.90**	**66.43 ± 1.22****	**44.53 ± 7.70****
hMC4R+G31V	**136.80 ± 28.98**	**140.90 ± 13.34**	**2.54 ± 0.75**	**59.03 ± 7.19*****	**37.04 ± 4.72****
hMC4R+F62C	**84.79 ± 27.85**	**127.50 ± 17.52**	**1.14 ± 0.48**	**55.15 ± 6.85*****	**73.80 ± 12.50**
hMC4R+N77S	**187.30 ± 23.54**	**90.37 ± 7.81**	**2.91 ± 1.06**	**57.98 ± 7.20*****	**37.33 ± 8.05****
hMC4R+K102*	**194.90 ± 68.00**	**107.40 ± 19.73**	**2.23 ± 0.63**	**69.29 ± 6.39***	**56.20 ± 10.72***
hMC4R+P195L	**134.20 ± 18.96**	**91.72 ± 19.41**	**2.27 ± 0.94**	**60.52 ± 8.79****	**70.40 ± 18.66**

Results are presented as the mean ± SEM (n = 3-4).

* Significant difference from the parameter of hMC4R, $P < 0.05$.

** Significant difference from the parameter of hMC4R, $P < 0.005$.

***Significant difference from the parameter of hMC4R, $P < 0.001$.

Table 3.2 Effects of hMRAP2a mutants on ligand binding and α-MSH-stimulated cAMP signaling properties of hMC5R

	Binding		α-MSH stimulated cAMP		
	IC_{50} (nM)	B_{max} (% hMC5R)	EC_{50} (nM)	R_{max} (% hMC5R)	Basal (%)
hMC5R	**42.71 ± 5.39**	**100**	**217.30 ± 59.66**	**100**	**100**
hMC5R+WT MRAP2a	**42.10 ± 11.25**	**61.74 ± 5.05**	**250.20 ± 69.78**	**67.80 ± 7.91**	**30.12 ± 6.02******
hMC5R+G31V	**40.06 ± 12.55**	**84.71 ± 10.49**	**291.20 ± 52.90**	**103.70 ± 10.26**	**53.98 ± 6.81*****
hMC5R+F62C	**40.63 ± 10.99**	**71.61 ± 12.53**	**219.00 ± 40.80**	**60.34 ± 11.26***	**27.40 ± 4.74******
hMC5R+N77S	**50.21 ± 9.52**	**100.60 ± 12.60**	**283.10 ± 59.84**	**93.95 ± 11.79**	**55.05 ± 8.23*****
hMC5R+K102*	**39.01 ± 8.08**	**120.20 ± 12.84**	**149.90 ± 15.73**	**88.74 ± 10.38**	**54.55 ± 10.28*****
hMC5R+P195L	**30.16 ± 6.19**	**43.80 ± 7.40****	**238.30 ± 58.10**	**53.58 ± 6.97****	**26.24 ± 5.76******

Results are presented as the mean ± SEM (n = 3-4).

***Significant difference from the parameter of hMC4R, $P < 0.001$.

****Significant difference from the parameter of hMC4R, $P < 0.0001$.

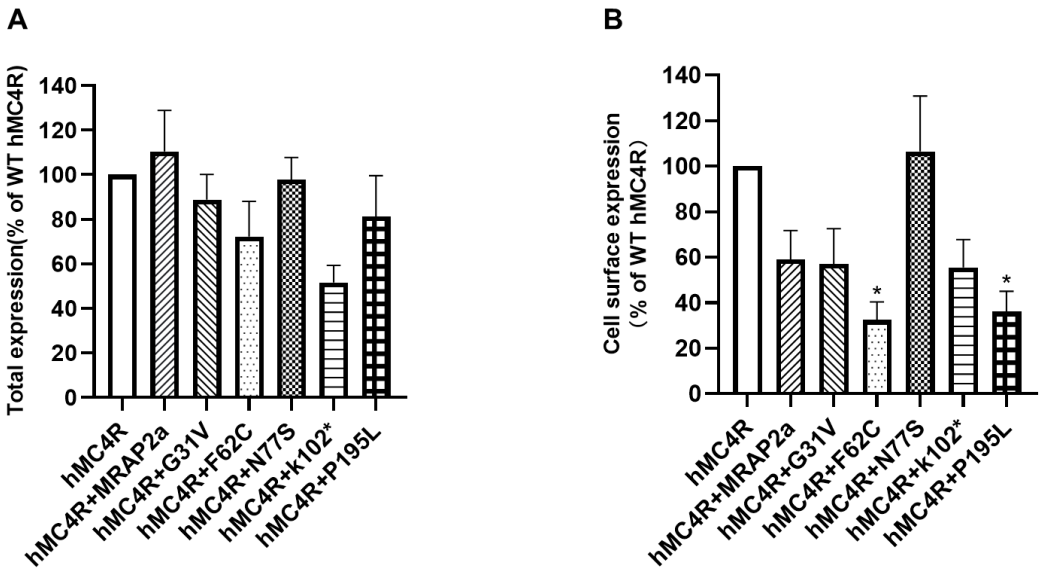


Figure 3.1 Regulation of hMC4R expression by hMRAP2a mutants. The total (A) and cell surface (B) expression of hMC4R were measured by flow cytometry. HEK293T cells were co-transfected with hMC4R and hMRAP2a mutants (1:5). The empty vector pcDNA3.1 fluorescence was used for background staining. The results are calculated as % of 1:0 group. Each data point represented as the mean ± SEM (n = 4). Different letters indicate significant difference ($P < 0.05$) (One-way ANOVA followed by Dunnett's test). * Significant difference from the parameter of hMC4R, $P < 0.05$.

