Emerging roles of melanocortin receptor accessory proteins (MRAP and MRAP2) in physiology and pathophysiology

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Abstract:

Melanocortin-2 receptor accessory protein (MRAP) has an unusual dual topology and influences the expression, localisation, signalling and internalisation of the melanocortin receptor 2 (MC2); the adrenocorticotropic hormone (ACTH) receptor. Mutations in MRAP are associated with familial glucocorticoid deficiency type-2 and evidence is emerging of the importance of MRAP in adrenal development and ACTH signalling. Human MRAP has two functional splice variants: MRAP-α and MRAP-β, unlike MRAP-β, MRAP-α has little expression in brain but is highly expressed in ovary. MRAP2, identified through whole human genome sequence analysis, has approximately 40% sequence homology to MRAP. MRAP2 facilitates MC2 localisation to the cell surface but not ACTH signalling. MRAP and MRAP2 have been found to regulate the surface expression and signalling of all melanocortin receptors (MC1-5). Additionally, MRAP2 moderates the signalling of the G-protein coupled receptors (GCPRs): orexin, prokineticin and GHSR1a; the ghrelin receptor.

Whilst MRAP appears to be mainly involved in glucocorticoid synthesis, an important role is emerging for MRAP2 in regulating appetite and energy homeostasis. Transgenic models indicate the importance of MRAP in adrenal gland formation. Like MC3R and MC4R knockout mice, MRAP2 knockout mice have an obese phenotype. In vitro studies indicate that MRAP2 enhances the MC3 and MC4 response to the agonist αMSH, which, like ACTH, is produced through precursor polypeptide proopiomelanocortin (POMC) cleavage. Analysis of cohorts of individuals with obesity have revealed several MRAP2 genetic variants with loss of function mutations which are causative of monogenic hyperphagic obesity with hyperglycaemia and hypertension. MRAP2 may also be associated with female infertility. This review summarises current knowledge of MRAP and MRAP2, their influence on GPCR signalling, and focusses on pathophysiology, particularly familial glucocorticoid deficiency type-2 and obesity.

Keywords:

MRAP; MRAP2; MC2; MC1R; MC2R; MC3R; MC4R; MC5R; melanocortin; ACTH; dual topology; ghrelin; alpha-MSH; adrenal gland; glucocorticoid; female infertility; familial glucocorticoid deficiency; melanocortin 2; receptor accessory protein; melanocortin receptor; MC; obesity

Introduction

Genetic variants of melanocortin-2 receptor accessory protein (*MRAP*) are associated with severe glucocorticoid deficiency (Metherell, et al., 2005). Adrenocorticotropic hormone (ACTH) is the endogenous agonist for the melanocortin receptor 2 (MC_2), a class A G-protein coupled receptor. MRAP is a small transmembrane protein which promotes the assembly of melanocortin receptor 2 in the endoplasmic reticulum and

stabilises MC_2 in the plasma membrane. MRAP influences the binding of ACTH to MC_2 and following agonist stimulation, MC_2 via Gs protein, activates adenylate cyclase generating cyclic adenosine monophosphate (cAMP).

Effective ACTH signalling is vital to produce steroids including glucocorticoid, hence genetic variants of *MRAP* are linked to familial glucocorticoid deficiency. *MRAP* expression is high in the brain and the adrenal gland and MRAP has been demonstrated to affect the differentiation of progenitor cells within the adrenal gland (Novoselova, et al., 2018).

MRAP2 shares approximately 40% sequence homology with *MRAP* but unlike MRAP, which facilitates MC_2 localisation and signalling; MRAP2 has less receptor fidelity, influencing the signalling of melanocortin receptors ($MC_{1\text{-}5}$), as well as orexin receptor, ghrelin receptor and growth hormone secretagogue receptor 1a (GHSR-1a) (Srisai, et al., 2017). MC_3 and MC_4 both have important roles in appetite control with deficiencies linked to obesity (Chen, et al., 2000). The MC_4 agonist setmelanotide is currently in phase 3 clinical trials for rare genetic disorders of obesity caused by mutations in proopiomelanocortin (*POMC*) (Kühnen, et al., 2016) and leptin receptor (*LEPR*) (Clément, et al., 2018). Evidence from *in vitro* analyses, MRAP2 transgenic animals and *MRAP2* variants in populations with metabolic syndrome (Baron, et al., 2019), indicate that MRAP2 has an important role regulating appetite control, with *MRAP2* genetic variants causing obesity.

This article will review the structures of melanocortin receptor accessory proteins, physiological roles, and emerging evidence about the *MRAP* and *MRAP2* genetic variants which shed light upon pathophysiological conditions.

Protein structure

MRAP is a hydrophobic single pass transmembrane protein which is encoded for by a single gene located on chromosome 21q22.11 (Xu, et al., 2002) composed of 6 exons. There are two functional MRAP splice variants: MRAP-α derived from exon 1-5 which is 172 amino acids (19 kDa) and MRAP-β derived from exon 1-4, 6, which is 102 amino acids (14.1 kDa). These splice variants have conserved N-terminal homology but differ at the C-terminus (Xu, et al., 2002; Metherell, et al., 2005)

When first described, it was predicted that the MRAP dimer would orientate with intracellular N-terminus and extracellular C-terminus (Metherell, et al., 2005). However, through epitope immunoprecipitation and live cell imaging studies the quaternary structure of the MRAP was found to form a stable anti-parallel homodimer with N-termini orientating both intracellularly and extracellularly, which is still thought to be unique in eukaryotes (Sebag and Hinkle, 2007).

MRAP is partially glycosylated and this is dependent upon the Asn-X-Ser/Thr motif facing the luminal surface of the endoplasmic reticulum (Sebag and Hinkle, 2007),

however, the creation of functional MRAP glycosylation mutants in this study determined that glycosylation was not necessary for dual topology.

A further series of mutational studies identified the N-terminal 31-37 sequence (LKANKHS) adjacent to the transmembrane region to be essential for this dual topology (Sebag, and Hinkle, 2009). Using bimolecular fluorescence complementation, Sebag and Hinkle demonstrated that this dual topology occurs in the endoplasmic reticulum; once formed this stable orientation is retained when the protein is expressed on the plasma membrane (Maben, et al., 2016).

