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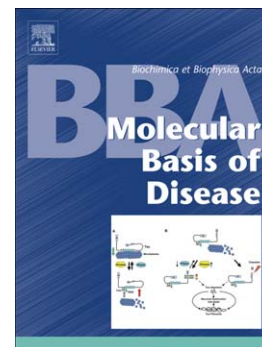
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Melanocortin Receptor Accessory Proteins (MRAPs):

Functions in the Melanocortin System and Beyond.

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Abstract

G-protein coupled receptors (GPCRs) are regulated by numerous proteins including kinases, G-proteins, β -arrestins and accessory proteins. Several families of GPCR accessory proteins like Receptor Activity Modifying Proteins, Receptor Transporting Proteins and Melanocortin Receptor Accessory Proteins (MRAPs) have been identified as regulator of receptor trafficking, signaling and ligand specificity. The MRAP family contains two members, MRAP1 and MRAP2, responsible for the formation of a functional ACTH receptor and for the regulation of energy homeostasis respectively. Like all known GPCR accessory proteins, MRAPs are single transmembrane proteins, however, they form a unique structure since they assemble as an anti-parallel homodimer. Moreover, the accepted idea that MRAPs are specific regulators of melanocortin receptors was recently challenged by the discovery that MRAP2 inhibits the activity of prokineticin receptors. Recent studies are starting to explain the role of the unusual structure of MRAPs and to illustrate the importance of MRAP2 for the maintenance of both energy and glucose homeostasis.

Introduction

The Melanocortin Receptor Accessory Protein (MRAP) family contains two members, MRAP1 and MRAP2. In humans, alternative splicing of the MRAP1 gene produces two isoforms MRAP1 α and MRAP1 β (1). MRAPs are small single transmembrane proteins that interact with and regulate the trafficking and signaling of several G-protein coupled receptors. In the adrenals, MRAP1 is essential for the trafficking of the ACTH receptor (Melanocortin-2 receptor) (1-3) to the plasma membrane and mutations in MRAP1 result in the loss of functional ACTH receptors (3). Consequently, mutations in either MRAP1 or the ACTH receptor clinically result in familial glucocorticoid deficiency (1). MRAP2 is expressed in several tissues including the brain where it plays an important role in the regulation of energy homeostasis (4-6). In fact, deletion of MRAP2 causes severe obesity in mice (6) and mutations in MRAP2 have been identified in obese patients (6,7). Both MRAP1 and MRAP2 display an unusual structure since they form anti-parallel homodimers (2,3), a structure unique to MRAPs in the eukaryotic proteome. In this article we will review recent findings on the structure and roles of MRAPs.

The Role of MRAPs in the Adrenal Gland

The adrenocorticotrophic hormone (ACTH) is issued from the processing of the proopiomelanocortin in the anterior pituitary gland and released into circulation where it acts mainly on the adrenal glands to stimulate glucocorticoid synthesis (8-11). ACTH mediates its corticotropin action through the activation of the melanocortin-2 receptor (MC2R a.k.a. ACTH receptor) in the adrenal cortex. MRAP1 was identified as an obligatory accessory protein of the MC2R (1) and was shown to be required for both surface expression (1,2) and signaling of the receptor

(2,3). The MC2R/MRAP1 complex exists primarily in the zona fasciculata of the adrenal cortex (12), where glucocorticoids are produced. Because MRAP1 is required for MC2R activity, mutations in either MC2R or MRAP1 result in the manifestation of the adrenal disease, familial glucocorticoid deficiency. MRAP1 is not only required for trafficking the MC2R to the cell surface but also for ACTH binding to the receptor (3). The second member of the MRAP family, MRAP2, can form a complex with MC2R and promote its trafficking to the plasma membrane (12,13), however, the affinity of MC2R for ACTH when expressed with MRAP2 is very low (13). Indeed, whereas sub-nanomolar concentrations of ACTH stimulate cAMP production in cells expressing MC2R and MRAP1, micromolar concentrations of ACTH are required to stimulate MC2R signaling when co-expressed with MRAP2 (13). Because MRAP2 fails to produce a signaling competent MC2R, the role of MRAP2 in adrenal glands could be to compete with MRAP1 for MC2R binding, therefore inhibiting MC2R activity.