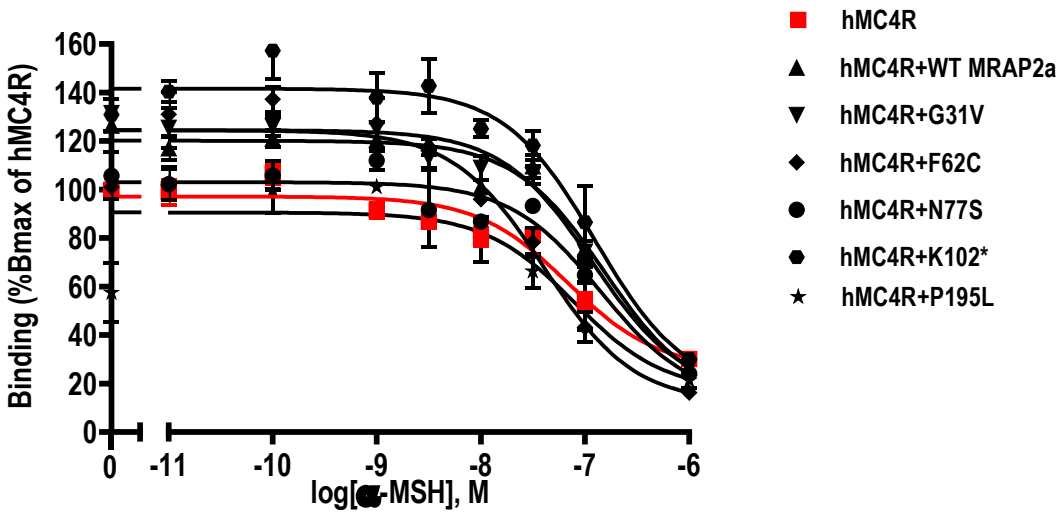


Figure 3.2 Ligand-binding properties of hMC4R regulated by hMRAP2a mutants to α-MSH. HEK293T cells were transiently transfected with hMC4R with or without hMRAP2a, hMRAP2a mutants (1:5). Binding properties were measured 48 h later by displacing the binding of ^{125}I-NDP-MSH using different concentrations of unlabeled α-MSH. Data are expressed as % of hMC4R binding ± range from duplicate measurements within one experiment. The curves are representative of at least four independent experiments.

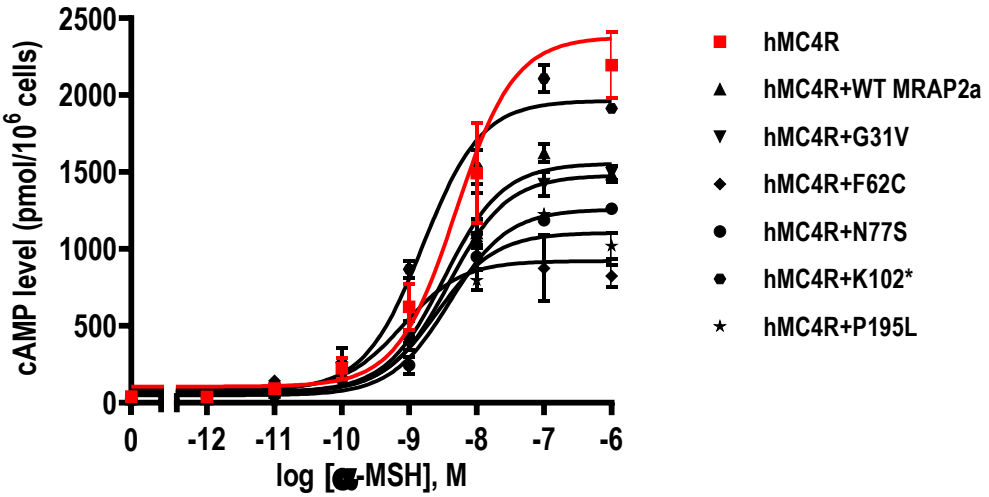


Figure 3.3 Effects of hMRAP2a mutants on α-MSH-stimulated cAMP signaling properties of hMC4R. HEK293T cells were transiently transfected with hMC4R with or without hMRAP2a, hMRAP2a mutants (1:5). Intracellular cAMP levels were measured by RIA after stimulation with different concentrations of α-MSH. The curves are representative of at least four independent experiments. All experiments were performed at least three times independently.

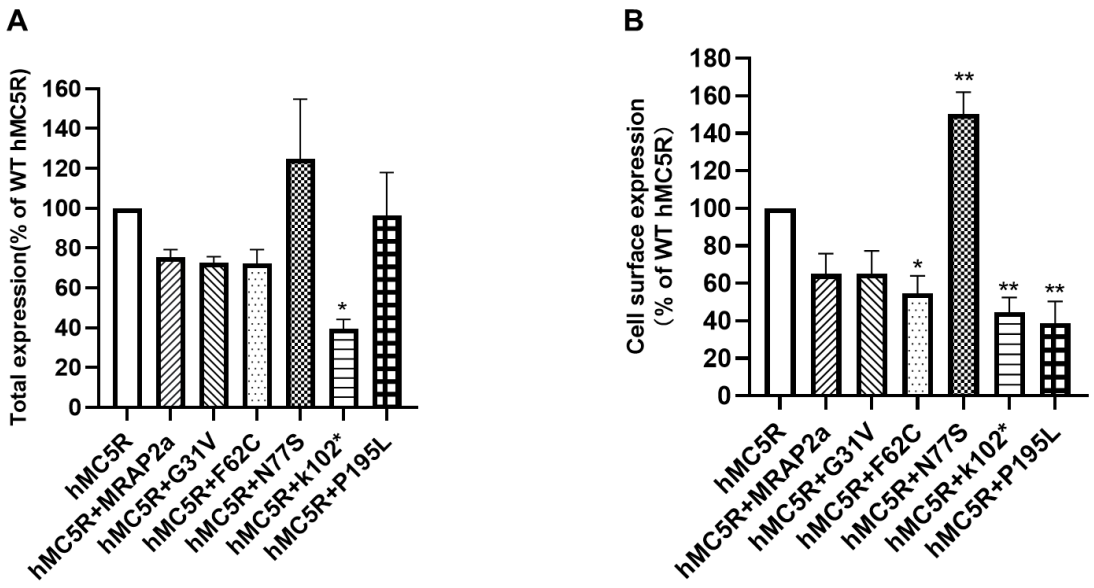


Figure 3.4 Regulation of hMC5R expression by hMRAP2a mutants. The total (A) and cell surface (B) expression of hMC5R were measured by flow cytometry. HEK293T cells were co-transfected with hMC5R and hMRAP2a mutants (1:5). The empty vector pcDNA3.1 fluorescence was used for background staining. The results are calculated as % of 1:0 group. Each data point represented as the mean ± SEM (n = 4). Different letters indicate significant difference ($P < 0.05$) (One-way ANOVA followed by Dunnett's test). * Significant difference from the parameter of hMC4R, $P < 0.05$.