The N-terminus and the transmembrane domains of MRAP are conserved between species however, the carboxyl region has greater variation (Agulleiro, et al., 2010). The 18-21 LDYI sequence is unique to the MRAP isoform and is required for the binding of ACTH to melanocortin receptor 2 (Sebag, and Hinkle, 2009) but MRAP C-terminus truncation experiments suggested this domain has less impact on ACTH signalling (Sebag, and Hinkle, 2009). However, truncated MRAP (with the removal of the C-terminal domain), expressed in HEK293/FRT cells, revealed diminished MC_2 surface expression and cAMP production compared to full length MRAP; suggesting that the C-terminus does modulate MC_2 cAMP generation in response to ACTH (Roy, et al., 2012). In addition serine and threonine residues in the C-terminus are necessary for MC_2 internalisation and recycling (Roy, et al., 2011). The exact residues necessary for MRAPs to interact with GPCRs have not been fully characterised; however, co-precipitation studies with MC_2 suggest that the MRAP transmembrane domain is necessary (Webb, et al., 2009).

Discovery, Structure and Characteristics of MRAP2

As a paralog to *MRAP, MRAP2* was identified in the human genome after sequence analysis. The *MRAP2* gene is located at 6q14.3 and encodes for a 205 amino acid protein, the sequence alignment of *MRAP2* indicates approximately 40% homology with *MRAP*. The greatest sequence conservation lies at the N-terminal and within the transmembrane domain, whilst the carboxyl terminal differs greatly between these two proteins (Chan, et al., 2009).

MRAP2 is a transmembrane protein that traverses the membrane once and as with the MRAP, MRAP2 could be found both on the surface membrane and subcellularly on the luminal surface of the endoplasmic reticulum (Sebag, and Hinkle, 2010). MRAP2 shares the dual orientation of the carboxyl and amino terminal forming an antiparallel homodimer (Figure 1). Interestingly, MRAP2 has been shown to form a heterodimer with the MRAP (Figure 1); a yellow fluorescent protein in bimolecular complementation studies revealed an antiparallel orientation of the MRAP-MRAP2 heterodimer (Sebag, and Hinkle, 2010).

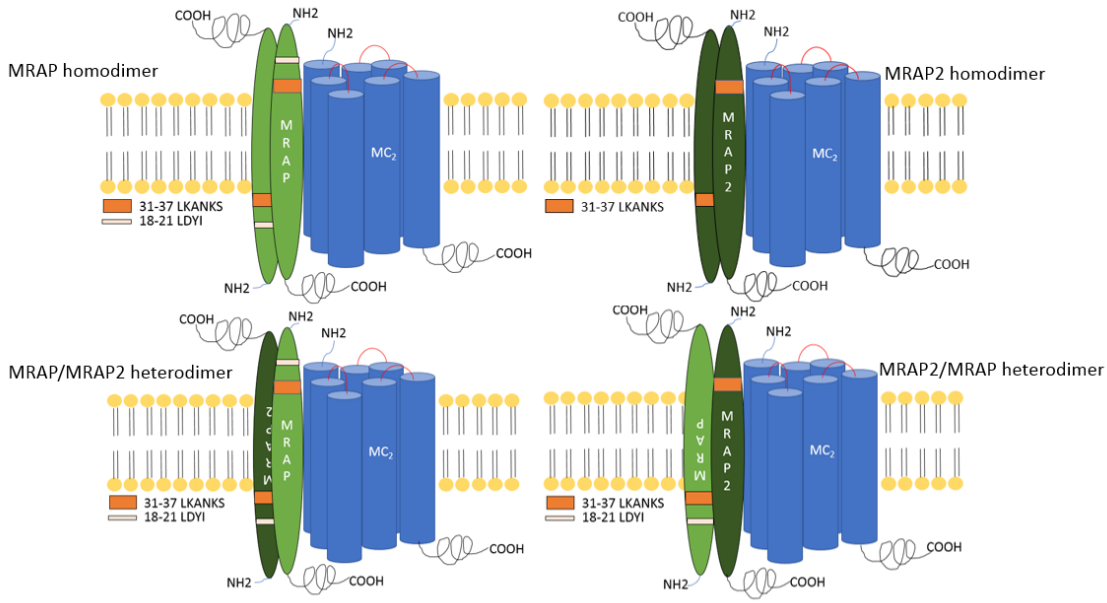

Figure 1: Dual topology of MRAP homodimer, MRAP2 homodimer and MRAP/MRAP2 heterodimer; the green transmembrane domain represents the structure and the dual topology with the amino (NH2) / carboxyl (COOH) both being located extracellularly and amino (NH2) / carboxyl (COOH) termini also intracellularly. This dual topology allows for homodimer (MRAP/MRAP; MRAP2/MRAP2) or heterodimer (MRAP/MRAP2; MRAP2/MRAP) formation. The $LKANKS_{31-37}$ (orange shading) immediately before the transmembrane region is required for dual topology, the $LDYI_{18-21}$ (yellow shading) is found only in MRAP and is required for ACTH binding to MC_2.

MRAP gene regulation

MRAP gene expression is upregulated by lipopolysaccharide (Gibbison, et al., 2015), ACTH (Liu, et al., 2013) and in adipocytes by PPARγ (peroxisome proliferator-activated receptor gamma) (Kim, et al., 2013). Diurnal *Mrap* expression has been observed in rats, with *Mrap* mRNA correlating with the expression of circadian regulating genes (*per1*, *per2* and *CLOCK*) (Spiga, et al., 2020). Since the *MRAP* mRNA half-life is shorter than *MC2R*, Clark and Chan, (2019) have proposed a model where MRAP may facilitate the transport of MC_2 to the plasma membrane without necessitating *de novo MRAP/MC2R* expression.

Gnomon automated computational analysis of the 21q22.11 region of the GRCh38.p13 (Souvorov, et al., 2010) has predicted the presence of a novel *MRAP* antisense RNA 1 (*MRAP-AS1*; XR_937664.1). Whether this non-coding RNA has a biological function and can regulate *MRAP* remains to be determined.

