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## Evaluating the Interaction Between the Human Melanocortin-2 Receptor and the Accessory Protein, Mrap1: Chimeric Receptor and Alanine Substitution Studies on Transmembrane Domain 4, Extracellular Loop 2, and Transmembrane Domain 5

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Evaluating the Interaction between the Human Melanocortin-2 Receptor and the  
Accessory Protein, Mrap1: Chimeric Receptor and Alanine Substitution Studies on  
Transmembrane Domain 4, Extracellular Loop 2, and Transmembrane Domain 5

A Dissertation

Presented to

the Faculty of Natural Science and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Perry Davis

November 2018

Advisor: Dr. Robert M. Does

Author: Perry Davis

Title: Evaluating the Interaction between the Human Melanocortin-2 Receptor and the Accessory Protein, Mrap1: Chimeric Receptor and Alanine Substitution Studies on Transmembrane Domain 4, Extracellular Loop 2, and Transmembrane Domain 5

Advisor: Dr. Robert M. Does

Degree Date: November 2018

## ABSTRACT

The melanocortin-2 receptor (MC2R) is the most complex due to its trafficking and ligand selectivity requirements for proper activation. The MC2R requires the melanocortin receptor accessory protein-1 (MRAP1) for proper trafficking and activation of the receptor by the melanocortin hormone, ACTH. MRAP1 is a single transmembrane-spanning domain protein that creates a homodimer with another MRAP1 protein. Furthermore, MRAP2 creates a heterodimer with the MC2R. Previous studies have shown that the MRAP1 protein contains an activation motif required for activation of MC2R and this activation motif located on the extracellular space side of the plasma membrane of the cell. The objective of this dissertation was to analyze potential contact sites between the extracellular space side activation motif of MRAP1 with the extracellular domains of the MC2R—the N-terminal, extracellular loop 1, extracellular loop 2, and extracellular loop 3. This analysis utilized a chimeric protein paradigm as well as alanine substitution experiments to observe potential contact sites between MRAP1 and the MC2R. By using these approaches, important residues required for trafficking or activation were identified in transmembrane 4, extracellular loop 2, and transmembrane 5 domains for MC2R. These results propose a revised mechanism for MC2R activation. Finally, the revised model suggests evolutionary implications for vertebrate MC2R activation.

## ACKNOWLEDGEMENTS

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## INTRODUCTION

The melanocortin receptors (MCRs) are a family of hormone-activated receptors that influence a number of physiological functions in vertebrates. The melanocortin receptor family consists of five different receptors, which were named in the numerical order in which they were cloned from the human genome (Cone, 2006). More so, each of these receptors is coded on its own gene, and these receptors are expressed in different cells and tissues (Cone, 2006) throughout an organism. Melanocortin receptors are G protein-coupled receptors (GPCRs) that belong to the rhodopsin/ $\beta_2$ -adrenergic-like family of GPCRs. G protein-coupled receptors are the largest group of cell surface receptors (Wettschureck and Offermanns, 2005).

The MCRs appear to be the smallest GPCRs within their subfamily in terms of amino acid length, and have relatively short N- and C- terminal ends (Cooray & Clark, 2011). In terms of the location and function of the melanocortin receptors (Cone, 2006), MC1R is located on melanocytes, in areas of the brain, and on macrophages. This MCR plays a role in pigmentation (melanocytes), body temperature regulation (CNS), and has anti-inflammatory properties. MC3R is predominantly expressed in the brain, but can be found in the placenta, stomach and pancreas; its main function lies in energy metabolism. MC4R is expressed mainly in the brain, as well as the autonomic nervous system, and



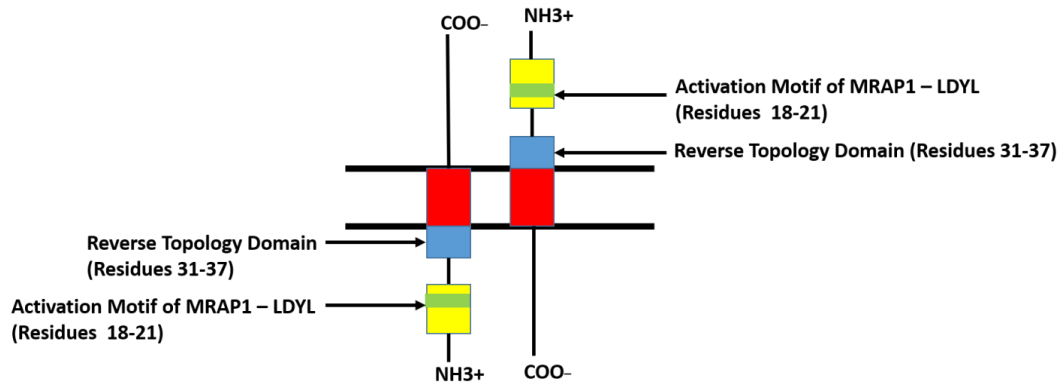
spinal cord where it plays a role in the regulation of food consumption and energy output. MC5R is expressed in many different tissues including skin, adrenal and exocrine glands. It is thought to play a role in the production of lipids by sebaceous glands of the skin, as well as some regulation of the immune system. Finally, MC2R is located in the adrenal cortex, and is involved in the initiation of steroidogenesis; the production of the glucocorticoid, cortisol. However, this receptor is also expressed in melanocytes, as well as, in adipocytes. MC2R is unlike any of the other melanocortin receptors because of its unique intracellular trafficking properties and ligand selectivity.

The MCRs can be activated by different peptide hormones derived from the proopiomelanocortin (POMC) gene. This gene is a member of the opioid/orphanin gene family (Dores & Baron, 2010). POMC is expressed in the pituitary gland and is responsible for the production of POMC propeptides in the corticotrophic and melanotrophic cells of the pituitary. Selective post-translational cleavage in the anterior and intermediate pituitary produces the melanocortin hormones adrenocorticotropin (ACTH),  $\gamma$ -MSH,  $\alpha$ -MSH, and  $\beta$ -MSH (Dores & Baron, 2010). Of the five melanocortin receptors (i.e., MC1R, MC2R, MC3R, MC4R, MC5R) that have been characterized in vertebrate genomes, activation of these receptors by melanocortin peptides (i.e., ACTH or the MSH-sized ligands) is fairly uniform with one exception: MC2R, as demonstrated by the human ortholog of this receptor (Cone, 2006; Sebag & Hinkle, 2007). First, one of the unique characteristics of the human melanocortin-2 receptor (hMC2R) is that this receptor has a strict requirement for interaction with the melanocortin-2 receptor accessory protein 1 (MRAP1) to facilitate receptor trafficking to the plasma membrane,

and activation of the receptor by ACTH (Metherell et. al, 2005). However, the other melanocortin receptors (MCRs) do not have an obligatory requirement for MRAP1 to facilitate trafficking or activation (Sebag and Hinkle, 2009).