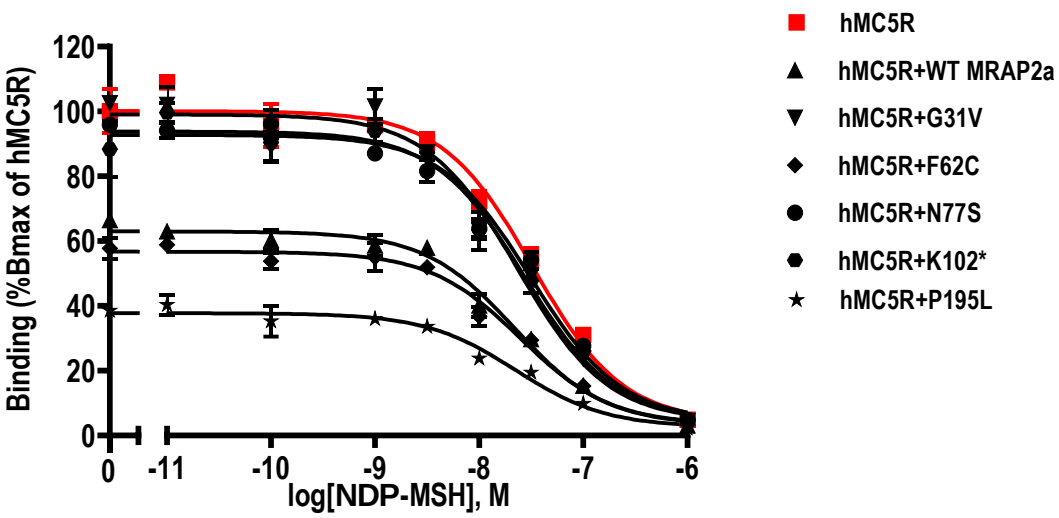


Figure 3.5 Ligand-binding properties of hMC5R regulated by hMRAP2a mutants to NDP-MSH. HEK293T cells were transiently transfected with hMC5R with or without hMRAP2a, hMRAP2a mutants (1:5). Binding properties were measured 48 h later by displacing the binding of ^{125}I-NDP-MSH using different concentrations of unlabeled NDP-MSH. Data are expressed as % of hMC5R binding ± range from duplicate measurements within one experiment. The curves are representative of at least four independent experiments.

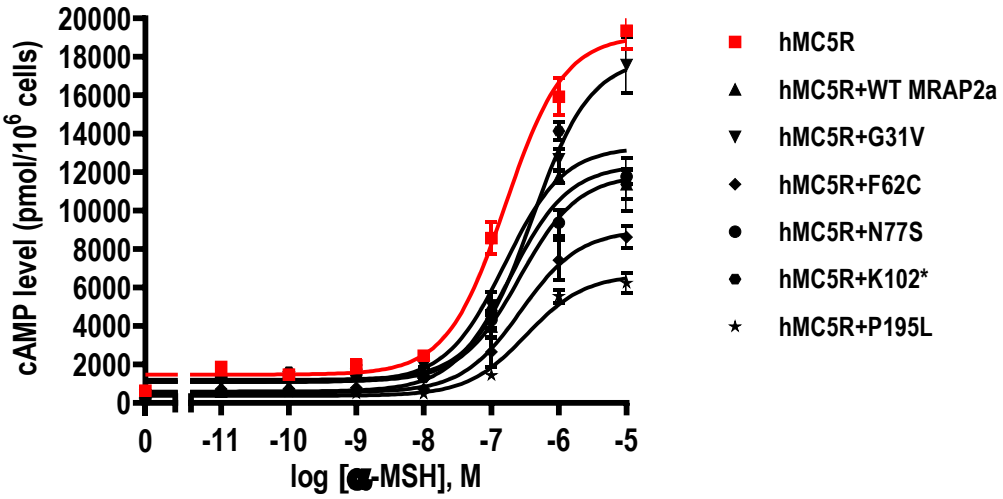


Figure 3.6 Effects of hMRAP2a mutants on α-MSH-stimulated cAMP signaling properties of hMC5R. HEK293T cells were transiently transfected with hMC5R with or without hMRAP2a, hMRAP2a mutants (1:5). Intracellular cAMP levels were measured by RIA after stimulation with different concentrations of α-MSH. The curves are representative of at least four independent experiments. All experiments were performed at least three times independently.

Chapter 4: Pharmacological analyses of twelve naturally occurring human melanocortin-5 receptor mutations

4.1. Introduction

G protein-coupled receptors (GPCRs) are the most numerous membrane proteins, regulating almost all known physiological processes by transducing diverse extracellular signals into intracellular space (Tao, 2020). Melanocortin receptors (MCRs) belong to rhodopsin-like family A GPCRs and signal primarily through coupling to G-proteins after agonist activation. This coupling stimulates the production of intracellular cyclic adenosine monophosphate (cAMP) via adenylyl cyclase, leading to protein kinase A (PKA) activation (Dores et al., 2014; Rodrigues et al., 2015). The well-known endogenous agonists for MCRs include α-melanocyte-stimulating hormone (MSH), β-MSH, γ-MSH, and adrenocorticotropin (ACTH). Five melanocortin receptor subtypes have been cloned, each with its own profile regarding the relative potency of different endogenous ligands (Brzoska et al., 2008; Schioth et al., 1995; Yang and Harmon, 2017). Additionally, each melanocortin receptor subtype has a distinct pattern of tissue expression and is involved in various physiological functions, including pigmentation, steroidogenesis, energy homeostasis, exocrine secretion, sexual function, and inflammation (Cone, 2006).

As the most recently cloned MCR, MC5R has been demonstrated to express in central

nervous system and a variety of peripheral tissues, including adrenal glands, pituitary glands, adipose tissue, liver, lung, skin, and skeletal muscle, contributing to its diverse functions (Ji et al., 2022; Xu et al., 2020). MC5R has the highest affinity for α-MSH but lowest for γ-MSH (Schioth et al., 1995). Once activated by α-MSH, MC5R couples to stimulatory G protein and mediates its physiological effects primarily through cAMP-dependent signaling pathway like other MCRs (Rodrigues et al., 2015). The study on glucose metabolism modulated by pituitary α-MSH have found that activation of MC5R-cAMP-PKA pathway increases muscle glucose uptake and thermogenesis (Enriori et al., 2016). The functional role of MC5R in promoting lipolysis and repressing re-esterification upon α-MSH stimulation has been observed in adipocytes (Rodrigues et al., 2013). Additionally, α-MSH, by activating MC5R, increases cAMP levels and inhibits leptin secretion in adipocytes, thereby indirectly regulating food intake and energy expenditure (Norman et al., 2003). An et al. also demonstrated that MC5R plays an important role in increasing fatty acid oxidation in skeletal muscles (An et al., 2007). Therefore, MC5R is a potential target for treating patients with obesity and diabetes mellitus (Ji et al., 2022).

Human genetic studies confirmed that MC4R is important for maintaining body weight and energy homeostasis (Girardet and Butler, 2014). Mutations in MC4R gene are the most common monogenic form of obesity (Farooqi et al., 2003; Hill and Faulkner, 2017), with about 170 distinct mutations identified in obese patients (Hinney et al., 2013). Extensive functional studies have been conducted on some of these mutant receptors and revealed that

most inactivating MC4R mutants are retained intracellularly (Lubrano-Berthelier et al., 2003b; Tao and Segaloff, 2003, 2005; Yeo et al., 2003), with only a few mutants being defective in ligand binding or G protein coupling/activation (Biebermann et al., 2003; Gu et al., 1999; Yeo et al., 2003). However, some mutant receptors do not have obvious defects, exhibiting normal cell surface expression, ligand binding, and cAMP production (Tao and Segaloff, 2003, 2005). MC5R mutations in Quebec families and Finns exhibit significant linkage or association with obesity phenotype (Chagnon et al., 1997; Valli-Jaakola et al., 2008). Numerous naturally occurring MC5R mutations have been identified by recent extensive genomic studies (Ji et al., 2022). However, whether these *MC5R* mutations, like obesity-associated *MC4R* mutations, lead to defective receptors and the exact molecular defects remain to be studied.