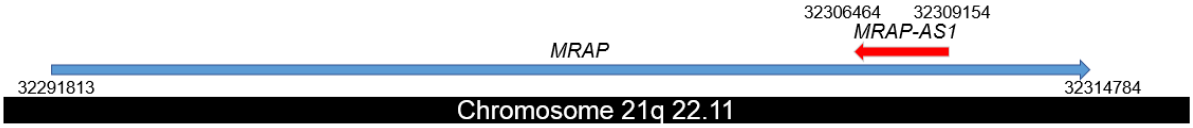

Figure 2: Predicted non-coding RNA, *MRAP-AS1* (XR_937664.1, shown in red) on chromosome 21q22.11 in relation to *MRAP* (blue)

The evolution of MRAPs

Phylogenetic studies have revealed the existence of the *MRAPs* orthologues in different piscine species: zebrafish, tetrapod and some bony fish subspecies (Valsalan, et al., 2012). Interestingly, whilst *MRAP2* was found in the sea lamprey and cartilaginous fish, *MRAP* was absent which lead to a theory that *MRAP2* gene duplication may have given rise to *MRAP* (Västermark, and Schiöth, 2011). However, cDNA blast analysis from the elephant shark genome project located the *MRAP* sequence (Venkatesh, et al., 2014) discrediting an *MRAP2* ancestral role. Considering all these findings, the origin of the *MRAPs* genes is thought to be as a result of R2 genome duplication events according to the evolution theory (Ohno et al., 1968); a comprehensive review of melanocortin receptor evolution has been presented by Dores (Dores, et al., 2016).

The presence of both *MRAPs* has been described in most mammals and chicken (Valsalan et al., 2012), the dual topology feature of MRAP and MRAP2 was conserved through the appearance of the motifs LKANKH (MRAP) and LKAHKY (MRAP2) in the orthologs of mice, chicken and zebrafish. Additionally, sequence alignment studies of all linages revealed the MRAP sequence to be shorter than the MRAP2 sequence, with the transmembrane domain being well conserved amongst all species examined.

Distribution of the MRAPs

In humans reverse transcription PCR (RT-PCR) indicated that both isoforms are present at high levels in the adrenals, testis and breasts; with low expression in in the digestive system, heart, fat and skin (Metherell, et al., 2005). In humans, *MRAP-*α and *MRAP-*β were found expressed in the adipose tissue, breast, testis and adrenal cortex (see table 1).

Examining the “Human Protein Atlas (HPA)- RNA-*seq*” data of 95 human samples reveals a wide distribution of *MRAP*, with the highest expression levels observed in the adipose tissue and the adrenal gland. This is followed by the skin and the male reproductive organs whilst brain, heart, ovary and the kidney had a lower expression (Fagerberg, et al., 2014). Table 1 lists human tissues expressing the *MRAP-α* and *MRAP-*β. Along with the adrenal gland expression, *MRAP2* was expressed in different parts of the brain at high levels, as shown in the HPA-RNA-*seq* data (Fagerberg, et

al., 2014). *MRAP2* was also found to be highly expressed in the female and male reproductive systems and stomach whilst endocrine glands, liver and peripheral blood had the lowest *MRAP2* expression (Fagerberg, et al., 2014).

As discussed above, the *MRAPs* are present in other species, including chicken, fish and mice (Zhang et al., 2017; Agulleiro et al., 2010; Asai et al., 2013; Valsalen et al 2012). While the *MRAP* was widely distributed in the tissues of those species, the *MRAP2* expression is mainly localised to the brain.

Table 1: Expression of *MRAP-α, MRAP-β* and *MRAP2* in human tissues. +++: high expression; +: low expression.

Tissue	*MRAP-α*	*MRAP-β*	*MRAP2*	Reference
Adrenal gland	+++	+++	+++	Metherell et al*.,* 2005 Chan et al., 2009
Testis	+++	+++	+	Metherell et al., 2005
Adipose	+	+	+	Xu et al., 2002 Metherell et al., 2005 Fagerberg et al., 2014
Breast	+++	+++	-	Metherell et al., 2005
Ovary	+++	+	+	Fagerberg et al., 2014
Endometrium	+	+	+	Fagerberg et al., 2014Fagerberg *et al*., 2014
Digestive tract	+	+	+	Fagerberg et al., 2014
Liver	+	-	-	Metherell et al., 2005
Heart	+	-	-	Metherell et al., 2005 Fagerberg et al*.,* 2014
Skin	+	+	+	Metherell et al., 2005
Brain: Pre-frontal cortex Cerebellum Hippocampus Hypothalamus Spinal cord	-	+ + + + - +	+++ +++ +++ +++ +++ +++	Gardinar et al., 2002 Chan et al., 2009 Fagerberg et al., 2014 Chaly et al., 2016
Pituitary gland	+	-	+++	Chaly et al., 2016
Thyroid gland	+++	-	-	Metherell et al., 2005

MRAPs and the melanocortin system

MRAP and MC_2

MC_2 was initially difficult to express on the surface of cells until an adrenal cell line was utilised, revealing that MRAP is an essential accessory protein (Metherell et al., 2005). The MC_2 receptor only responds to ACTH; ACTH activation of MC_2 results in glucocorticoid-, mineralocorticoid- and steroid-genesis in the adrenal gland. Genetic variants of *MC2R* result in severely impaired glucocorticoid production which is discussed in more detail below. MRAP was found to assist in MC_2 transport from the endoplasmic reticulum to the cell surface (Metherell, et al., 2005), whilst MC_2 has reported to move unassisted to the cell surface (Roy et al., 2007), this finding has been criticised since extremely low endogenous MRAP2 present in HEK293 cells may facilitate the MC_2 movement (Sebag and Hinkle, 2007). MRAP increases both the density of surface MC_2 and the binding affinity of ACTH to MC_2. Measurement of cAMP in response to Gs activation revealed that MRAP-α elicits a 4-fold greater dose response than MRAP-β but the maximal cAMP response was higher in the MC_2/MRAP-β cells compared to the MC_2/MRAP-α (Roy, et al., 2007). The interaction of MRAP and MC_2 also has an effect on receptor internalisation and recycling (Roy, et al., 2011).