Of these five melanocortin receptors, MC2R orthologs require interaction with MRAP1, to facilitate trafficking to and activated at the plasma membrane of a respective target cell (Hinkle and Sebag, 2009). MRAP1 is a single-chain polypeptide with a single transmembrane spanning domain. MRAP monomers form an antiparallel homodimer (Sebag & Hinkle, 2007), hence the homodimer has reverse topology (Figure 1). In humans, there are two isoforms of MRAP1,  $\alpha$ - and  $\beta$ -, which are the result of alternative splicing of exons of the MRAP1 primary transcript (Metherell et al., 2005). Interestingly, in other tetrapods and teleosts the MRAP1 mRNA does not appear to undergo alternative splicing. Furthermore, mutations in MRAP1 cause familial glucocorticoid deficiency type II (FGD II) (Webb & Clark, 2010).

In terms of the primary sequence of MRAP1 orthologs, there are three distinct domains that are required for successful trafficking and activation of MC2R. For example, in mouse MRAP1 (Figure 1), residues 38-61 (transmembrane domain) are involved with trafficking

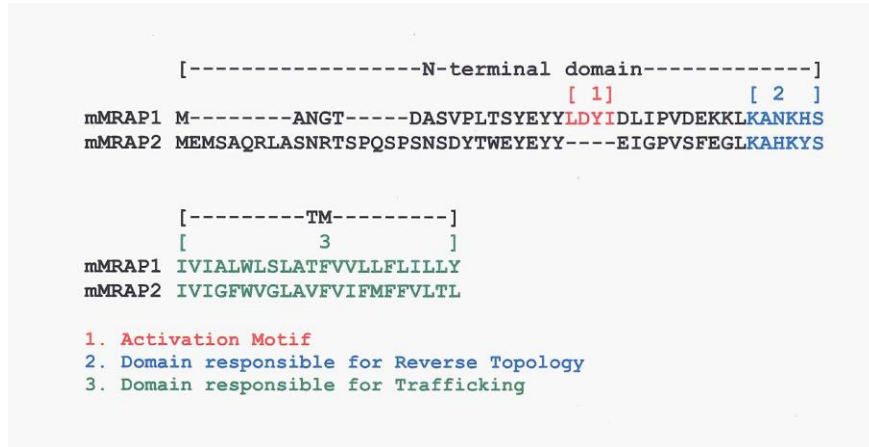


**Figure 1: Schematic of mammalian MRAP1.** Adapted from Webb and Clark (2010). MRAP1 forms a homodimer with reverse topology. In the N-terminal domain residues 18-21 (dark green) are the activation motif. Residues 31-37 are responsible for reverse topology (blue). Residues 38-61 in the TM region are responsible for trafficking (red).

of MC2R to the plasma membrane. In the N-terminal domain, residues 31-37 are required for the reverse topology of MRAP1 (Shown in Figure 1). Finally, the activation motif, LDYL (18-21), is located in the N-terminal of MRAP1 (Shown in Figure 1), and alanine substitution at these amino acid positions completely blocks activation of MC2R (Sebag and Hinkle, 2009). This level of organization is found in the other tetrapod and teleost MRAP1 orthologs that have been characterized (Dores et al., 2016).

It should be mentioned that MRAP2, a paralog of MRAP1, is found in adrenal cortex cells (Chan et. al., 2009). The human MRAP2 gene, *C6orf117*, is found on a different chromosome than the MRAP1 gene (Chan et al. 2009). Like MRAP1, MRAP2 has a single membrane spanning domain. Since the dimerization domain (Figure 2) is conserved between MRAP1 and MRAP2, MRAP2 is able to create an antiparallel homodimer (Webb and Clark, 2010). Sebag and Hinkle (2009) showed that the mouse MRAP2 (mMRAP2) is able to traffic hMC2R to the plasma membrane, but mMRAP2 could not facilitate the activation of hMC2R because it lacks an activation domain. As

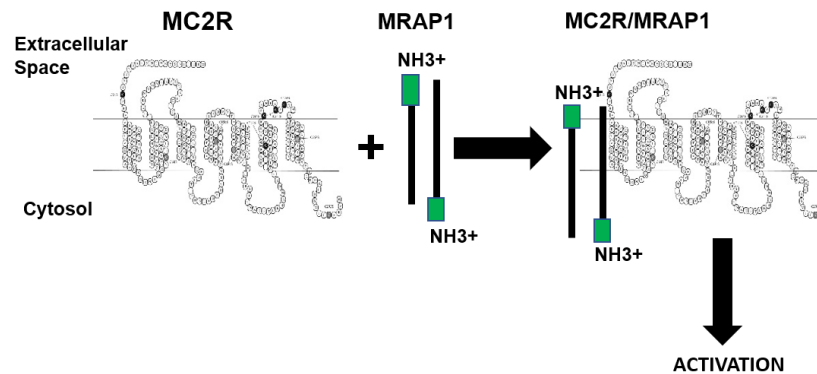
seen in Figure 2, sequence alignments of mMRAP1 and mMRAP2 indicate that the N-terminal and transmembrane domains are conserved, but the MRAP2 paralog lacks the activation motif (Sebag & Hinkle, 2007).



**Figure 2: Alignment of mMRAP1 and mMRAP2 sequences.** Mouse (m) MRAP1 (Accession #: NM\_029844) mMRAP2 (Accession #: NP\_001094952.2) were aligned as described in Dores et al. (1996).

Studies by Hinkle (Sebag and Hinkle, 2007, Sebag and Hinkle, 2009) and Clark (Cooray et al., 2008; Webb and Clark, 2010, Cooray et. al., 2011) have shown that MC2R and MRAP1 form a heterodimer at the ER. The heterodimer traffics to the plasma membrane, and at the plasma membrane an ACTH binding event activates the MC2R/MRAP heterodimer. Sebag and Hinkle (2009) found that the TM region of MRAP1 is responsible for trafficking by making contact with a TM region of MC2R. However, it is unknown which TM on MC2R is making contact with the TM of MRAP1 to facilitate trafficking to the plasma membrane in Figure 3. In addition, since MRAP1 is a homodimer with reverse topology, and there is an activation motif on each MRAP monomer (Figure 1) does the MRAP1 homodimer make contact with an extracellular

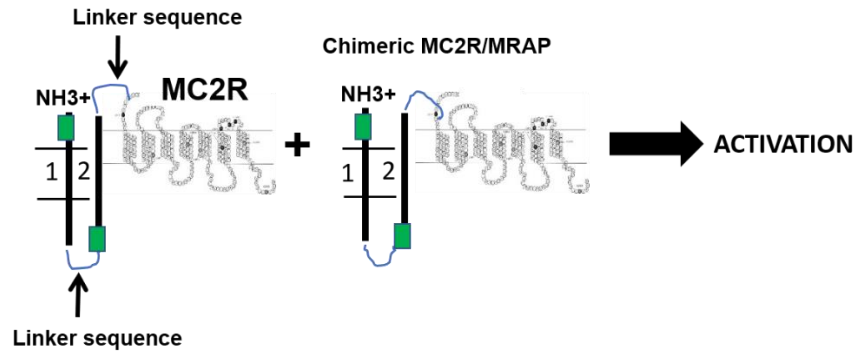
loop on MC2R, an intracellular loop on MC2R, or extracellular and intracellular loop of MC2R to facilitate activation of the receptor? In Figure 3, the MRAP1 homodimer is shown alone as well as creating a heterodimer with the MC2R. Note that the MRAP1 activation motifs are shown in green with one motif oriented towards the extracellular space while the other motif is positioned on the intracellular space.