The Unusual Structure of MRAPs

MRAP1 and MRAP2 are small single transmembrane proteins that contain a single glycosylation site in the extreme N-terminus. MRAPs were originally predicted to display a type II topology (N_{in}/C_{out})(1), however, using both biochemical and imaging approaches, we showed that MRAPs actually have a dual topology, meaning that MRAPs are inserted in the membrane in both N_{in}/C_{out} and N_{out}/C_{in} orientations (2,3,13) (Fig. 1A). Additionally, inversely oriented protomers of MRAP1 and MRAP2 assemble to form anti-parallel homodimers and heterodimers (2,3,13). This unique structure of MRAPs was later confirmed by several other groups (14,15). Early experiments using antibodies specifically directed against the N- or C-terminus of MRAP1

showed that both ends of the endogenous protein were detectable on the extracellular side of the plasma membrane in non-permeabilized Y1 cells, an adrenal cell line expressing endogenous MRAP1 (2). Utilizing bi-molecular fluorescence complementation, in which two complementary fragments of YFP were fused on either the N-terminus or the C-terminus of MRAP1 respectively demonstrated, that the anti-parallel dimer of MRAP1 was formed in the ER early after translation (3,16).

While the dual topology of MRAPs was established, the stability of the protomer's orientation was unclear and the ability of MRAP1 molecules to change their orientation once inserted in the membrane remained unknown. A recent study from Maben et al. elegantly showed that the dual topology of MRAP1 is not only achieved in the ER, but that once inserted in the membrane, the orientation of each protomer is fixed (17). Maben et al. demonstrated this property of MRAP1 by selectively inserting a biotinylation site on either the N-terminus or C-terminus of MRAP1 and determining the biotinylation state of each protomer when the biotin transferase BirA was specifically targeted to the lumen of the ER or in the cytosol. Results from this experiment showed that the extracellular N or C-terminal regions of MRAP1 were only biotinylated when BirA was targeted to the lumen of the ER, but not with BirA localized in the cytosol, thus demonstrating that once inserted in the ER membrane the orientation of MRAP1 remains stable (17). Maben et al. also showed that cytosol localized BirA could only label the C-terminal biotinylation site of the N_{out} protomer and the N-terminal biotinylation site of the C_{out} protomer, thus further demonstrating that the orientation of MRAP1 across the membrane is stable (17). Whereas all the available evidence so far demonstrated that MRAPs form stable

anti-parallel oligomer, the mechanisms responsible for the formation of this unique structure as well as its stoichiometry remain to be established.

Specific roles of MRAP domains.

MRAP1 is a small single transmembrane protein containing. While the N-terminal and transmembrane regions of MRAP1 are well conserved in evolution, the C-terminal region is extremely variable. Using site-specific deletions and mutagenesis, we have identified several critical and functionally distinct regions of MRAP1. Amino acids 31-37 (LKANKHS) preceding the transmembrane domain are required for the dual topology of MRAP1 and their deletion results in MRAP1 being inserted almost exclusively in the N_{out}/C_{in} orientation (3) (Fig. 1B). Interestingly, while amino acids 18-21 (LDYI) are not necessary for MRAP1-mediated ACTH receptor trafficking, deletion or mutation of those four amino acids in MRAP1 result in an ACTH receptor that is unable to bind ACTH or signals (3) (Fig. 1B). This finding establishes that the roles of MRAP1 in promoting ACTH receptor trafficking and signaling are distinct and rely on different regions of MRAP1. This exact LDYI motif is missing in MRAP2, thus explaining why expressing MRAP2 can promote ACTH receptor trafficking but not signaling, except at very high agonist concentration (1 μ M) (13). The specific sequence of the transmembrane (TM) domain is not required for the dual topology of MRAP1 but is functionally critical since replacing the TM of MRAP1 with the TM of the Receptor Activity Modifying Protein 3 produces a chimeric protein with dual topology but results in diminished MC2R trafficking and loss of ACTH-stimulated signaling (13). Malik et al. further demonstrated that only the TM region of the N_{out} protomer of MRAP1 is required for MC2R signaling (18). This was made evident after the TM of MRAP1 was replaced by the trans-

membrane region of CD8 exclusively in the N_{out} protomer causing a loss in MC2R-mediated cAMP production. Importantly, the same substitution of transmembrane domain in the C_{out} protomer of MRAP1 did not result in impaired MC2R signaling (18).