Sequence comparison of human MCRs shows high homologies, with a 60% identity between MC4R and MC5R (Catania et al., 2004). Additionally, two crucial motifs, D/ERY motif in TM3 and N/DPxxY motif in TM7, are highly conserved among MCRs (Tao and Segaloff, 2004). The D/ERY motif is critical for constraining the receptor in its inactive conformation (Huang and Tao, 2014; Yang and Tao, 2020), while N/DPxxY motif is important for receptor expression, ligand binding, signal transduction and internalization (Yang et al., 2015). Additionally, hundreds of naturally occurring *MC5R* mutations are registered in gnomAD database (https://gnomad.broadinstitute.org/). Based on that, in the present study, we generated twelve naturally occurring hMC5R mutants at highly conserved residues and investigated their pharmacological properties through expression, ligand

binding and α-MSH-stimulated cAMP production. This could shed new light on potential strategies for treating obesity in humans.

4.2. Materials and Methods

4.2.1. Reagents and supplies

The ligands used in this study included NDP-MSH, obtained from Peptides International (now Vivitide, Louisville, KY, USA), and α-MSH, purchased from Pi Proteomics (Huntsville, AL, USA). [^{125}I]-NDP-MSH and [^{125}I]-cAMP were iodinated using chloramine T method (Steiner et al., 1969). N-terminal c-myc-tagged wild-type (WT) hMC5R and hMC5R mutants (P70A, D119Y, Y141F, R158H, R158L, P253L, D291H, P292S, I294T, I294M, Y295C, and Y295H) (Fig. 1) were subcloned into pcDNA3.1(-) vector and commercially synthesized by Synbio Technologies (Monmouth Junction, NJ, USA) to generate plasmids for cell transfection. Paraformaldehyde 16% w/v solution (EMS, Hatfield, PA, USA) was used to prepare 4% PAF for flow cytometry. Flowmi™ Cell Strainers (SP BEL-ART, Wayne, NJ, USA) were used for filtering small volume samples prior to flow analysis.

4.2.2. Cell culture and DNA transfection

Human embryonic kidney (HEK) 293T cells from American Type Culture Collection

(ACTT, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 2% fetal bovine serum (ATCC, Manassas, VA, USA) and 12.5% newborn calf serum (R&D Systems, Flowery Branch, GA, USA), 10mM HEPES, 0.25 μg/mL amphotericin B, 50 μg/mL gentamicin, 100 IU/mL penicillin, and 100 μg/mL of streptomycin in a cell incubator (37 °C and 5% CO_2). For experiments, cells were plated into 6-well plates pre-coated with 0.1% gelatin. When cells reached 50-70% confluency, WT or mutant MC5Rs were transiently transfected into cells respectively using calcium phosphate precipitation method (Chen and Okayama, 1987). Forty-eight hours after transfection, cells were used to investigate cell surface and total expression, ligand binding and cAMP signaling properties.

4.2.3. Flow cytometry assay

Flow cytometry is a high-throughput single-cell analytical method that has evolved from identifying cell surface proteins to characterizing intracellular proteins. To investigate total (including cell surface and intracellular proteins) and cell surface expression of WT and mutant hMC5Rs, flow cytometry (Accuri Cytometers, Ann Arbor, MI, USA) was used as described previously (Wang and Tao, 2011; Zhang et al., 2019). The C6 Accuri Cytometer (Accuri Cytometers, Ann Arbor, MI, USA) was used to collect fluorescence signals. Fluorescence from cells transfected with empty vector (pcDNA3.1) was used for correction of background staining. The expression of each mutant was calculated as a percentage of WT

hMC5R expression using the formula: (mutant fluorescence - pcDNA3.1 fluorescence) / (WT fluorescence - pcDNA3.1 fluorescence) ×100%.

4.2.4. Ligand binding assays

A competitive binding assay was used to investigate binding properties of WT and mutant hMC5Rs as described previously (Tao and Segaloff, 2003). Forty-eight hours after transfection, cells were treated with different concentrations of unlabeled NDP-MSH (ranging from 10^{-10} to 10^{-5} M) and a fixed amount of radioactive [^{125}I]-NDP-MSH (~100,000 cpm) as the competitor. The concentration of ligand that results in 50% displacement of radiolabeled ligand (IC_{50}, an indicator of binding affinity) and maximal binding capacity (B_{max}) were calculated.

4.2.5. Ligand-stimulated cAMP assays

The intracellular cAMP levels were determined by radioimmunoassay (RIA) as described previously (Steiner et al., 1969). Forty-eight hours after co-transfection, cells were treated with different concentrations of α-MSH (ranging from 10^{-10} to 10^{-4} M). The maximal response (R_{max}) and concentration of agonist that results in 50% maximal response (EC_{50}, an indicator of agonist potency) were calculated.

4.2.6. Statistical Analysis

All data were shown as mean ± S.E.M. The parameters and significance of differences were calculated using GraphPad Prism 8.3 software (GraphPad, San Diego, CA, USA). The significance of differences in flow cytometry, ligand binding and cAMP signaling parameters were analyzed by one-way ANOVA followed by Dunnett's post hoc test, with $p < 0.05$ set as the threshold for significant.

4.3. Results

4.3.1. No mutant with misfolding defect was identified

Tao and Segaloff proposed a classification scheme (classes I to V) to categorize MC4R inactivating mutations based on the life cycle of GPCRs (Tao, 2006). This scheme mimics the classification proposed for low-density lipoprotein receptor (Hobbs et al., 1990) and cystic fibrosis transmembrane conductance regulator (CFTR) mutations (Welsh and Smith, 1993).

Mutations that belong to Class I include those that terminate the receptor prematurely, resulting in defective receptor biosynthesis. Mutants synthesized relatively normally but retained inside cell is the predominant defect in all GPCR inactivating mutations (class II).

Therefore, we performed flow cytometry to quantify the expression of WT and mutant hMC5Rs. Our results showed that all the mutants had similar total expression levels as WT hMC5R, except for Y295H, which had a statistically significant decrease in total expression level (**Fig. 4.2A**). In addition, I294T had significantly increased cell surface expression compared to the WT control, whereas the rest of mutants had similar cell surface expression levels as WT hMC5R (**Fig. 4.2B**).