**Figure 3: MC2R and MRAP1 create a heterodimer at the plasma membrane of a cell.** The MRAP1 activation motifs are shown in green. One monomer’s activation motif is oriented on the extracellular space side while the other monomer’s activation motif is positioned on the intracellular side of the cell.

Recently, Malik et al. (2015) using a chimeric protein paradigm strategy, found that the activation motif on the N-terminal of mMRAP1 facing the extracellular space side of the plasma membrane is responsible for the activation of MC2R. This conclusion was based on the following set of experiments. In this study (Figure 4), the “wild-type” chimeric receptor consisted of two MRAP1 proteins that were connected to each other by a linker region while another linker region connected the hMC2R to the second MRAP1 protein. When this “wild-type” chimeric protein was expressed alone in HEK-293 cells and stimulated with human ACTH(1-24), activation of the chimeric receptor was observed. Figure 4 illustrates the “wild-type” chimeric receptor. The linker regions

between the MRAP monomers and the MC2R are shown in blue, and the activation motifs of MRAP are shown in green.

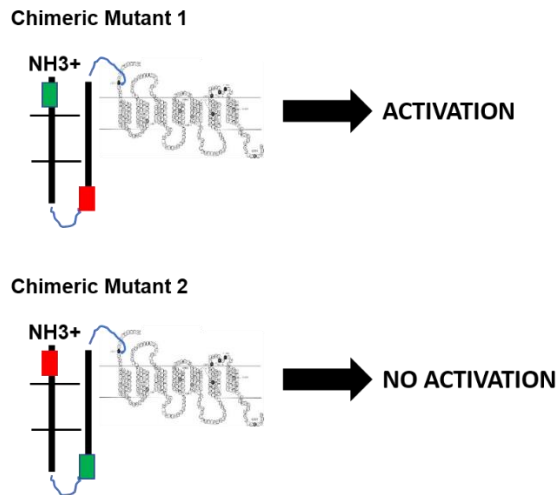


**Figure 4: The “wild type” chimeric protein.** The activation motifs of the MRAP1 monomer 1 and monomer 2 are shown in green while the linker regions of the chimeric protein are shown in blue. When the “wild type” chimeric protein was expressed alone in HEK-293 cells and stimulated with human ACTH(1-24), activation was observed.

When the “wild type” chimeric MRAP1/MC2R cDNA was expressed in HEK293 cells the corresponding chimeric receptor trafficked to the plasma membrane, and could be activated by ACTH.

However, this initial experiment did not resolve whether the extracellular or intracellular activation motif was making contact with hMC2R. Therefore, two different chimeric mutant receptors were made to address this question (Figure 5). In the first mutant chimeric (Mutant 1), the activation motif positioned on the intracellular space side was replaced with alanines (Figure 4). For the second mutant chimeric (Mutant 2), the activation motif positioned on the extracellular space side was replaced with alanines (Figure 5). Figure 5 illustrates the two chimeric receptors where the alanine substitutions at the two activation motifs of the MRAP monomers are shown in red. Each chimeric mutant receptor was expressed alone in HEK-293 cells and stimulated with human

ACTH(1-24). Interestingly, the mutant 1 chimeric receptor could be activated, however, the mutant 2 chimeric receptor could not be activated in this experiment.

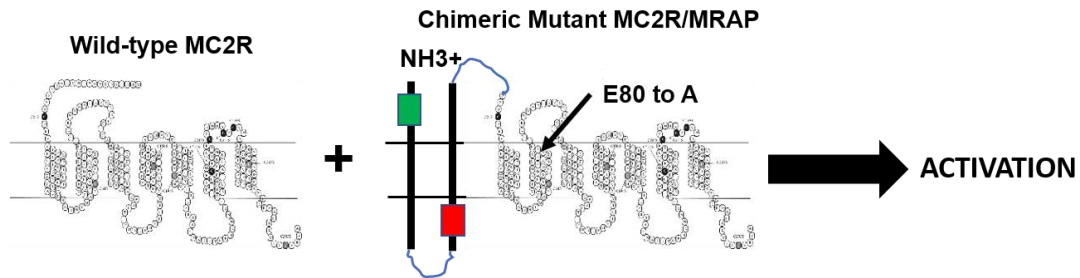


**Figure 5: The mutant 1 and 2 chimeric proteins.** The alanine substitutions of the MRAP1 monomer activation motifs are shown in red. The first mutant substituted alanines at the activation motif oriented on the intracellular space side of the cell while the second mutant substituted alanines positioned on the extracellular space side of the cell. Each of these mutants were expressed in HEK-293 cells and stimulated with human ACTH(1-24). The mutant 1 chimeric protein resulted in activation while the mutant 2 chimeric protein resulted in no activation.

To confirm that the activation motif on the mutant 1 chimeric receptor was responsible for the activation observed in the previous experiment, a new mutant chimeric receptor was made. The final mutant chimeric receptor had the following features: the MRAP1 activation motif oriented on the intracellular space was replaced with alanines; and an alanine was substituted for the glutamic acid (E) at position 80 in MC2R. The design for the new chimeric mutant receptor was based on an earlier site-directed mutagenesis study. Chen et al. (2007) found that substitution of an alanine at position E80 completely blocked activation of hMC2R. Figure 6 illustrates the most important chimeric experiment in the Malik et al. (2016) study. The green shows the

activation motif of the MRAP1 monomer facing the extracellular side whereas the red shows the alanine substitution of the MRAP1 monomer activation motif on the intracellular side. Furthermore, an arrow points to the critical amino acid position, E80, in hMC2R. When this mutant chimeric receptor was expressed alone in HEK-293 cells there was no activation following stimulation of ACTH(1-24). Hence, the mutant chimeric receptor is completely inactivated. However, cell surface ELISA analysis showed that this new mutant chimeric receptor was on the plasma membrane. In a second experiment, wild type MC2R was co-expressed with the new mutant chimeric receptor as shown in Figure 6 and stimulated with human ACTH(1-24). Note that when wild type MC2R is expressed alone in HEK-293 cells the receptor does not traffic to the plasma membrane and there is no activation. However, when wild type MC2R is co-expressed with the new mutant chimeric MC2R/MRAP as shown in Figure 6 activation was observed following stimulation with human ACTH(1-24). This experiment provides additional evidence that the activation motif of MRAP1 is interacting with an extracellular domain of MC2R.