The C-terminus of MRAP1 is not required since its truncation does not affect the dual topology or function of MRAP1 (3). The C-terminus of MRAP1 may however, have a role in the fine tuning of MC2R signaling since small differences in the efficacy and potency of ACTH were measured when MC2R was expressed with different isoforms of MRAP1 (15) (Fig. 1). Little is known regarding the dimerization site(s) between MRAP1 and MC2R, however a fragment of MRAP1 mostly composed of the transmembrane domain was shown to co-immunoprecipitate with the MC2R (19). This finding suggests that the transmembrane domain of MRAP1 interacts with one or several of the transmembrane domains of MC2R, however, the tendency of highly hydrophobic peptides to be promiscuous warrants further investigation to prove the specificity of the described interaction.

Because of the dual topology of MRAP1 it was originally unclear if the required LDYI and LKANKHS motifs needed to be localized on the extracellular or intracellular side of the membrane. Malik et al. answered this question by fusing tandem dimers of MRAP1 to the ACTH receptor. Fusion of MRAP1-MRAP1 tandems to the N-terminal region of the ACTH receptor forced the orientation of the first protomer to be N_{in}/C_{out} and the orientation of the second protomer to be N_{out}/C_{in}. Using those fusion proteins, Malik et al. could specifically mutate or delete the LDYI or LKANKHS motifs in one protomer but not the other. Deleting or mutating those motifs in the N_{in}/C_{out} MRAP1 without modifying the N_{out}/C_{in} protomer did not alter ACTH receptor signaling. In contrast, mutating either motif in the N_{out}/C_{in} protomer caused an almost complete

loss of ACTH-stimulated signaling (18). Therefore, it was established that the ACTH receptors requires the N-terminal region of MRAP1 on the outside of the cell to function. They also demonstrated that the anti-parallel dimer of MRAP1 was required for ACTH receptor function since fusing a monomer of MRAP1 in either orientation to the ACTH receptor did not promote trafficking or signaling of the receptor (18).

Unlike MRAP1, MRAP2 shows significant evolutionary conservation throughout its sequence including the C-terminal region (Fig. 2), thus suggesting that, in contrast to MRAP1, the C-tail of MRAP2 may play an important functional role.

MRAP2: a Novel Regulator of Energy Homeostasis:

Until recently the physiological role of MRAP2 was unclear. As it is abundantly expressed in the hypothalamus and was known to interact with melanocortin receptors in-vitro (20), we investigated its impact on MC4R pharmacology and physiology. MC4R is a GPCR expressed in the hypothalamus, especially in the paraventricular nucleus (PVN) where it controls food intake and energy expenditure (21-23). Its endogenous agonists, α - and β -MSH, are the products of proopiomelanocortin (POMC) processing in the POMC neurons of the arcuate nucleus (ARC)(24). Mutations in the MC4R are the most common cause of monogenic early onset obesity and deletion of MC4R in mice recapitulate the obesity phenotype and increased linear growth observed in humans (23). In zebrafish, MC4R is critically involved in the larval growth and increased MC4R signaling leads to slower growth and shorter larvae (25).

Zebrafish have 2 isoforms of MRAP2, MRAP2a and MRAP2b, that both interact with the MC4R. However, their effect on MC4R pharmacology is very different. Whereas MRAP2a

strongly and dose dependently inhibits MC4R signaling by inhibiting agonist binding, MRAP2b potentiates α MSH-stimulated cAMP production downstream of zMC4R (4). Another clear difference between MRAP2a and MRAP2b is the onset of their expression. MRAP2a is expressed early in the embryo but MRAP2b is only expressed later in the life of the zebrafish. Consistent with the inhibitory activity of MRAP2a on MC4R, we showed that knocking-down the expression of MRAP2a in WT zebrafish embryos caused a significant growth delay that was not observed when MRAP2a was knocked-down in MC4R KO fish, thus demonstrating in-vivo that MRAP2 is an important regulator of the MC4R (4). In an elegant study conducted in mice, Asai et al. showed that MRAP2 KO mice develop obesity, and that mammalian MRAP2 potentiates MC4R signaling in-vitro (6). They also showed that specifically deleting MRAP2 in Sim1 positive hypothalamic neurons, some of which express MC4R, is sufficient to largely replicates the obesity phenotype of the global MRAP2 KO animals.