The three zones of the adrenal cortex synthesise different steroids: zona glomerulosa, mineralocorticoids; zona fasciculata, glucocorticoids and zona reticularis, androgens. The binding of ACTH to MC_2 results in the production of cAMP, that in turn stimulates the protein kinase A (PKA) signalling pathway. In the adrenal gland, this signalling pathway is responsible for the transcription of genes necessary for steroid synthesis, such as steroidogenic acute regulatory protein (*StAR*) (Manna, et al., 2003). The interaction of MC_2 with either of the MRAP isoforms and the response variation (Roy, et al., 2007) reflects the physiological requirements of these steroids in different physiological conditions, such as the response to stress or blood pressure changes.

In addition to *MRAP*, *MRAP2* is expressed in human and rat adrenal glands (Gorrigan, et al., 2011; Fagerberg, et al., 2014). The response of MC_2 depends on which MRAP it is coupled with, *in vitro* studies revealed that MC_2/MRAP2 transiently transfected into HEK293 cells required higher concentrations of ACTH to elicit a response compared to MC_2/MRAP transfected cells (Gorrigan, et al., 2011). *MRAP* and *MRAP2* expression has been described in the foetal adrenal gland suggesting a role of MC_2/MRAP and or MC_2/MRAP2 in the adrenal differentiation and proliferation (Gorrigan, et al., 2011). This is supported by the phenotype of $MRAP^{-/-}$ mice which have disrupted adrenal gland development with no apparent zonation in the adrenal cortex, without *in utero* corticosterone supplementation, the majority of these $MRAP^{-/-}$ mice died at birth (Novoselova, et al., 2018). $MRAP^{-/-}$ mice had disrupted adrenal progenitor cell differentiation due to impaired sonic hedgehog and WNT4/β-catenin signalling (Novoselova, et al., 2018).

MRAPs and other melanocortin receptors

The melanocortin receptors are expressed throughout the body and have established roles in different tissues. There is great interest in the involvement of MRAPs and the surface expression of melanocortin receptors, $MC_{1,3\text{-}5}$. These G-coupled protein receptors are activated by melanocortin peptides: proopiomelanocortin (POMC) and the peptides derived from POMC: adrenocorticotropic hormone (ACTH), alpha-, beta- or gamma-melanocyte stimulating hormone (α-MSH, β-MSH and γ-MSH). ACTH is further cleaved into: $ACTH_{(1\text{-}39)}$, $ACTH_{(1\text{-}17)}$, $ACTH_{(1\text{-}10)}$, α-MSH and deacetyl α-MSH (Wakamatsu, et al., 1997). Unlike MC_2 which only responds to ACTH, these MCs, respond with varying affinity to the endogenous melanocortin peptides and activating different signalling pathways, accounting for MC functional variability. Agouti related protein (AgRP) is an endogenous antagonist of MC_3 and MC_4 (Ollmann, et al., 1997; Fong, et al., 1997). MRAPs regulate both the expression and the response of the other MCs. Although MRAPs had no effect on the MC_1 and MC_3 cell surface expression in *in vitro* studies with CHO cells; MC_1, MC_3, MC_4 and MC_5 became less sensitive to the synthetic α-MSH analogue, NDP-MSH, when co-expressed with either MRAP or MRAP2 (Chan, et al., 2009).

As described above, MC_2 and the MRAPs are important for adrenal gland development. MC_5 expression has been described in the foetal adrenal gland and is suggested to have a role in foetal development (Nimura, et al., 2006). The role of MRAP and MRAP2 regulating adrenal differentiation, may be through interaction with either MC_2, MC_5 or a heterodimer of both, since heterodimerisation of MC receptors has been reported (Mandrika, et al., 2005).

MC_1 is well characterised in stimulating melanin synthesis, skin (or retinal) pigmentation occurs through the oxidation of tyrosine resulting in the formation of either eumelanin (brown pigmentation) or pheomelanin (yellowish-red pigmentation). MC_1 is expressed in both melanocytes and keratinocytes, as are POMC, ACTH and the prohormone convertases 1 and 2. α-MSH is the MC_1 agonist, activating cAMP and PKA signalling pathways which ultimately activate the microphthalmia transcription factor (MITF) and tyrosinase transcription (Beaumont, et al., 2009). The resultant pigmentation protects the skin from the DNA damaging effects of the ultraviolet radiation. The variability in skin colour is due to loss of function alleles in *MC1R* single gene mutation which also increase the incidence of skin cancer and melanoma (Sánchez-Laorden, et al., 2007). Whilst mutations of *MRAP2* do not cause disturbances in skin pigmentation, the phenotypic characteristics of *MRAP* mutations have included skin hyperpigmentation, as well as high plasma ACTH concentrations (Jain et al*.,* 2011). Expression of MRAP and MRAP2 in skin is very low (Table 1) a lack of functional MC_2 in *MRAP* mutation may result in higher circulating ACTH and consequently MC_1 hyperactivity impacting upon skin pigmentation.

The ratio of MRAP2 to the melanocortin receptor may have an important effect on the melanocortin signalling; a 1:4 ratio of MC_4 :MRAP2 gave greatest cAMP response to α-MSH whilst a 4:1 ratio of MC_4 :MRAP2 reduced cAMP below mock transfected cells (Schonnop, et al., 2016). Further work is required to elucidate the role of MRAP2 on MCs the *MRAP2* construct design, cell type, melanocortin peptide used to stimulate transfected cells and technique to measure MC activation are important factors to consider in interpreting this complex interaction.

In zebrafish two MRAP2 isoforms have been reported (mrap2a and mrap2b) and the expression of these varies during development; the expression of *mrap2a* was found to be present from day 1 of embryogenesis, as was *mc4r,* however there was little expression of *mrap2b* (Sebag, et al., 2013). Knockdown of *mrap2a* with antisense morpholinos significantly decreased growth, however, when the *mrap2a* was knocked down in *mc4r* null fish the effect was less marked; in the embryo, mrap2a inhibits MC_4 signalling. AgRP is an MC_4 antagonist, if both *AgRP* and *mrap2a* were knocked down there was a significant impairment of growth and they are proposed to collaborate inhibiting MC_4 constitutive activity (Sebag, et al., 2013).