4.3.2. Five mutants had defects in binding and consequent signaling (class III)

Mutant receptors belonging to class III are produced and transported onto cell surface but are defective in ligand binding (Tao, 2006). NDP-MSH was used to investigate whether MC5R mutations cause alterations in the ligand binding properties of receptor. Competitive binding assay results showed that D119Y, P253L, P292S, I294T, and I294M had no detectable binding compared to WT hMC5R (**Fig. 4.3A and Table 4.1 & 4.2**). To further investigate whether these hMC5R mutations alter the signaling properties, α-MSH-induced intracellular cAMP production was measured. WT hMC5R showed a dose-dependent increase in intracellular cAMP accumulation upon stimulation with α-MSH, with an EC50 of 27.96 nM. However, these five mutants were devoid of cAMP production in response to ligand stimulation (**Fig. 4.4A, Table 4.1 & 4.2**).

4.3.3. Three mutants had significantly decreased maximal binding and signaling (class III)

Competitive binding assays found that P70A, Y141F, and R158H bound NDP-MSH with normal binding affinity (IC_{50}), while their maximal binding (B_{max}) was significantly decreased compared to WT hMC5R (**Fig. 4.3B and Table 4.1 & 4.2**). The ligand-stimulated cAMP assay results showed that EC50s of Y141F and R158H were significantly increased, whereas P70A showed a similar EC_{50} to WT hMC5R. Additionally, P70A, Y141F, and R158H had significantly decreased R_{max} values (**Fig. 4.4B and Table 4.1 & 4.2**). These findings suggest that partially defective ligand binding of P70A, Y141F, and R158H results in reduced ligand-stimulated cAMP production.

4.3.4. Three mutants had significantly decreased maximal binding and signaling (class III)

Class IV mutants are produced and transported to cell surface and can bind to the ligand normally but are defective in signaling, showing a reduced maximal response and/or decreased sensitivity (increased EC_{50}) (Tao, 2006). Our results showed that D291H, Y295C and Y295H had increased, normal and decreased maximal binding (B_{max}), respectively, while all of them showed similar binding affinity (IC_{50}) compared to WT hMC5R (**Fig. 4.3C & D and Table 4.1 & 4.2**). However, all these three mutants were defective in α-MSH-stimulated $G\alpha_s$-cAMP signaling pathway, indicating a clear defect in G protein binding and/or activation (**Fig. 4.4A and Table 4.1 & 4.2**).

4.3.5. R158L was fully functional on binding and signaling (class V)

Class V mutants have no known defects because these mutant receptors are expressed on cell surface, bind to the ligand normally, and generate a normal response to ligand stimulation (Tao, 2006). Competitive binding assay found that R158L had similar IC_{50} and B_{max} values compared to WT hMC5R (**Fig. 4.3B and Table 4.1 & 4.2**). Additionally, R158L showed a dose-dependent increase in intracellular cAMP accumulation upon stimulation with α-MSH, with an EC_{50} of 39.58 nM. Both the potency (EC_{50}) and maximal response of R158L were like WT hMC5R (**Fig. 4.4B and Table 4.1 & 4.2**).

4.4. Discussion

GPCRs consist of seven transmembrane α-helices (TMs) connected by three intracellular (IL) and three extracellular loops (EL), with C terminus intracellular and N terminus extracellular (Tao, 2006). They convert extracellular signals into various intracellular responses, which forms the basis for their participation in many physiological processes. To date, many naturally occurring mutations in GPCRs have been identified. These mutations can alter the function of GPCRs, leading to loss of function, gain of function, constitutive activation, or changes in ligand specificity, thereby causing various genetic diseases (Vassart and Costagliola, 2011). MC5R is an important GPCR that plays multiple

roles in regulating exocrine gland function, immune responses, energy homeostasis, and other physiological processes (Ji et al., 2022; Lee and Taylor, 2013; Xu et al., 2020). Determining specific effects of MC5R mutations will help elucidate the diverse roles of MC5R in physiology and disease, paving the way for potential therapeutic interventions targeting this receptor.

The highly conserved DRY motif at TM3 and N/DPxxY motif at TM7 are critical for receptor function in GPCRs. The DRY motif forms salt bridges with surrounding residues and with TM6, creating an ionic lock that constrains the receptor in an inactive conformation. Upon ligand binding, the ionic lock is broken, and the DRY forms new interactions with TM5, stabilizing the receptor in an active conformation (Palczewski et al., 2000; Rosenbaum et al., 2009). We observed that Y141F (class III) represented a functionally impaired mutant of hMC5R, with significantly reduced maximal binding and signaling. This suggests a conformational change that interferes with ligand binding, leading to defective signaling. The same phenomenon was reported in MC3R, where Y180A in DRY motif resulted in a significant decrease in R_{max} and an increase in EC50 (Huang and Tao, 2014).

The N/DPxxY motif has Important functions In GPCR expression, conformational transitions after GPCR activation, G protein coupling, and GPCR internalization (Yang et al., 2015). The proline in this motif causes a distortion in the α-helical structure, whereas tyrosine faces into a pocket lined by TM2, TM3, TM6 and TM7 (Rosenbaum et al., 2009).

Our results found that although P292S-mutated and I294M-mutated hMC5Rs (class III) were successfully trafficked to the cell surface, no detectable binding and signaling was found. Therefore, we speculate that P292S caused damage to the structurally important helical deformation, resulting in the receptor's inability to bind to ligand. Interestingly, unlike I294M mutant hMC5R, I335S mutation in N/DpxxY motif of hMC3R lead to a complete loss of ligand binding and signaling due to intracellular retention (Tao, 2007). This demonstrates the importance of isoleucine and shows that when isoleucine is mutated to different amino acids, it may produce varying pharmacological effects on different receptors.

Amino acid tyrosine in N/DpxxY motif is essential for receptor-G protein interactions and signaling, and mutations of tyrosine residues in some receptors result in a lack of ligand binding or signaling defects (Roth et al., 2009). Examples include type 1 angiotensin II receptor (Hunyady et al., 1995; Laporte et al., 1996), α1B-adrenergic receptor (Wang et al., 1997), the gonadotropin-releasing hormone receptor (Arora et al., 1996), and the B2 bradykinin receptor (Kalatskaya et al., 2004). Our study found that Y295C and Y295H (class IV) exhibited similar ligand binding to WT hMC5R but were defective in signaling. Additionally, we replaced Asp291$^{7.49}$ with histidine (D291H, class IV) at DPLIY motif of hMC5R and observed a functional coupling defect but no defect in ligand binding. This suggests that Asp291 plays an important role in the coupling of MC5R to G proteins and subsequent effector activation. Mutagenesis and signaling studies have shown that amino acid at position 7.49 (Asn or Asp) is considered to contribute to the binding of positively

charged sodium ions in an interaction network between water molecules and the highly conserved negatively charged aspartate at position 2.50 in TM2. An interaction with a monovalent sodium cation is thought to be a common mechanism of allosteric modulation of GPCRs, which appears to be specifically associated with the maintenance of an inactive state (Kleinau et al., 2020). This interaction influences the orientation of TM7 and receptor dynamics, implying regulation of transitions between different receptor activity states (Zarzycka et al., 2019).