Whereas, both MC4R KO mice and MRAP2 KO animals develop severe obesity, significant differences exist between the two animal models. While the onset of obesity in MC4R KO mice is very early and is largely due to hyperphagia and decreased energy expenditure (23), MRAP2 KO mice display a later onset of obesity without measurable increase in food intake or impairment in energy expenditure (6). Additionally, MRAP2 KO animals retain a normal anorexigenic response to central injection of the MC4R agonist MTII, thus suggesting that the MC4R remains functional. Interestingly, the double MC4R/MRAP2 KO mice develop obesity however, they weigh less than MC4R KO mice (6), suggesting that MRAP2 likely regulates energy homeostasis through both MC4R-mediated and MC4R-independent pathways.

The role of MRAP2 in MC4R signaling is also supported by recent genetic studies that identified non-synonymous mutations in the MRAP2 gene of obese patients. In particular the mutation MRAP2-Q174R, detected in an individual with extreme obesity but not in control subjects, was reported to have lost its potentiating effect on MC4R when tested in-vitro (7). This finding suggests that a decreased MC4R activity caused by the loss of MRAP2 function may contribute to the obesity phenotype observed in the carrier.

A new mechanism of MC4R signaling has recently been identified by Ghamari-Langroudi et al. in hypothalamic neurons. They showed that MC4R directly couples to the Kir7.1 potassium channel in a G-protein independent manner (26). It will be interesting to determine if, in addition to regulating MC4R signaling through G-proteins, MRAP2 is capable of modulating MC4R signaling through Kir7.1.

In support of the hypothesis that MRAP2 regulates energy homeostasis through pathways independent of MC4R, our group showed that MRAP2 interacts with, and regulates the activity of, prokineticin receptors (PKRs) (5). PKR1 is a $G\alpha_s$ and $G\alpha_{q/11}$ coupled receptor expressed in several tissues including POMC neurons of the hypothalamus (27,28) where it regulates food intake (27,29). Like MC4R, PKR1 stimulation potently decreases food intake, and PKR1 KO mice have been shown to develop obesity (30). Surprisingly, in contrast to its activity on MC4R, MRAP2 inhibits PKR1 trafficking and signaling in-vitro (5). In addition, the anorexigenic effect of the PKR1 agonist, PK2, is enhanced in MRAP2 KO mice compared to WT siblings, thus demonstrating the inhibitory role of MRAP2 on PKR1 in-vivo (5). The mechanism through which PKR1 regulates food intake is still not well understood. Whereas studies from Gardiner et al. suggest that the anorexigenic action of PKR1 is at least partly mediated through the melano-

cotin system (27), our results suggest that the contribution of the MC4R may not be essential to the anorexigenic action of PKR1 since the inhibition of food intake caused by central PK2 injection is not decreased in MC4R KO mice compared to WT siblings (5).

Ultimately, the identification of MRAP2 as a regulator of PKR1 illustrates that MRAP2 can modulate the trafficking and signaling of GPCRs outside of the melanocortin receptor family and that MRAP2 can either potentiate or inhibit GPCRs depending on the target. MRAP2 also prevents the trafficking and signaling of PKR2 (5), a receptor closely related to PKR1. Mutation in PKR2 have mainly been implicated in the development of the Kallmann syndrome (31-33), however, PKR2 is expressed in the hypothalamus, therefore a role of PKR2 in the regulation of energy homeostasis is possible.