**Figure 6: Final chimeric experiment.** The final chimeric substituted alanines at the activation motif of the MRAP1 monomer positioned on the intracellular side of the cell. Furthermore, an alanine was substituted at position E80 on the MC2R of the chimeric protein. Previous studies have shown that substitution at this position completely inactivates MC2R. Wild-type MC2R was co-expressed with the final chimeric protein in HEK-293 cells. The cells were stimulated with human ACTH(1-24), and activation was observed.

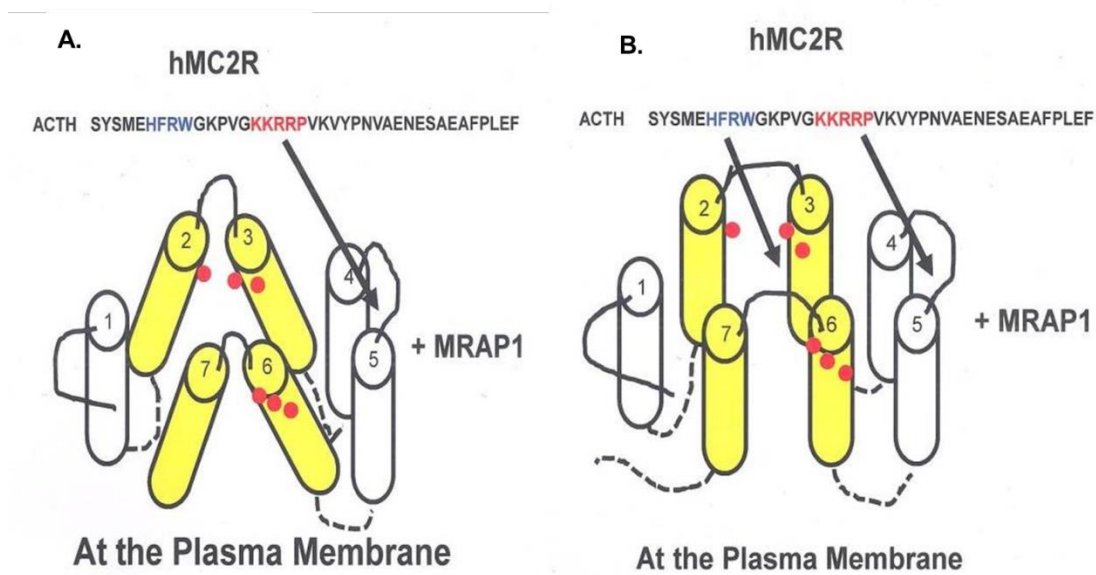
While the preceding experiments by Malik et al. (2016) clearly demonstrate the importance of the activation motif in MRAP1 for orienting the MC2R in a confirmation that allows for ACTH binding, it is important to identify the motifs in ACTH that are making contact with the MC2R. Shown in the figure below, ACTH is a polypeptide hormone that is 39 amino acids in length. Schwyzer (1977) found that only the first 24 amino acids of ACTH, [ACTH(1-24)], are needed to stimulate glucocorticoid production. Note that the sequence for  $\alpha$ -MSH is found in the first 13 amino acids of ACTH(1-24). While both melanocortin peptides contain the critical amino acid sequence HFRW which is required for activating all melanocortin receptors,  $\alpha$ -MSH cannot activate the hMC2R. Interestingly,  $\alpha$ -MSH is not able to activate either tetrapod or teleost MC2R orthologs (Mountjoy et al., 1994; Chen et al., 2007; Agulleiro et al., 2010). Schwyzer (1977) addressed this paradox by observing that the KKRR motif (Figure 7) present in ACTH(1-24) was also required for activating the ACTH receptor (i.e. MC2R). Hence, in ACTH

there are two amino acid motifs involved in the activation process: the HFRW motif and KKRR motif (Schwyzer, 1977).

ACTH (1-39) SYSMEHFRWGKPVGKKRRPVKVYPNVAENESAEAFPLEF  
ACTH (1-24) SYSMEHFRWGKPVGKKRRPVKVYP  
ACTH (1-16) SYSMEHFRWGKPVGKK  
 $\alpha$ -MSH SYSMEHFRWGKPV-NH<sub>2</sub>

**Figure 7: Human ACTH, ACTH Analogs, and  $\alpha$ -MSH Amino Acid Sequences.** The HFRW motif is shown in green while the KKRR motif is shown in red. ACTH and  $\alpha$ -MSH share the important HFRW motif, but not the KKRR motif. Schwyzer (1977) showed that the KRR motif is required for activation of the MC2R.

The current model for the activation of hMC2R is presented in Figure 8. When hMC2R is co-expressed with a mammalian MRAP1, the heterodimer will form in the ER and will move to the plasma membrane (Figure 8A). However at this stage, the HFRW binding site (shown in yellow) is closed. When ACTH(1-24) makes contact with hMC2R, the current model proposes that the KKRR motif on ACTH(1-24) binds to a site within the TM4/EC2/TM5 domain (Figure 8A). This binding event triggers a conformational changes in the receptor that results in the opening of the HFRW binding site (Figure 8B) so that the HFRW motif on ACTH(1-24) can dock with this binding site. This event results in an additional conformational change in the receptor that activated the G protein to initiation the intracellular communication event (Dores, 2017). Therefore, activation of the receptor appears to be a two-step process.



**Figure 8: Proposed model of hMC2R activation by ACTH binding.** **A.** The human MC2R takes on a barrel-like shape at the plasma membrane of a cell. When stimulated with human ACTH, the KKRR motif (shown in red) of ACTH docks at the proposed TM4/EC2/TM5 domain of MC2R (Dores et al., 2016; Liang et al., 2013; Davis et al., 2013; Dores, 2018). This binding even causes a conformational change in the receptor. **B.** This conformational change in the receptor causes the HFRW binding pocket to be exposed for the HFRW motif of ACTH to bind to the receptor. The HFRW binding site of the human MC2R is shown in yellow; the HFRW binding site of MC2R is located in TM2, TM3, TM6, and TM7 (Pogosheva et al., 2005,; Chen et al., 2007; Dores, 2009).

Support for this model comes from multiple studies. All melanocortin receptors have an HFRW binding site that involves amino acid positions located on TM2, TM3, TM6, and TM7; these residues are located close to the extracellular space (Pogosheva et al., 2005,; Chen et al., 2007; Dores, 2009). With respect to the KKRR binding site, work in our lab using a single alanine substitution paradigm for human, frog, and rainbow trout MC2R orthologs indicated that critical residues in Extracellular Loop 2 (EC2) and TM5 of MC2R appear to provide the binding site for the KKRR motif of ACTH (Dores et al., 2016; Liang, 2013; Davis, 2013).