In contrast the *mrap2b* isoform was highly expressed in adult zebrafish, and reduced MC_4 constitutive activity and sensitized MC_4 to the agonist α-MSH; the *mrap2b* has homology to mouse *MRAP2* (Sebag, et al., 2013). It was suggested that this switch in the *mrap2* expression may facilitate a change in metabolism to enable free-feeding once embryonic development was complete.

MRAP and familial glucocorticoid deficiency (FGD)

Glucocorticoids modulate the stress response through the hypothalamic-pituitary-adrenal (HPA) axis and mediate negative feedback control over the corticotropic releasing hormone (CRH) in the hypothalamus, as well as ACTH release from the pituitary gland. *MRAP2*, expression has been reported in hypothalamus, adrenal and pituitary glands (see table 1) alongside evidence of the melanocortin ligands (Chaly, et al., 2016). These findings suggest the control of the response may be moderated by the melanocortin receptors, with the assistance of the MRAPs, at all three levels of the HPA axis.

Familial glucocorticoid deficiency (FGD) is characterised by a poor response by the adrenal cells to adrenocorticotropic hormone (ACTH) leading to cortisol deficiency. As described above, the MRAPs have an important role stabilising the MC receptors and facilitating ACTH signalling. FGD is an autosomal recessive disease with 25% of the cases due to different *MC2R* mutations (FGD-1), 5% of cases relate to the steroidogenic acute regulatory protein (*StAR*) but around 50% of the FGD cases had an unidentified genetic background. Single nucleotide polymorphism (SNP) array genotyping of FGD cases revealed that 20% were linked to mutations in the *MRAP*

sequence (FGD-2) (Metherell, et al., 2005). The discovery of MRAP as an essential accessory protein for MC_2 prompted further investigations into the mechanisms underlying FGD-1.

The *MC2R* coding sequence lies predominantly in one exon, therefore, splicing mutations are very rare and missense mutations preserve a residual function, thus, they are associated with a milder or later onset of the disease. FGD-1 occurs in early childhood with hypoglycaemia, developmental delay and frequent infections, all due to low cortisol levels. The reduced receptor sensitivity to ACTH leads to increased circulating ACTH concentrations which are associated with hyperpigmentation of the skin and tall stature. These latter signs are thought to result from increased MC_1 stimulation and estradiol synthesis by excessive circulatory ACTH levels (Imamine, et al., 2005).

The cell lines HEK293 and CHO do not express melanocortin receptors and provide a useful *in vitro* model for transfection with the MRAPs and the MCs. Chung and colleagues investigated 24 reported MC_2 polymorphisms with MRAP using cAMP reporter assays and cell surface analyses, they found that two thirds of these SNP mutations impaired MC_2 receptor transport to the cell surface, despite interacting with MRAP, and resulted in a failure of MC_2 receptor to respond to ACTH (Chung, et al., 2008).

Unlike *MC2R* mutations, almost all the mutations occurring in the *MRAP* are homozygous non-sense or splice-site mutations which cause severe truncation or ablation of the MRAP protein (Jain, et al., 2011), (See table 2). Those mutations were found to be associated with severe glucocorticoid deficiency immediately after birth or in early infancy with seizures due to hypoglycaemia. Cases of the FGD-2 have been reported where there is a later onset and a milder presentation of the symptoms. Sequencing the genome of individuals with later onset glucocorticoid deficiency revealed a homozygous missense (c.175T>G) mutation that caused an aspartame to tyrosine substitution at position 59 (N59Y). Whilst another case was reported a missense (c.76T>C) which led to an alanine to valine substitution at position 26 (V26A) (Hughes, et al., 2010).

MRAP$^{-/-}$ mice do not survive after birth without the intervention of *in utero* corticosterone and had impaired adrenal gland development (Novoselova, et al., 2018). MRAP is likely to have a major role in adrenal gland development and the severity of the FGD-2 could be due to underdevelopment of this gland. Although *MRAP2* is also expressed in the adrenal gland, it did not seem to compensate for absent or mutated *MRAP* and therefore, might not have a role in the pathogenesis of FGD. However, as there are still about 50% of FGD cases with unidentified mutations, further nucleotide sequencing and genome analysis of FGD individuals may reveal a role of MRAP2.

Whilst *MRAP* genetic mutations leading to glucocorticoid deficiency are rare, the use of synthetic glucocorticoids for inflammatory disorders and autoimmune diseases is

widespread. A side-effect of synthetic glucocorticoid treatment is adrenal insufficiency, characterised by the failure of the adrenal gland to respond to ACTH. In a rat model, five day exposure of methylprednisolone reduced ACTH and corticosterone concentrations and downregulated both *MRAP* and *StAR* expression (Spiga, et al., 2020).

Table 2: Human *MRAP* mutations which have been described to be associated with familial glucocorticoid deficiency-type 2. * This mutation in *MRAP* sequence causes primary adrenal insufficiency.

Type of mutation	Codon substitution-Codon number	Amino acid substitution	FGD-2 Mutation phenotype	Reference
Missense	G >A-1	Met1Ile	Disruptive	(Metherell, et al., 2005)
Missense	C >A-11	Tyr11Termination	Disruptive	(Metherell, et al., 2005)
Missense	T >C-26	Val26Ala	Disruptive	(Hughes, et al., 2010)
Missense	T >G-59	Tyr59 Asp	Disruptive	(Hughes, et al., 2010)
Missense	T >C-76	Val76Ala	Disruptive	(Hughes, et al., 2010)
Deletion	c.-17_23del	-	Disruptive	(Modan-Moses, et al., 2006)
Deletion	p.R451W in CYP11A1	-	*Disruptive (Primary adrenal insufficiency)	(Guran, et al., 2016)
Deletion	c.IVS3ds+1delG	-	Disruptive	(Metherell, et al., 2005)
Deletion	c.130del	Val44insTer	Disruptive	(Metherell, et al., 2005)