Promiscuity of MRAPs:

Until recently MRAPs were thought to specifically interact and regulate melanocortin receptors. Whereas MRAP1 was shown to be required for MC2R (1,2) and inhibitory for MC5R (16), MRAP2 was shown to potentiate the MC4R receptor (4,6). It is now clear that, at least in the case of MRAP2, those accessory proteins are able to interact and modulate the trafficking and signaling of GPCRs that do not belong to the melanocortin receptor family (5). As mentioned earlier, the first non-melanocortin receptors identified as being regulated by MRAP2 are PKR1 and PKR2 (5). Because of the obesity phenotype of MRAP2 KO animals (6), assessing the effect of MRAP2 on GPCRs known to be involved in the regulation of energy homeostasis may prove fruitful. While it appears that MRAP2 can regulate several families of GPCRs, it is important to note that it displays a high level of selectivity for its targets. Indeed, numerous GPCRs

including the Glucagon-like peptide-1 receptor, the MC3R, the neuropeptide receptors 2 and 5 and the β -adrenergic receptor, have been shown not to be modulated by MRAP2 (4,5). Some of the main challenges will be to understand how MRAPs recognize their different receptor partners and to identify the mechanisms involved in the specific potentiation or inhibition of each target.

MRAP2 and glucose homeostasis

While the importance of MRAP2 in regulating energy homeostasis has been demonstrated by several groups (4,6,7), its role in glucose homeostasis is still unclear. In fact, contradictory reports are present in the literature. The first study describing MRAP2 as a regulator of energy maintenance in rodents reported no difference in the ability of MRAP2 KO mice to clear glucose (6). A more recent study by Novoselova et al. using a different MRAP2 KO model obtained from the EUComm consortium (*Mrap2*^{tm1a(EUComm)Wtsi}), showed that deletion of MRAP2 results in glucose intolerance (34). The discrepancy observed between the two studies may be due to subtle genetic differences between the two models of MRAP2 KO mice. In our hands using the *Mrap2*^{tm1a(EUComm)Wtsi} animals, we find that mice lacking MRAP2 are both glucose intolerant and insulin resistant even at a young age (7 weeks old) and before they develop an obesity phenotype (Fig. 4A-C). We also find that the insulin resistance becomes more severe as the mice grew older (Fig. 4D-F). The discrepancy in the results obtained from the different mouse models used, as well as the lack of understanding of the mechanism through which MRAP2 regulates glucose homeostasis highlights the necessity of more in depth studies. Because MRAP2 is not significantly expressed in tissues directly involved in glucose uptake or release like skelet-

al muscle, adipose tissue or the liver, it is likely that MRAP2 centrally mediates its actions on glucose homeostasis.

Conclusions

MRAP proteins are essential for the function of several GPCRs. In contrast with the original theory, the roles of MRAP proteins, or at least of MRAP2, are not limited to the regulation of melanocortin receptors. Additionally, several aspects of MRAPs biology remain unclear. For example, the reasons behind the requirement for the unique structure of MRAPs is not understood and may suggest the existence of distinct functions for MRAPs on either side of the plasma membrane. Also, the mechanisms involved in the modulation of GPCR trafficking, ligand binding and MRAPs selectivity for their specific GPCR targets are unknown. It is also not understood how MRAPs can potentiate a group of GPCRs (MC2R, MC4R) while inhibiting another (MC5R, PKR1, PKR2). It is likely that understanding the mechanism of action of MRAPs will unveil important characteristics of GPCR accessory protein biology and GPCR pharmacology that may ultimately prove instrumental for pharmaceutical effort aimed at modulating the activity of various receptors for therapeutic applications.