Based on the preceding observations, there are four working assumptions for this thesis. First, there are three distinct sites in MC2R: an HFRW binding site, a KKRR binding site, and a MRAP1 activation motif binding site. Second, the MRAP1 activation motif (LYDL/I) is located on the N-terminal of the MRAP1 monomer that is positioned on the extracellular space side of the plasma membrane (Figure 1). **Therefore, MRAP1 must make contact with an extracellular domain of MC2R.** Third, the KKRR binding site is predicted to be in the TM4/EC2/TM5 domain (Dores et al., 2016). Hence, two hypotheses are proposed to explain the interaction between MRAP1 and MC2R. For Hypothesis 1, the potential targets for MRAP1 interaction with MC2R could be: the N-terminal domain, EC1, or EC3. To test this hypothesis chimeric protein experiments will be conducted to determine whether the N-terminal domain, the EC1 domain, or the EC3 domain contain the contact site for interaction with the activation motif of MRAP1. Based on the results of these experiments, multiple and single alanine substitution paradigms will be used to determine which amino acid positions within the predicted receptor contact site interact with the activation domain of MRAP1. Finally, if none of these three domains can be shown to interact with MRAP1, then the alternative hypothesis (Hypothesis 2) is that the MRAP1/MC2R contact point is within the TM4/EC2/TM5 domain, and as a result MRAP1 and the TM4/EC2/TM5 domain may interact to create the KKRR binding site to facilitate activation of the MC2R. Collectively, these two hypotheses should resolve the interaction between MRAP1 and MC2R.

## CHAPTER 1: Designing Chimeric MC2R receptors

In order to identify which extracellular domain on the human melanocortin-2 receptor (hMC2R) is making contact with melanocortin receptor accessory protein 1 (MRAP1), a chimeric receptor paradigm was used. In order to use this paradigm it was essential to identify a melanocortin receptor that does not require MRAP1 for activation. The rationale for using the chimeric protein paradigm is that the exchange of an extracellular domain from the non-MRAP-dependent melanocortin receptor with an extracellular domain from the MRAP1 dependent receptor (i.e. hMC2R) may make it possible to identify the MC2R/MRAP1 extracellular loop contact site. To create these chimeric receptors, two criteria needed to be met. First, the receptor's extracellular domains needed to be similar in length to the extracellular domains of hMC2R. Second, within the receptor and hMC2R extracellular domains, primary sequence identity needed to be low.

First, a screening process was implemented to compare the length of the hMC2R extracellular domains to the extracellular domains of other melanocortin receptors. An alignment of the hMC2R, *Xenopus tropicalis* MC1R (xtMC1R), xtMC3R, and *Callorhinchus milii* or elephant shark esMC3R (esMC3R) are shown in Figure 9. These receptors were selected because previous studies in our lab indicated that none of these

receptors required co-expression with MRAP1 for activation when expressed in CHO cells (Dores unpublished data). This figure shows that the extracellular domain 1 (EC1), extracellular domain 2 (EC2), and extracellular domain 3 (EC3) of xtMC1R, xtMC3R, and esMC3R are all similar in length when compared to the hMC2R. However, when comparing the N-terminal (NT) of these receptors to hMC2R there is a difference in the length (Figure 9). The NT of hMC2R is 26 amino acids in length. The xtMC1R is shown to have 30 amino acids in its NT domain. The NT of xtMC3R is 47 amino acids in length, and esMC3R is shown to have 37 amino acids in its NT domain. In an earlier study that used hMC2R and hMC4R chimeric receptors, it was shown that swapping out the N-terminal and TM1 of hMC2R with the N-terminal and TM1 of hMC4R resulted in interference with activation of the chimeric receptor (Hinkle et al., 2011). In this experiment, it appeared that the larger size of the N-terminal of this chimeric receptor was thought disrupt folding of the chimeric receptor and non-specifically block activation. To negate this issue, the xtMC1R was used to make this study's chimeric receptors because of its similar length in the N-terminal to the N-terminal of the hMC2R, and other features of this receptor.

```

N-terminal
[-----TM1-----]
h MKH-----IINSYENINNTARNNSDCPRVVLPEEIFFTTISIVGVLEN 42
x1 MLH-----STVNSTNATINVGTELKPTNTSDTVMDVPEELFLFLCVFSLLEN 47
x3 MSAKAFAMNTTNVFSVQAVLANATLDPNETLFLSNLSRIGFCEQVLIKTEVFLTLGIIISLLEN 63
e3 M-----NSTHFLFDLQLNGSGDLNKSSILNRSNPGFCEQVPIKSEVFLTLGIIISLLEN 53

-----] IC1 [-----TM2-----] EC1 [--

h LIVLLAVFKNKNLQAPMYFFICSLAISDMGLSGLYKILENIIILRNMGYKPRGSFETTADDI 105
x1 ILVVIAIFRNHNLHSPMYFFICCLAASDMLVSSSNLGETLIIFMLKQGIKSEPLLKMDYI 110
x3 ILVILAILKKNLHSPMYFFLCSLAVADMLVSVSNALETIVI-AIQNKYLVIGDYLLQHLDDV 126
e3 ILVILAILKKNLHSPMYFFLCSLAVADMLVSVSNALETIVMALLNNGYLVANDQFIQIMDNV 116

-----TM3-----] IC2 [-----TM4-----]

h IDSLFVLSLLGSIFLSLVIAADRYITIFHALRYHSIVTMRRTVVVLTVIWTFCTGTGITMVIF 168
x1 FDTMICCSLVTSLSFLGAIAIDRYITIFYALRYHSIMTLRRVVIAIGVIWSVSLVCAAFIVY 173
x3 FDSMICISLVASICNLLVIAIDRYITIFYALRYHSIMTVKKAIALIVVIWTSCIICGIVFIVF 189
e3 IDSLICISLVASICNLLVIAIDRYITIFYALRYHSIMTVKRALLLIIVIWIACIFCGIIFIIY 179

EC2 [-----TM5-----] IC3 [-----

h SHHVPTVITFTSLFPLMLVFILCLYVHMFLARSHTRKISTLPRANN-----MKGAIT 221
x1 HESRAVILCLIVFFFLMVALMVALYIHMFAARQHARSISALQKGSRRITPHQARAMKGAIT 236
x3 SESKTIVIVCLITMFFTMLVLMATMYVHMFLEFARLHVKRIAALPVDGVVQORTC----MKGAIT 248
e3 SNSKTVIIICLITMFFTMLVLMATMYVHMFLEFARLHVKRIAALPVDGIVRPRTC----MKGAIT 238

---TM6-----] EC3 [-----TM7-----]

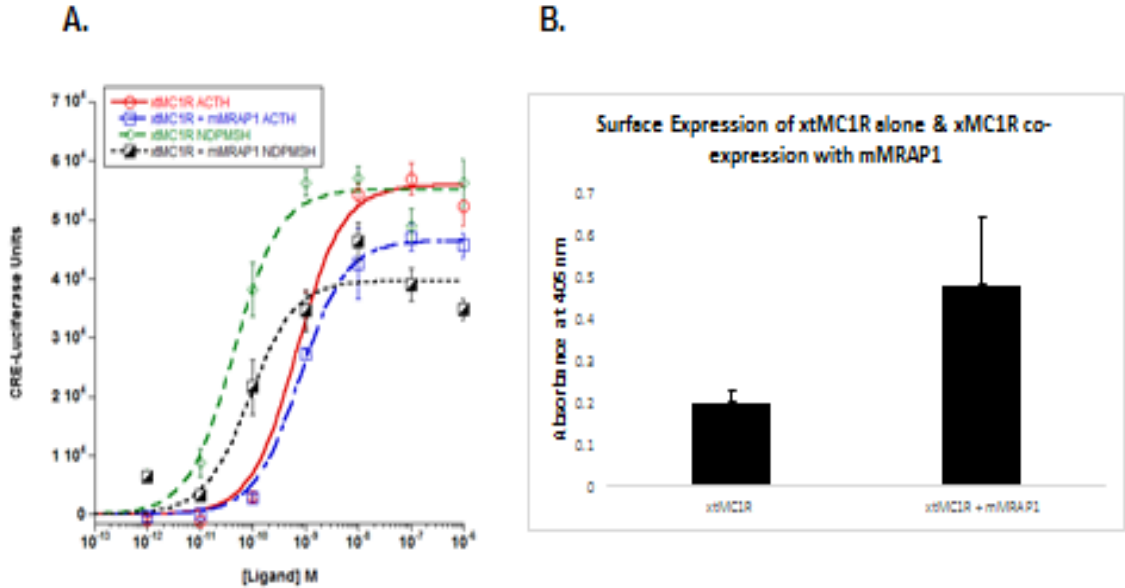
h LTILLGVFIFCWAPFVLHVLLMTFCPSNPYCACYMSLFQVNGMLIMCNAVIDPFIYAFRSPEL 284
x1 LTLILLGVFFFLCWGPLFLHLTLFVSCPGHHICNSYFYFNIIYLLLVICNSVIDPLIYAFRSQEL 299
x3 ITILLGVFVVCWAPFFLHLILIIISCPNSYCVCYTSYFNTYLILIMCNSIIDPLIYAFRSLEM 311
e3 ITILLGIFIICWAPFFLHLILIIISCPKNAYCICYTSHFNTYLILIMCNSVIDPMIYAFRSQEM 301

C-terminal

h RDAFKKMIFCSRYW 298
x1 RKTLKEIVWCSW 311
x3 RKTFFKEIICCYGMNFGKCG 330
e3 RKTFFKEIACCYGMNLSRFSVHRINAETERTSESSCHCDFGKTSVFNQVALS 354