MRAP2 and Appetite control

Appetite control comprises a complicated neuroendocrine circuit involving several hormones, the sympathetic and parasympathetic nervous system and some elements of the limbic system. MC_4 regulates energy expenditure and appetite control and has been found in areas of the brain associated with energy homeostasis: *MC4R* mRNA has been detected in the paraventricular nucleus (PVN), ventromedial, medial preoptic and the supraoptic nucleus of the hypothalamus; all of which are integrated in controlling feeding behaviour (Siljee, et al., 2013). In the PVN, *MC4R* was detected in transcription factor single minded 1 (*SIM1*$^{+}$) expressing neurones, a population of neurones known to regulate food intake and mediate MC_4 signalling through a glutamatergic pathway (Xu, et al., 2013). The MC_4 agonist setmelanotide is in clinical trials for treating severe obesity with underlying genetic causes (Kühnen, et al., 2016; Clément, et al., 2018). *MC4R*$^{-/-}$ transgenic mice are severely obese and studies with these animals supported the involvement of MC_4 in appetite control, they had hyperphagia and were non-responsive to melanotan II (MTII), an α-MSH analogue (Marsh, et al., 1999). α-MSH is released by POMC neurons in the arcuate nucleus in response to sensory input from the periphery, this in turn stimulates PVN-MC_4 signalling leading to reduced appetite. Whilst leptin hunger signalling to the counteracting agouti-related peptide neurons causes the inactivation of the same PVN, leading to increased appetite. This is a simplified account of how MC_4 is involved in controlling feeding behaviour.

MRAP2 was found to be expressed at high levels in the brain and in areas that are associated with appetite control, where MC_4 has been comprehensively described (Liang et al., 2018; Bruschetta et al., 2018; Silijee et al 2013). Similar to transgenic *MC4R*$^{-/-}$ mice, both whole body *MRAP2*$^{-/-}$ and brain targeted *MRAP2*$^{-/-}$ mice were morbidly obese, however, *MRAP2*$^{-/-}$ mice do not show signs of hyperphagia or reduced energy consumption (locomotion and body temperature), they have normal expression of *POMC* but reduced *AgRP* levels; and they respond to MTII treatment (Asai, et al., 2013). Deletion of both *MC4R* and *MRAP2* produced mice that were less obese than single deletion *MC4R*$^{-/-}$ mice. Exposure of *MC4R/MRAP2* transfected cells with α-MSH increases cAMP production compared to *MC4R* expressing cells, further suggesting an interaction between MC_4 and MRAP2 (Asai, et al., 2013). MRAP2 was also found to interact with MC_3, cAMP production increased in *MC3R /MRAP2* transfected cells in response to α-MSH compared to *MC3R* expressing cells (Asai, et al., 2013).
MRAP2 might be involved in an energy homeostasis/feeding behaviour circuit independently of MC_4. MC_3 is perceived to have a minor role in energy homeostasis, *MC3R* knockout mice have an obese phenotype but with a reduced lean body mass and reduced appetite (Chen, et al., 2000). *MC3R* is expressed on the POMC neurons in the arcuate nucleus and thought to be exerting autocrine feedback control (Lee, et al., 2008). *MC3R, MC4R* and *MRAP2* have been observed in chicken hypothalamus; chicken (*c)MRAP* (a homolog of *MRAP2*) was found to enhance the constitutive

activity of ACTH treated c *MC3R* /c *MC4R* transfected cells in a CRE-luciferase reporter assay (Zhang, et al., 2017).

MRAP2 interactions with non-melanocortin receptors

The role of MRAP2 in mediating energy homeostasis and feeding behaviour extends beyond the melanocortin receptors. *MRAP2* is expressed in the AgRP neurones, aforementioned AgRP is an MC_4 antagonist; MRAP2 may regulate the AgRP neuronal activity, since deletion of *MRAP2* resulted in reduced *AgRP* expression and inhibited AgRP orexigenic activity in response to starvation (Srisai, et al., 2017). The MRAP2-AgRP effect may explain the reduced food intake reported in *MRAP2*$^{-/-}$ mice. GHSR-1a (growth hormone secretagogue receptor 1a) is a receptor found in the arcuate nucleus of the hypothalamus and binds ghrelin, which is released by the stomach, sending sensory input to stimulate feeding via AgRP neurones. Srisai et al described MRAP2 to enhance the signalling of GHSR-1a (Srisai, et al., 2017), via $G\alpha_{q/11}$ however, MRAP2 independently inhibits β-arrestin recruitment and signalling (Rouault et al., 2020). Whilst MRAP2 was found to stimulate GHSR-1 and thus feeding, it was also found to induce feeding by a counteracting mechanism, suppressing the prokineticin receptor 1 (PKR-1). *PKR-1* has a wide tissue distribution and mediates several functions including suppressing food intake. Measurement of surface density and surface expression of tagged-PKR-1 in *MRAP2* positive and negative cells revealed lower PKR-1 surface expression in MRAP2 expressing cells; MRAP2 may not contribute to PKR-1 transport to the cell surface (Chaly, et al., 2016). In the presence of MRAP2, PKR-1 was found to have reduced signalling, as lower production of cAMP and IP_3 were observed, respectively in the CRE-luciferase and IP-One assays (Chaly, et al., 2016).

An MRAP2/orexin receptor (OX1R) interaction has been described, OX1R is a hypothalamic receptor expressed in the PVN which stimulates food intake. MRAP2 was found to downregulate the surface expression of *OX1R* and inhibit OX1R signalling (Rouault et al., 2017). This study mapped the MRAP2 domains and found the C-terminal domain of MRAP2 inhibited signalling of both OX1R and PKR-1. The MRAP2 N-terminal 23-33 domain was involved in inhibiting the OX1R transport to the cell surface; and the N-terminal 34-43 domain was suggested to exert negative regulatory control over the MRAP2 activity (Rouault, et al., 2017). A distinct region of the MRAP2 N-terminus, from that required for OX1R and PKR-1, is suggested to regulate GHSR-1a signalling (Rouault et al., 2020).