E131$^{2.40}$, D154$^{3.25}$ and D158$^{3.29}$ in hMC3R, corresponding to E100$^{2.40}$, D122$^{3.25}$, and D126$^{3.29}$ in hMC4R, potentially form a salt bridge with Arg in pharmacophore (HFRW) of the ligand (Hruby et al., 1987; Liu et al., 2022). Since all agonist ligands share the pharmacophore that is essential for receptor recognition, the interaction of SHU9119 with MC4R observed in the complex crystal structure is likely related to MSH binding, such as the interactions between H6/T101 and R8/D126 in the α-MSH/receptor complex (Kleinau et al., 2020). In this study, we replaced Asp119$^{3.29}$ with tyrosine (D119Y, class III) in hMC5R, and detected no measurable ligand binding or signaling, like the findings with D158$^{3.29}$E, and D158$^{3.29}$ Q in the hMC3R structure-function study (Wang et al., 2008). This suggests that Asp119 is also important for ligand binding and signaling in hMC5R. Additionally, P260$^{6.50}$ in MC4R is a highly conserved amino acid in all MCRs. Naturally occurring mutation P260$^{6.50}$ Q in hMC4R is intracellularly retained, resulting in reduced receptor activity (Beckers et al., 2010). However, P253$^{6.50}$ L (class III) in hMC5R exhibited normal cell surface

expression but was unable to bind to ligand, resulting in subsequent signaling defects. We speculate that different mutations of the highly conserved proline in TM6 in different MCRs may have different pharmacological effects on the receptor.

P78$^{2.38}$L and R165$^{4.41}$ W/Q/G are well-known MC4R mutations associated with obesity. P78$^{2.38}$L mutation resulted in no ligand binding or signaling due to a significant decrease in cell surface expression (Nijenhuis et al., 2003; Tao and Segaloff, 2003). In contrast, P70$^{2.38}$A-mutated hMC5R (class III) had similar binding affinity to WT hMC5R but exhibited significantly reduced signaling, indicating the importance of proline in hMC5R signaling. R165W, R165Q and R165G were known to lead to partial loss of function (Calton et al., 2009; Farooqi et al., 2000; Lotta et al., 2019; Thearle et al., 2012), consistent with our findings (R158$^{4.41}$H-mutated hMC5R). Interestingly, R158$^{4.41}$L-mutated hMC5R was expressed on cell surface, bound to ligand normally, and generated a normal response to ligand stimulation. It is generally agreed that different mutations at conserved sites have varying pharmacological effects on MCRs. However, whether these mutations in hMC5R are directly related to obesity still requires extensive medical data and research.

4.5. Conclusion

Like MC4R, naturally occurring mutations in MC5R cause functional impairment. The highly conserved DRY motif on TM3 and N/DPxxY motif on TM7 are essential for

receptor function in hMC5R. Mutations in these motifs, which resulted in insufficient ligand binding or signaling defects (class III or class IV), include P292S, I294T/M, Y141F, D291H, Y295C/H. Asp119 is important for hMC5R to recognize the core pharmacophore of agonist ligands, similar to its role in hMC3R and hMC4R. However, sometimes different mutations of highly conserved residues have different pharmacological effects on MCR. Although R158H-mutated hMC5R caused partial loss of function like MC4R, R158L was fully functional on binding and signaling. In addition, unlike the signaling defects caused by intracellular retention of P70A and P253L in hMC4R, P70A and P253L mutations in hMC5Rs mainly caused signaling defects by affecting the binding of receptor to ligand.

Table 4.1 The ligand binding and signaling (B) properties of WT and mutant hMC5Rs

	Binding		α-MSH stimulated cAMP	
	IC_{50} (nM)	**B_{max} (% WT)**	**EC_{50} (nM)**	**R_{max} (% WT)**
WT	**5.03 ± 0.53**	**100**	**27.96 ± 7.71**	**100**
P70A	**3.65 ± 0.61**	**12.78 ± 2.01******	**44.13 ± 7.98**	**65.52 ± 9.73***
Y141F	**4.94 ± 0.89**	**29.76 ± 1.60******	**228.3 ± 38.58***	**64.50 ± 8.84***
R158H	**3.69 ± 0.41**	**12.91 ± 1.88******	**213.5 ± 75.10***	**54.51 ± 9.57****
R158L	**5.40 ± 0.35**	**73.07 ± 4.22**	**39.58 ± 14.73**	**83.46 ± 5.45**
D291H	**9.19 ± 2.46**	**140.80 ± 10.72****	**ND**	**ND**
Y295C	**6.64 ± 1.87**	**83.15 ± 16.09**	**ND**	**ND**
Y295H	**6.17 ± 2.14**	**33.73 ± 6.80******	**ND**	**ND**

Results are presented as the mean ± SEM (n = 3 - 4).

ND means not detected.

*a significant difference from the parameter of WT hMC5R, $P < 0.05$.

** a significant difference from the parameter of WT hMC5R, $P < 0.005$.

**** significant difference from the parameter of WT hMC5R, $P < 0.0001$.

Table 4.2 Summary of functional properties of 12 mutant hMC5Rs

MC5R	Surface expression	Binding	Signaling	Class
P70A	+	↓	↓	**III**
D119Y	+	−	−	**III**
Y141F	+	↓	↓	**III**
R158H	+	↓	↓	**III**
R158L	+	+	+	**V**
P253L	+	−	−	**III**
D291H	+	↑	−	**IV**
P292S	+	−	−	**III**
I294M	+	−	−	**III**
I294T	↑	−	−	**III**
Y295C	+	+	−	**IV**
Y295H	+	↓	−	**IV**

"+": normal expression / ligand-stimulated response / cAMP signaling as WT hMC5R.

"↑": increased expression / ligand-stimulated response / cAMP signaling as WT hMC5R.

"↓": defective ligand-stimulated response / cAMP signaling compared to WT hMC5R.

"-": no ligand-stimulated response / cAMP signaling were detected.

"Class" column is based on the classification from previous article (Tao, 2006).