```

**Figure 9: Sequence Alignment of hMC2R, xtMC1R, xtMC3R, and esMC3R** This figure shows an alignment of the hMC2R, xtMC1R, xtMC3R, and the esMC3R. This alignment was used to compare the extracellular domains of xtMC1R, xtMC3R, and esMC3R to the extracellular domains of hMC2R. xtMC1R, xtMC3R, and esMC2R were selected because none of these receptors required co-expression with an MRAP1 for activation when expressed in CHO cells (Dores unpublished data). The extracellular domains of interest included the N-terminal, extracellular domain 1, extracellular domain 2, and extracellular domains 3. Abbreviations: x – *Xenopus tropicalis*; e – Elephant Shark.



**Figure 10A: xtMC1R co-expressed with or without mMRAP1 stimulated with hACTH(1-24) or NDP-MSH** A) This figure shows the xtMC1R expressed alone (the curves shown in red or green) or co-expressed with mMRAP1 (the curves shown in blue or black). The reactions were either stimulate with hACTH(1-24) or NDP-MSH at concentrations of  $10^{-6}$  to  $10^{-12}$  M. B) **Cell surface ELISA analysis of xtMC1R co-expressed with mMRAP1.** xtMC1R +/- co-expression with mMRAP1 was analyzed using a cell surface ELISA assay and statistically evaluated using Student's t-test. n = 3.

Although our lab has shown that the xtMC1R does not require MRAP1 for activation, there is still the question of whether MRAP1 interfering with the activation of this receptor. Figure 10A illustrates an activation assay of xtMC1R expressed alone or co-expressed with mouse MRAP1 (mMRAP1). The receptor was stimulated with either



hACTH(1-24) or NDP-MSH at concentrations ranging from  $10^{-6}$  to  $10^{-12}$  M. These results show no significant difference in activation of the xtMC1R in the absence of mMRAP1 (shown in red) or presence of mMRAP1 (shown in blue) when xtMC1R was stimulated with hACTH(1-24). The  $EC_{50}$  value for xtMC1R expressed alone was  $6.8 \times 10^{-10}$  M  $\pm$   $1.5 \times 10^{-10}$ . The  $EC_{50}$  value when the receptor was co-expressed with mMRAP1 and stimulated with hACTH(1-24) is  $7.8 \times 10^{-10}$  M  $\pm$   $9.3 \times 10^{-11}$ . A F-test indicated a p-value of 0.84, which is not statistically significant. Similarly, there was little difference in activation of xtMC1R in the absence of mMRAP1 (shown in green) or presence of mMRAP1 (shown in black) when stimulated with NDP-MSH. The  $EC_{50}$  value for xtMC1R expressed alone was  $4.5 \times 10^{-11}$  M  $\pm$   $1.4 \times 10^{-11}$ , and when the receptor was co-expressed with mMRAP1 the  $EC_{50}$  value was  $8.6 \times 10^{-11}$  M  $\pm$   $4.1 \times 10^{-11}$ . A F-test calculated a p-value of 0.95 showing no statistical difference. Overall, these results illustrate two important conclusions. First, they show that xtMC1R expression and activation is not dependent on co-expression of mMRAP1. Second, they show that xtMC1R expression and activation is not negatively affected by co-expression of mMRAP1. In addition, co-expression with mMRAP1 does not have any effect, positive or negative, on the trafficking of xtMC1R (Figure 10B;  $p = 0.10$ ; t-Test).