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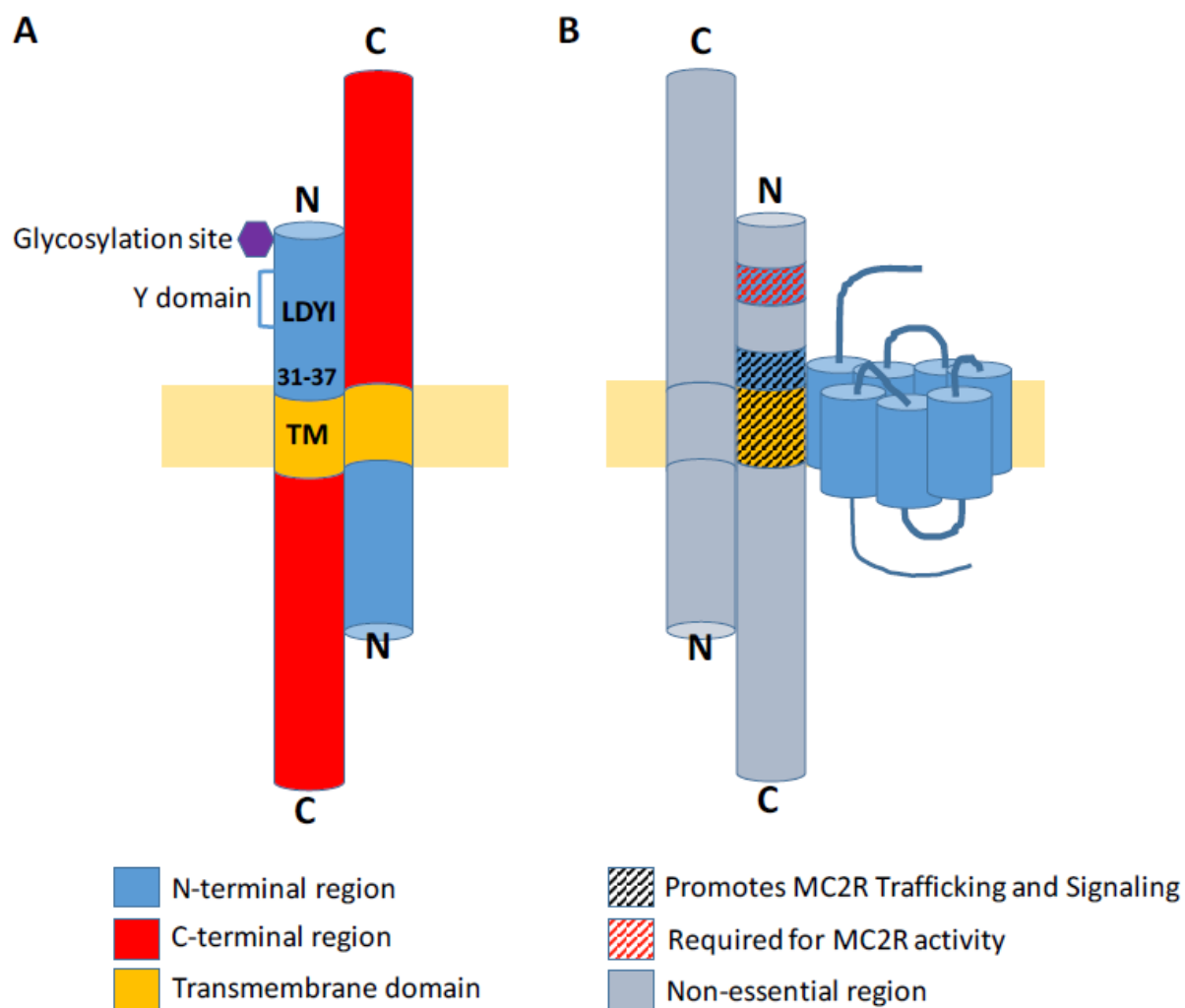


Figure 1: Schematic representation of MRAP structure and domains.
A. Representation of MRAP anti-parallel homodimer. **B.** Localization and function of important MRAP domains for MC2R trafficking and/or signaling