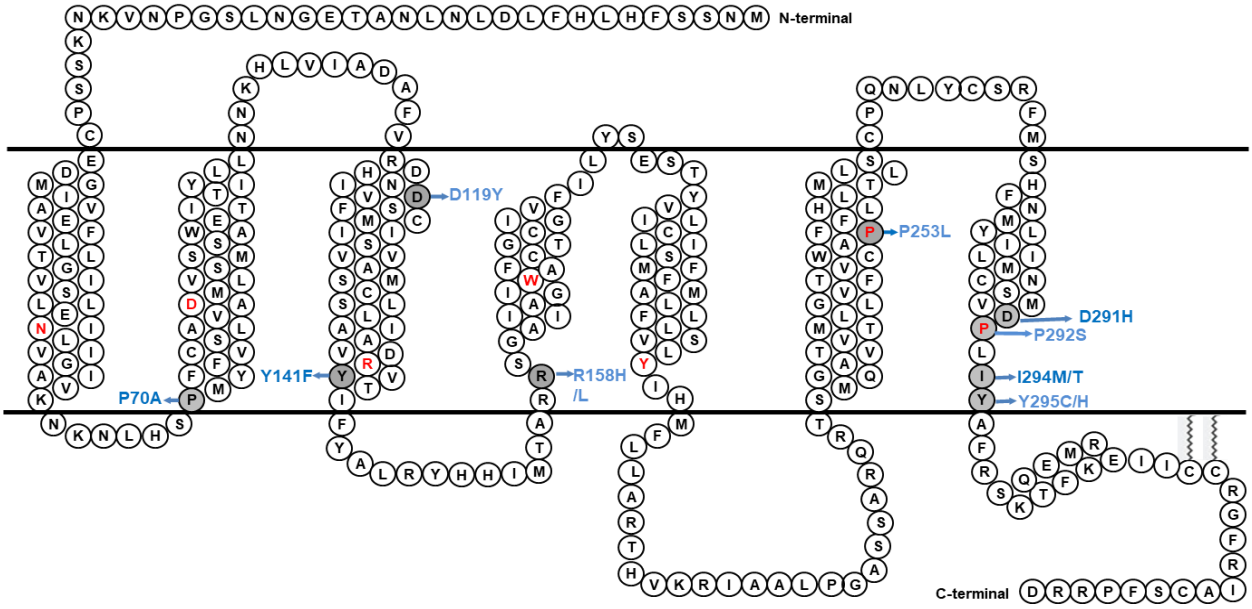


Figure 4.1 Schematic model of the human *MC5R*. The naturally occurring mutations characterized in this study are highlighted with gray background. Most highly conserved residues in each TMs are highlighted with red.

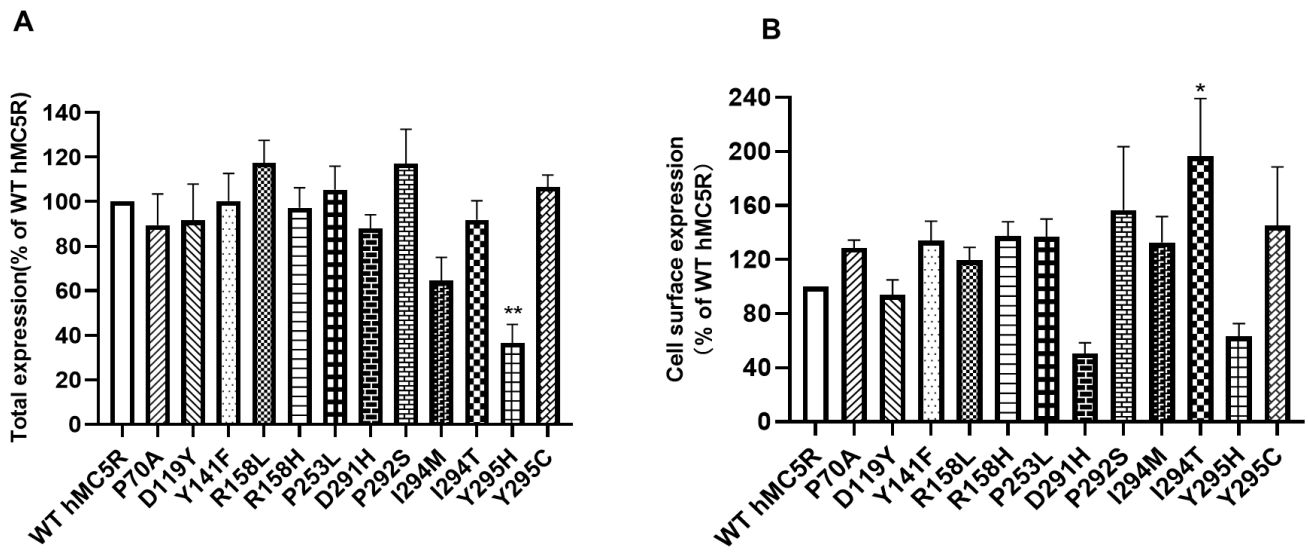


Figure 4.2 Total (A) and cell surface (B) expression of WT and mutant hMC5Rs. HEK293T cells were transfected with hMC5R or mutant hMC5Rs. Cell surface and total expression levels of the WT and mutant hMC5Rs were quantitatively determined by flow cytometry. The empty vector pcDNA3.1 fluorescence was used for background staining. The results are expressed as percentage of total or cell surface expression levels of the WT hMC5R. Each data point represented as the mean ± SEM (n = 3). Star (*) indicates significant difference from WT hMC5R ($P < 0.05$).