Figure 11 illustrates the amino acid sequence alignment of hMC2R and xtMC1R as well as the sequence identity between hMC2R and xtMC1R in their respective transmembrane domains and extracellular loops. First, Figure 11A shows the alignment of the entire amino acid sequences for hMC2R and xtMC1R. The extra cellular loops of

interest are denoted in red; this includes the N-terminal (NT), extracellular loop 1 (EC1), extracellular loop 2 (EC2), and extracellular loop 3 (EC3) of the receptors.

**A. Alignment of Human MC2R and *Xenopus tropicalis* MC1**

```

                                N-terminal                                [-----TM1-----]
hMC2 MKH-----IINSYENINNTARNNSDCPRVVLPEEIFFTISIVGVLENLIVLLAVF
xMC1 MLH-----STVNSTNATINVGTELKPTNTSDTVMDVPEELFLFLCVFSLLENILVVIAIF
      IC1  [-----TM2-----]                                EC1  [-----TM3-----]
hMC2 KNKNLQAPMYFFICSLAISDMLGSLYKILENILIILRNMGYLKPRGSFETTAADDIIDSFLVLSLLGSIF
xMC1 RNHNHLSPMYFFICCLAASDMLVSSSNLGETLIIFMLKQGIKSEPLLVKMDYIFDTMICCSLVTSLS
-----]                                IC2  [-----TM4-----]                                EC2  [-----TM5-----]
hMC2 SLSVIAADRYITIFHALRYHSIVTMRRTVVVLTVIWTFCTGTGITMVI FSHHVPTVITFTSLFPLMLVF
xMC1 FLGAI AIDRYITIFYALRYHSIMTLRRVIAIGVIWSVSLVCAAIFIV YHESRAVILCLIVFFLFMLAL
-----]                                IC3  [-----TM6-----]                                C-terminal
hMC2 ILCLYVHMFLARSHTRKISTLPRANN-----MKGAITLTILLGVFIFCWAPFVLHVLLMTFCP
xMC1 MVALYIHMFAARQHARSISALQKGRSRITPHQARAMKGAITLTLLLVGFVFLCWGPLEFLHLLTFVSCP
      EC3  [-----TM7-----]                                C-terminal
hMC2 SNPYCACYMSLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKMIFCSRYSW
xMC1 GHHICNSYFYFNIIYLLLVICNSVIDPLIYAFRSQELRKTTLKEIVWCSW

```

**B. Sequence Identity for hMC2R and xMC1R by domain**

	%Identity						
N-terminal	10%	EC1	16%	EC2	0%	EC3	33%
TM1	38%	TM3	33%	TM5	38%	TM7	52%
IC1	64%	IC2	67%	IC3	28%	C-terminal	39%
TM2	39%	TM4	26%	TM6	63%		

**Figure 11: Alignment of hMC2R & xtMC1R** **A.** The sequence alignment of hMC2R and xtMC1R. The extracellular domains of interested are shown in red. Also, **B.** illustrates the sequence identity between the hMC2R and xtMC1R. Again, the extracellular domains of interest are shown in red.

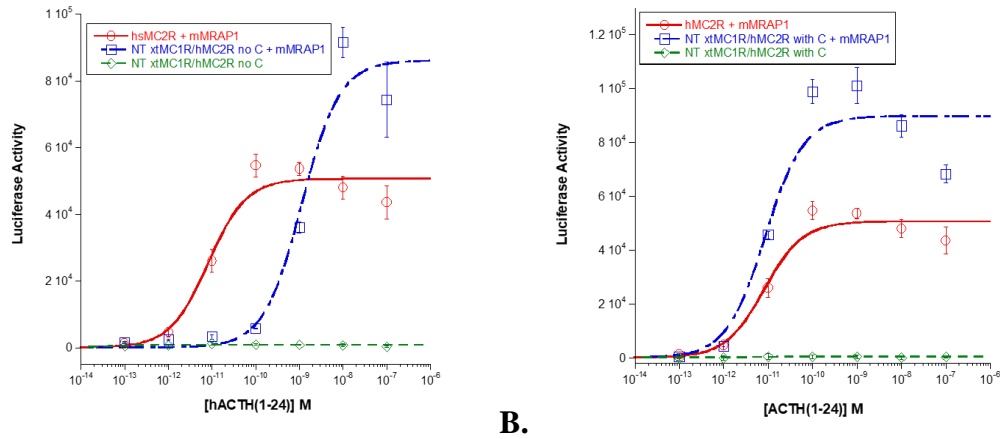
Second, Figure 11B shows primary sequence identity between all domains of hMC2R and xtMC1R. The domains denoted in red are the extracellular loops of interest. The N-terminal domains of hMC2R and xtMC1R have 10% primary sequence identity. The EC1 domains of hMC2R and xtMC1R have 16% primary sequence identity. The EC3 domains of hMC2R and xtMC1R have 33% primary sequence identity. Most interestingly, the EC2 domain of xtMC1R has no primary sequence identity when compared with the EC2 domain of hMC2R.

## Conclusions

Based on the results presented in Figure 10, and the alignment of xtMC1R and hMC2R presented in Figure 11, xtMC1R appeared to be a very good candidate for the chimeric receptor experiment. xtMC1R has an N-terminal domain that is comparable in length to the N-terminal domain of hMC2R, and the relatively low primary sequence identity between xtMC1R and hMC2R extracellular domains was another favorable factor.

## CHAPTER 2: Evaluation of the N-terminal hMC2R Chimeric Receptor

For the first chimeric receptor experiment, the N-terminal domain of the hMC2R was replaced with the N-terminal of xtMC1R (Figure 12A). In this experiment the wild-type hMC2R (positive control) had an  $EC_{50}$  value of  $8.1 \times 10^{-12} \text{ M} \pm 3.3 \times 10^{-12}$ , and the xtMC1R **N-terminal**/hMC2R chimeric receptor had an  $EC_{50}$  value of  $1.1 \times 10^{-9} \text{ M} \pm 4.7 \times 10^{-10}$ ; a nearly 1000 fold decrease in sensitivity for stimulation by hACTH(1-24) that was statistically significant (T-test;  $p = 0.04$ ).



**Figure 12: Analysis of the xtMC1R/hMC2R N-terminal Chimeric receptors.** A) The N-terminal domain of hMC2R was replaced with the N-terminal of xtMC1R (Figure 11) to make the xtMC1R/hMC2R N-terminal chimeric receptor. Wild-type hMC2R (red dose response curve) and the chimeric N-terminal receptor (blue dose response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. The chimeric N-terminal receptor was also expressed alone (green dose response curve. (n=3). B) A second xtMC1R/hMC2R chimeric receptor was made in which D<sup>26</sup> (xtMC1R; Figure 11) was replaced with C<sup>26</sup>. Wild-type hMC2R (red dose response curve) and the chimeric N-terminal receptor (C<sup>26</sup>) (blue dose response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. The chimeric N-terminal receptor C<sup>26</sup> was also expressed alone (green dose response curve. (n=3).