MRAP2 and obesity

Polygenic obesity is affected by genetics, life-style and environmental factors which predispose to increased appetite and reduced energy expenditure; whole genome and exome sequencing have been invaluable to identify genetic factors. Monogenic

obesity has a negligible environmental influence, obesity is usually very severe and occurs in early childhood. A role for MRAP2 in the pathogenesis of obesity was suggested following a realisation that it regulates MC_4, which, as discussed above, is essential for energy homeostasis and feeding behaviours. *MRAP2* knockout mice have severe early onset obesity (Asai, et al., 2013). Asai et al sequenced the *MRAP2* coding regions and the intron-exon boundaries, in both adults and children with obesity. They discovered four genetic variants, one which resulted in a non-functional MRAP2 (E24X, see Table 3). The other three rare variants were towards the C-terminus and considered to have less impact on obesity (Asai et al., 2013 - see table 3). The different functional domains in the MRAP2 observed by Rouault et al., (2017), might suggest that these variants could influence interactions of MRAP2 / MC_4. Eight more genetic variants of *MRAP2* have been detected (3 intronic; 2 synonymous; 2 coding and one synonymous; see table 3) following mutational sequencing of the *MRAP2* coding region of extremely obese individuals (Schonnop, et al., 2016), which were absent in the control group. This study also reported reduced MC_4 signalling when co-transfected with the nonsynonymous *MRAP2* mutant (p.Gln174Arg) in HEK293 cells (Schonnop, et al., 2016). Studies of the 6q14.1-6q16.3 region revealed *SIM1*$^+$ and *MRAP2* variants in patients with Prader-Willi like-syndrome (Geets, et al., 2016).

The mechanism by which *MRAP2* deletion causes obesity is not clearly understood and predicted to be due to effects on MC_4. However, RT-qPCR analysis of hypothalamus from *MRAP2*$^{tm1a/tm1a}$ (transgenic mice with targeted exon 4 deletion) showed normal *MC4R* PVN content. In contrast, there was a 50 % reduction in the *SIM1*$^+$ in the PVN and decreased PVN expression of oxytocin, arginine vasopressin, corticotrophin-releasing hormone and thyrotropin-releasing hormone (Novoselova, et al., 2016). *MRAP2*$^{tm1a/tm1a}$ mice also had raised cholesterol and lipoprotein (HDL and LDL) concentrations, consistent with observations in MC_4 deficient mice; there is a suggestion that SIM1$^+$ is either activated downstream of MC_4 (Novoselova, et al., 2016) or mediates the MC_4 function within the hypothalamic PVN (Xu, et al., 2013).

Although mutations in the *MRAP2* cause an early onset obesity like *MC4R* mutations, Asai et al. (2013) observed no change in the feeding or energy expenditure patterns in the *MRAP2* knockouts compared to the *MC4R* knockouts. The transgenic mice used by Novoselova et al which have a different genetic background, show some changes in the feeding behaviour. These two reports preceded reports that MRAP2 interacts with the non-melanocortin GPCRs: GHSR-1a, ORX1 and PKR-1. This raises a possibility that, the obesity pathologies observed in *MRAP2* transgenic mice could involve pathways outside of melanocortin signalling.

The *MRAP2* exon regions were sequenced in a cohort of 9418 subjects, and 23 rare heterozygous variants were identified which independently increase the risk of obesity (Baron, et al., 2019). To elucidate a molecular mechanism these variants were co-transfected in CHO cells with *MC4R* and the cAMP production recorded in response

to ACTH or α-MSH. Seven *MRAP2* variants (c.3-7 del, G31V, F62V, F62C, N77S, K102* and P195L) decreased cAMP produced in response to ACTH or α-MSH. The variants C5-5del, c.3-7 del, G31V, F62V, F62C, N77S, K102* and P195L were associated with obesity (table 3), the authors remarked that 75% of the carriers of *MRAP* variants also displayed an abnormal eating behaviour (Baron, et al., 2019). *Lep*, *LepR*, *MC4R*, *PCSK1*, *POMC* and *SIM1* are implicated in monogenic obesity, however, individuals with *MRAP2* variants also have higher hypertension and hyperglycaemia (Baron, et al., 2019). As mentioned above, Srisai *et al* reported that MRAP2 enhances the ghrelin signalling through GHSR-1a, ghrelin receptor has been described to affect blood pressure regulation (Mao, et al., 2016) and further investigation of MRAP2 and ghrelin signalling is required to understand the hypertension phenotype described in these individuals.

Table 3: *MRAP2* mutations associated with obesity in humans. 1- *MRAP2* heterozygous mutations detected using coding/intron-exon sequencing of *MRAP2* gene in coding exons; 2- two cohort studies with severe obesity; 3- patients with Prader-Willi like phenotype; 4- Genome -wide association study for severe early -onset obesity.

Type of mutation	Codon substitution-Codon number	Amino acid substitution	Mutation phenotype	Reference
Missense	G >A-3	Ala3Thr	Uncertain significance	(Baron, et al., 2019)
Missense	G >T-3	Ala3Ser	Uncertain significance	(Baron, et al., 2019)
Missense	C >G-13	Gln13Glu	Uncertain significance	(Baron, et al., 2019)
Nonsense	G >T-24	Glu24Term	Disruptive	(Asai, et al., 2013)
Missense	G >T-31	Gly31Val	Disruptive	(Baron, et al., 2019)
Missense	C >T-32	Pro32Leu	Uncertain significance	(Baron, et al., 2019)
Missense	G >T-40	Ala40Ser	Non-pathogenic	(Geets, et al., 2016)
Missense	T >G-62	Phe62Cys	Disruptive	(Baron, et al., 2019)
Missense	A >G-77	Asn77Ser	Disruptive	(Baron, et al., 2019)
Missense	A >T-88	Asn88Tyr	Non-pathogenic	(Asai, et al., 2013)
Missense	T >C-91	Val91Ala	Uncertain significance	(Baron, et al., 2019)
Missense	G >C-99	Glu99Gln	Uncertain significance	(Baron, et al., 2019)
Missense	A >T-102	Lys102X	Disruptive	(Baron, et al., 2019)
Missense	A >G-113	Arg113Gly	Uncertain significance	(Baron, et al., 2019)
Missense	T >G-114	Ser114Ala	Uncertain significance	(Baron, et al., 2019)
Missense	C >G-115	Leu115Val	Non-pathogenic	(Asai, et al., 2013)
Missense	A >G-121	Asn121Ser	Uncertain significance	(Baron, et al., 2019)
Missense	C >T-125	Arg125Cys	Uncertain significance	(Baron, et al., 2019)
Missense	G >A-125	Arg125His	Non-pathogenic	(Schonnop, et al., 2016)
Missense	C >T-133	His133Tyr	Uncertain significance	(Baron, et al., 2019)
Missense	G >A-137	Ala137Thr	Non-Pathogenic	(Schonnop, et al., 2016)