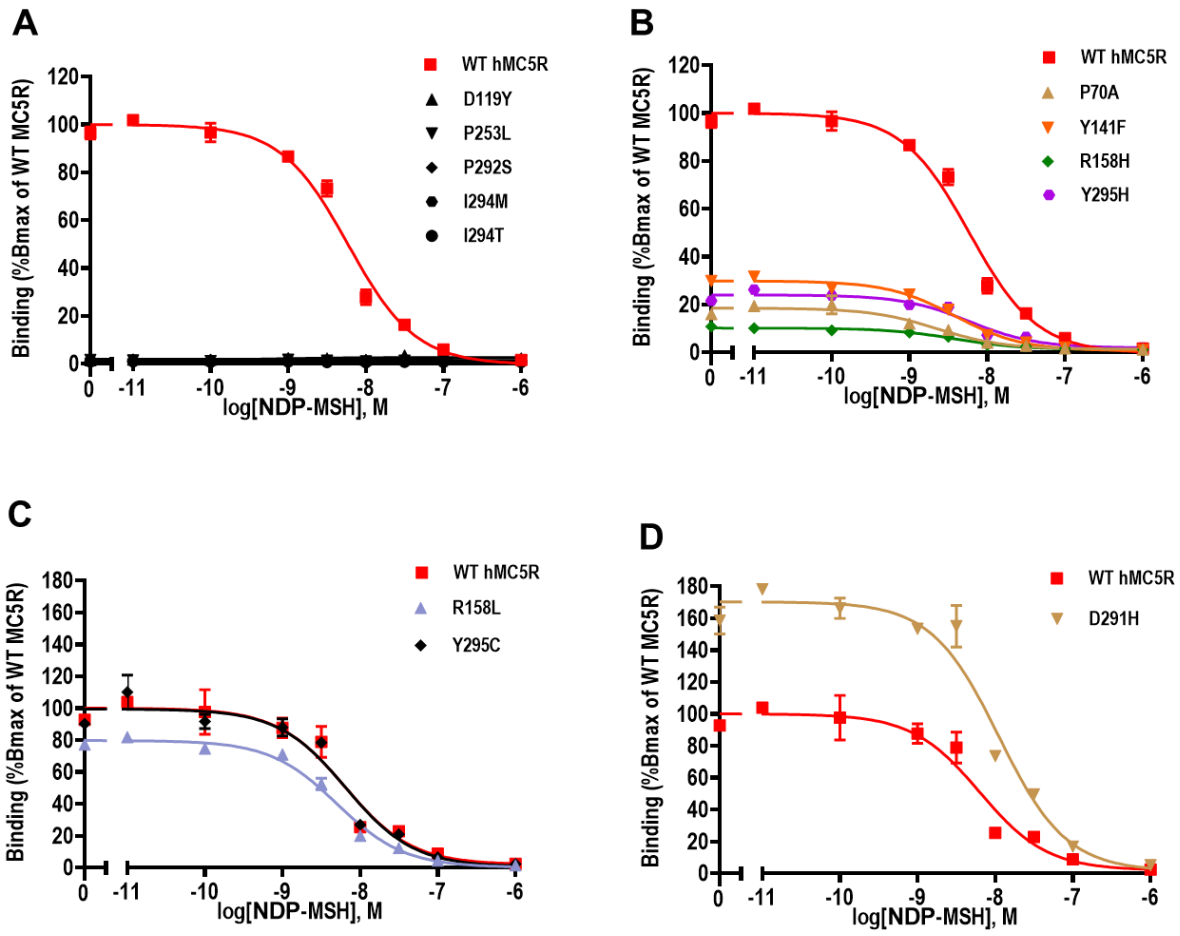


Figure 4.3 NDP-MSH binding assay of WT and mutant hMC5Rs transiently expressed in HEK293T cells. Different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I-NDP-MSH. Results are expressed as % of WT hMC5R binding ± range from duplicate determinations within one experiment. All experiments were repeated at least three independent times.

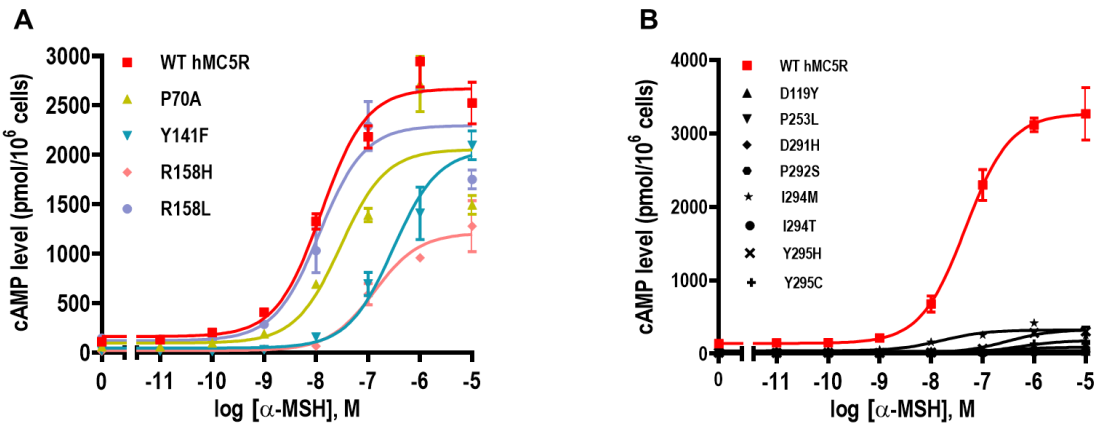


Figure 4.4 Signaling of the WT and mutant hMC5Rs transiently expressed in HEK293T cells. cAMP signaling properties of WT and mutant hMC5Rs to α-MSH were measured by RIA. Results are expressed as the mean ± S.E.M. of triplicate determinations within one experiment and all experiments were performed at least three times.

Conclusions and Future Prospective

The pharmacological regulation of melanocortin receptor accessory proteins (MRAPs) on melanocortin receptors (MCRs) is a fascinating area of study in endocrinology and pharmacology. MRAPs play a crucial role in modulating the activity and specificity of MCRs, which are involved in a wide range of physiological processes including energy homeostasis, inflammation, and pigmentation.

Our study further highlights the role of hMRAPs in regulating MC5R pharmacology. The results show that these accessory proteins, including the newly discovered isoforms hMRAP2b and hMRAP2c, affect not only MC5R signaling but also its cellular trafficking and ligand binding capacity. This provides valuable insights into the complexity of MC5R regulation and its pathway interactions, such as $G\alpha_s$-cAMP signaling and ERK1/2 activation, which is critical for understanding the physiological and potential therapeutic roles of MC5R. With the understanding that different MRAPs modulate MC5R differently, there is an opportunity to develop selective modulators that could target specific signaling pathways. Such modulators could be used to manipulate MC5R functions in a controlled manner, potentially leading to novel treatments for conditions where MC5R is implicated.

Furthermore, pharmacological study in Chapter 3 demonstrated that the identified obesity-associated MRAP2a mutants (G31V, F62C, N77S, K102*, and P195L) exhibit diverse regulatory effects across different melanocortin receptors (MCRs). This variability in

their influence highlights the complex role that MRAP2a mutations can play in modulating MCR function, which is crucial for understanding their contribution to obesity and potentially other metabolic disorders. Animal models expressing these MRAP2a mutants could be developed to study their effects in a whole-organism context. Such models would help in understanding the physiological consequences of these mutations and their interaction with dietary and environmental factors.

Study on naturally occurring mutations in MC5R underscores the critical roles of highly conserved motifs in MC5R function. Specifically, the DRY motif on transmembrane domain 3 (TM3) and the N/DPxxY motif on TM7 are vital for the receptor's basic operations, such as ligand binding and signal transduction. P292S, I294T/M, Y141F, D291H and Y295C/H lead to functional impairments classified as class III (insufficient ligand binding) or class IV (signaling defects). Furthermore, the residue Asp119 is pivotal for recognizing the core pharmacophore of agonist ligands, reflecting a conserved functional aspect seen in other MCRs. However, studies have shown that mutations in conserved residues at the same site across different MCRs result in varying pharmacological effects, indicating a complex interplay between the structural and functional dynamics within the receptor.

There is a significant opportunity to develop targeted therapies that can either correct or compensate for the dysfunctional receptor caused by these mutations. Small molecule modulators or peptide-based therapies that can enhance receptor function or mimic ligand

activity could be particularly effective. In addition, advanced structural studies, such as cryo-electron microscopy and X-ray crystallography, should be used to visualize the exact changes in receptor conformation due to mutations. This can provide a more precise understanding of why certain mutations disrupt receptor function and others do not.

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