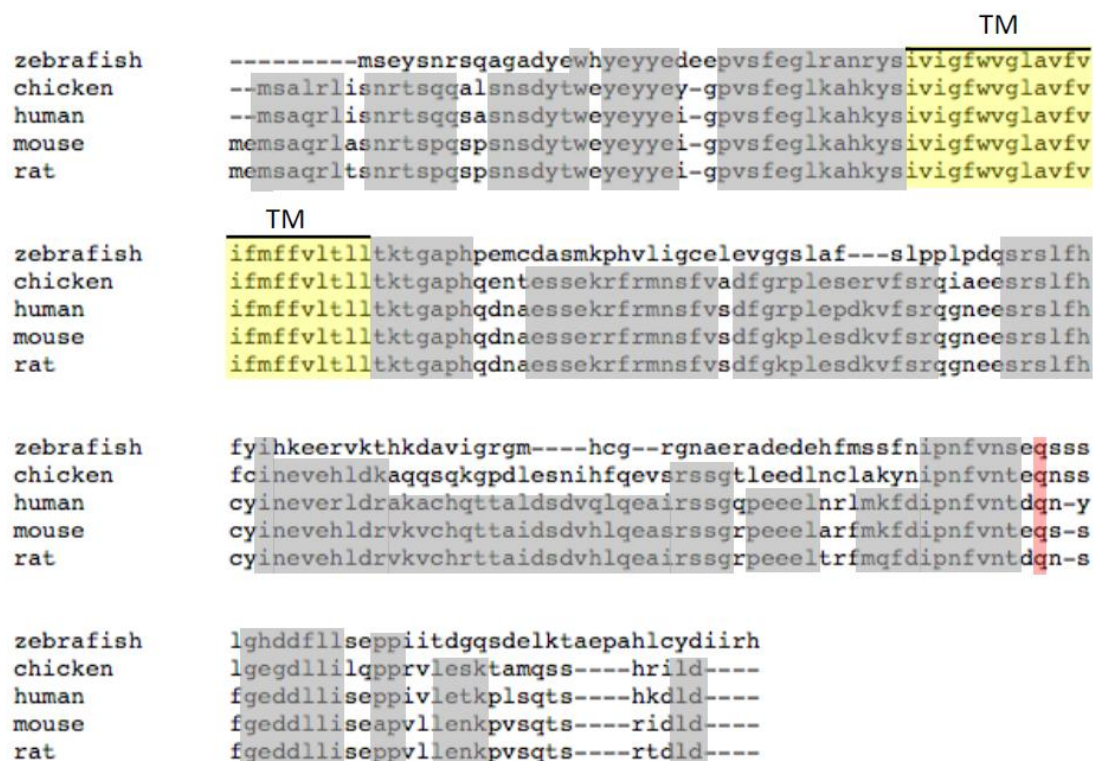


Figure 2: Sequence alignment of MRAP2 from several species. Yellow highlight depicts the position of the transmembrane domain. Gray highlights depict positions of high sequence homology. Red highlight shows the position of the mutated amino acid in the Q174R mutant.