Missense	T >C-162	Met162Thr	Uncertain significance	(Baron, et al., 2019)
Missense	A >G-174	Gln174Arg	Non-pathogenic	(Schonnop, et al., 2016)
Missense	A >G-193	Thr193Ala	Uncertain significance	(Baron, et al., 2019)
Missense	C > T-195	Pro195Leu	Disruptive	(Baron, et al., 2019)
Missense	G >T-203	Asp203Tyr	Uncertain significance	(Baron, et al., 2019)
Deletion	c.-5_5del	-	Loss of function	(Baron, et al., 2019)
Deletion	c.-3_7del	-	Loss of function	(Baron, et al., 2019)

MRAP and insulin signalling

The first description of MRAP came from murine 3T3L1 adipocytes; a novel low molecular weight protein was found in differentiated adipocytes but not pre-adipocytes, and in both in brown and white adipose tissue. Initially named fat tissue low molecular weight protein (FALP) (Xu, et al., 2002) it was subsequently renamed MRAP after recognition of the MC_2 interaction (Metherell, et al., 2005). PPARγ, a regulator of adipocyte differentiation, activates transcription of *MRAP* (Kim, et al., 2013). MRAP was observed in adipocytes at the peri-nuclear membrane, but if adipocytes were exposed to insulin the distribution changed, with discrete MRAP specks distributed throughout the cytoplasm (Xu, et al., 2002).

Baron et al reported that siRNA *MRAP2* knockdown in EndoC-βH1 cells reduced insulin secretion (Baron, et al., 2019). In adipocytes, MRAP is required for lipolytic responses to ACTH through MRAP interaction with Gαs (Zhang, et al., 2018). Increased insulin sensitivity in response to a high fat diet has been reported in transgenic mice with *MRAP* overexpression in adipose tissue, correlating with decreased plasma triglyceride and cholesterol concentrations. Insulin resistance and glucose intolerance has also been described in *MRAP2* knockout mice (Rouault, et al., 2017). Further research is required to elucidate these pathways.

Inflammation

MC_1 and MC_3 signalling through α-MSH and ACTH mediate anti-inflammatory responses downregulating and inhibiting the release of proinflammatory cytokines: TNF-α, INF-γ, IL-6; inducible nitric oxide synthase and the chemokine receptors CXC1 and 2 whilst stimulating IL-1 release (Catania, et al., 1998; Catania, et al., 2004; Getting, et al., 2003). MC_3 therapeutics targeted for rheumatoid arthritis and other chronic inflammatory conditions report side effects of hyperpigmentation and increased melanoma risk, through hyperstimulation of MC_1. Adrenal inflammation is associated with side effects of synthetic corticosteroids where MRAP2 is downregulated (Spiga, et al., 2020). Additional investigations may determine whether either MRAP isoform has an anti-inflammatory role.

MRAP2- Single nucleotide variations related to cancer

The BioMuta single-nucleotide variation (SNV) and disease database revealed *MRAP2* SNV to be linked to different types of cancers with varying frequencies. High frequency of SNV was seen with melanomas and uterine cancers followed by a lower variation frequency seen in colorectal, lung, stomach and liver cancers (Simonyan, and Mazumder, 2014). As discussed above *MC1R* mutations are associated with melanoma (Sánchez-Laorden, et al., 2007), although not affecting MC_1 surface expression, MRAP2 reduces responsiveness of MC_1 to the synthetic α-MSH agonist NDP-MSH and affects receptor signalling (Chan, et al., 2009). Other types of cancers that were showed a lower *MRAP2* SNV include breast, thyroid, prostate, pancreas, urinary tract and brain cancers (Simonyan, and Mazumder, 2014). SNV that are linked to *MRAP2* over-expression included liver, lung and pancreatic cancers, however, SNV leading to *MRAP2* suppression included breast, thyroid and prostate cancers. The tissue distribution of MRAP2 is shown in table 1; MRAP2 has not been reported in healthy human liver, thyroid or urinary tract. The association of MRAP2 to a wide variety of cancers is of interest. However, the potential pathogenesis and involvement of the melanocortin receptors or other GPCRs needs to be elucidated.

Female fertility

Recently in this journal, MRAP2 was described to be significantly up-regulated in women with unexplained infertility (D'Aurora, et al., 2019). *MRAP-α, MRAP-β* and *MRAP2* have low expression in the endometrium (Table 1). Hypertrophy of the uterine gland epithelium was observed in women with unexplained infertility. The increased *MRAP2* expression suggests that MRAP2 might influence the stability of the endometrium (D'Aurora, et al., 2019); understanding the roles of MRAP and MRAP2 in the uterus and female reproductive axis may reveal a role for melanocortin signalling in infertility.

Summary

In summary, genetic variants of *MRAP* are associated with severe familial glucocorticoid deficiency, however evidence is emerging that synthetic glucocorticoids may also disrupt ACTH and *MRAP* expression resulting in adrenal inflammation. *MRAP2* genetic variants are associated with severe obesity but the phenotypes are subtly different to the phenotypes observed with *MC4R* genetic variants. There is a great deal of interest in the development of melanocortin therapeutics to treat obesity, as evidenced by setmelanotide, and fully understanding the role of MRAP2 and the

interactions with both melanocortin receptors and other GCPR would be of great value. The use of the *MRAP2* knockout and transgenic models have aided understanding of interactions with the different MCs in metabolic regulation, feeding behaviour and energy homeostasis. *MRAP* is detected in areas with absent or extremely low MC_2 expression, potentially it also may bind to non-melanocortin receptors. Further studies with transgenic models and insights from studies of genetic variants may provide further insights into the MRAP physiological processes.

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MRAP: https://en.wikipedia.org/wiki/Melanocortin_2_receptor_accessory_protein
MRAP2: https://en.wikipedia.org/wiki/MRAP2.

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