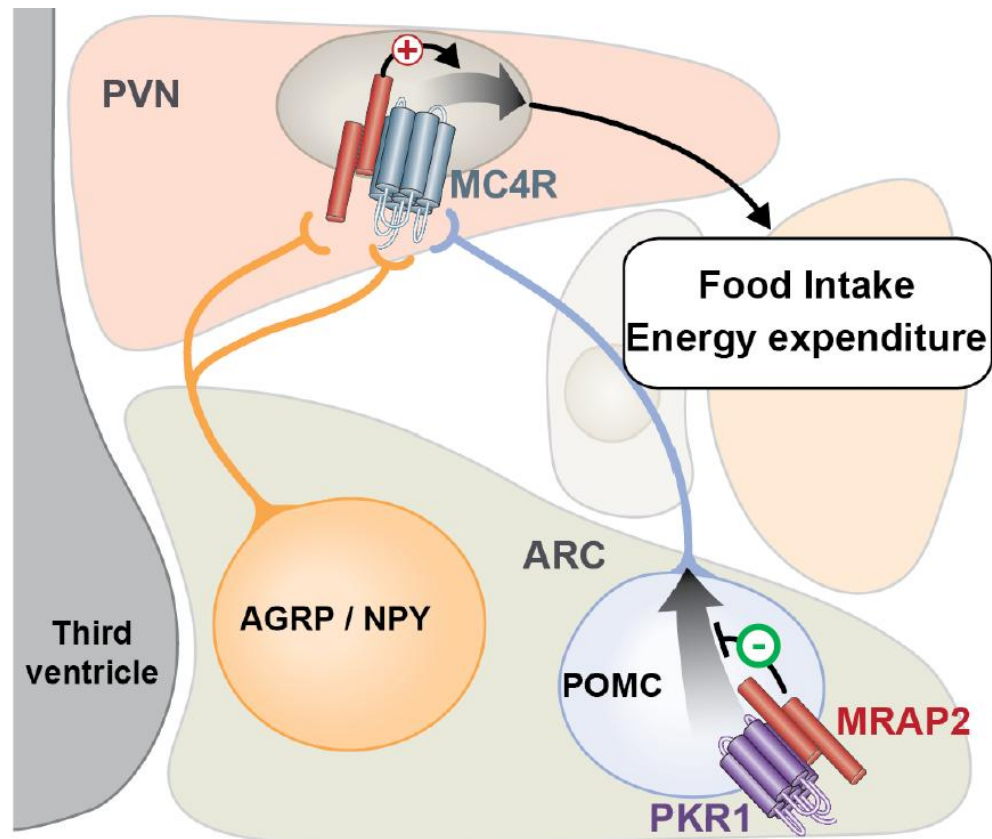


Figure 3: Regulatory action of MRAP2 on MC4R and PKR1 in the hypothalamus

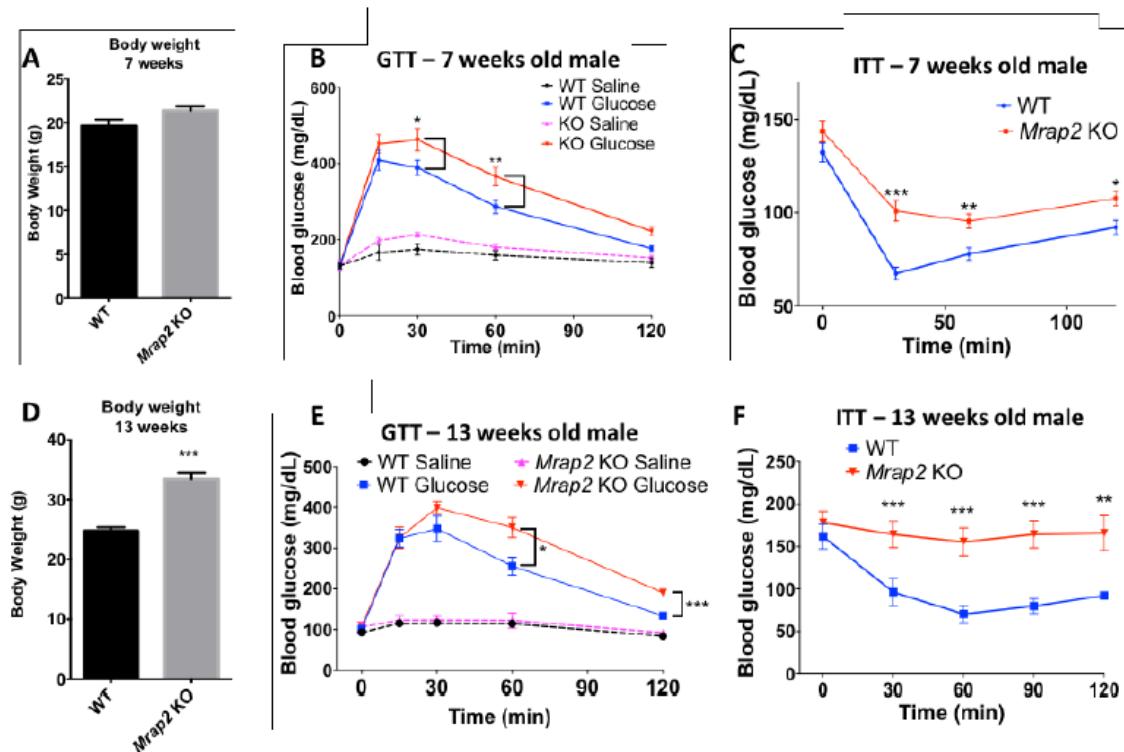


Figure 4: MRAP2 KO mice are insulin resistant and glucose intolerant. A. Body weight of 7 week old MRAP2 KO mice and WT siblings. **B.** GTT in of 7 week old MRAP2 KO mice and WT siblings. **C.** ITT in of 7 week old MRAP2 KO mice and WT siblings. **D.** Body weight of 13 week old MRAP2 KO mice and WT siblings. **E.** GTT in of 13 week old MRAP2 KO mice and WT siblings. **F.** ITT in of 13 week old MRAP2 KO mice and WT siblings. * $p < 0.05$

Highlights

- MRAP proteins display a unique antiparallel structure.
- The antiparallel structure of MRAP is achieved in the ER and is stable.
- MRAP2 is not specific to melanocortin receptors and interacts with other GPCRs.
- MRAP2 is an important player in the regulation of energy and glucose homeostasis.