However, when the xtMC1R N-terminal/hMC2R chimeric receptor was expressed in the absence of mMRAP1 no activation was evident (green curves shown in Figure 12A).

Hence, exchanging the N-terminal of xtMC1R with the N-terminal of hMC2R did not make the chimeric receptor MRAP independent.

Although Figure 12A seemed to indicate a role for the N-terminal domain of hMC2R in the activation of the receptor, an earlier study reported that there is an important cysteine residue located at position 21 in the N-terminal of hMC2R (Figure 11; Yang et. al, 2007) that is utilized for proper protein folding. However, xtMC1R does not

contain this residue in its N-terminal domain (Figure 11). Therefore, a new chimeric N-terminal xtMC1R/hMC2R was made with a cysteine inserted at residue D<sup>26</sup> in the N-terminal of xtMC1R. Shown in 12B, this experiment's results show the wild-type hMC2R (positive control) had an EC<sub>50</sub> value of  $8.1 \times 10^{-12} \text{ M} \pm 3.3 \times 10^{-12}$ , and the xtMC1R**N-terminal**/hMC2R chimeric receptor with the C<sup>26</sup> substitution had an EC<sub>50</sub> value of  $8.5 \times 10^{-12} \text{ M} \pm 5.1 \times 10^{-12}$ . The replacement of this important cysteine recovered activation of the xtMC1R/hMC2R N-terminal chimeric (t-test; p = 0.5). Furthermore, when the xtMC1R/hMC2R N-terminal chimeric with C<sup>26</sup> was expressed alone, no activation occurred.

## Conclusions

Based on the results of observed in Figure 12B, exchanging the N-terminal of hMC2R with the N-terminal of xtMC1R and replacing position D<sup>26</sup> with a cysteine residue did not interfere with the activation of the chimeric receptor, and did not make the chimeric receptor MRAP1 independent. All MC2R orthologs have a cysteine residue in their N-terminal domain, hence the N-terminal domain is important for maintaining the correct conformation of the MC2R, but the results presented in this chapter indicate that the N-terminal domain of hMC2R is not a likely site for making contact with the activation motif on MRAP1.

Interest in the N-terminal domain comes from the Fridmanis et al. (2010) study that proposed that the N-terminal of hMC2R contained a signal that blocked trafficking (referred to as a stop transfer sequence). The stop-transfer hypothesis was proposed to explain why when hMC2R was expressed alone (without MRAP1) in HEK-293 cells, the

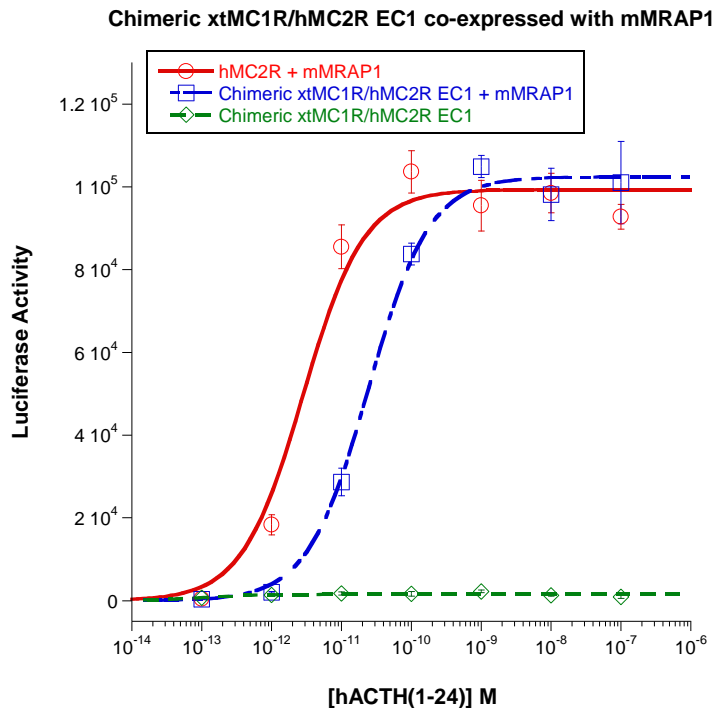
receptor was retained in the endoplasmic reticulum. The authors rationalized that when hMC2R was co-expressed with MRAP1, the “stop-transfer” sequence was blocked in some manner. To test this hypothesis, the N-terminal of human MC4R (43 amino acids in length) was exchanged with the N-terminal domain of human MC2R (25 amino acids in length) and the chimeric hMC2R **N-terminal** /hMC4R receptor was expressed in HEK-293 cells and did not move to the plasma membrane. Since wild-type hMC4R does not require co-expression with MRAP1 for trafficking to the plasma membrane, the authors concluded that the “stop-transfer” sequence in the absence of co-expression with human MRAP1 in HEK-293 cells preventing trafficking of the chimeric receptor. In their study, Fridmanis et al. (2010) did not do the reciprocal experiment and make an hMC4R **N-terminal** /hMC2R chimeric receptor that based on their hypothesis should be able to move to the plasma membrane without co-expression with MRAP1.

If the “stop transfer sequence” hypothesis is correct, then the chimeric receptor xtMC1R N-terminal/hMC2R should also traffic to the plasma membrane in the absence of co-expression with MRAP1 in CHO cells, and be activated following stimulation with hACTH(1-24). However, as indicated in Figure 12A and B replacing the N-terminal of hMC2R with either xtMC1R N-terminal sequence did not result in activation. A flaw in the Fridmanis et al (2010) study was that the authors did not consider the possibility that the inability of the hMC2R **N-terminal**/hMC4R chimeric receptor to traffic to the plasma membrane of HEK293 cells might have been due to the miss-folding and degradation of the hMC2r/hMC4R chimeric receptor. A re-evaluation of the Fridmanis et al (2010) study would be an appropriate follow-up project to this section of the thesis.

### CHAPTER 3: Evaluation of the EC1 Chimeric Receptor

The next chimeric receptor tested the exchange of the hMC2R EC1 domain with the EC1 domain of xtMC1R. Figure 13 shows the xtMC1**REC1**/hMC2R chimeric activation assay. The curve shown in red is the wild-type hMC2R co-expressed with mMRAP1.





**Figure 13: xtMC1REC1/hMC2R co-expressed with mMRAP1 and simulated with hACTH(1-24).** The extracellular 1 domain (EC1) of xtMC1R was replaced with the EC1 domain of xtMC1R (Figure 11) to make the xtMC1R/hMC2R EC1 chimeric receptor. Wild type hMC2R (red dose response curve) and the xtMC1R/hMC2R EC1 chimeric receptor (blue dose response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. Also, the xtMC1R/hMC2R EC1 chimeric receptor was expressed alone (green dose response curve) (n=3).

Following stimulation with hACTH(1-24), the wild type hMC2R had an EC<sub>50</sub> value of  $2.8 \times 10^{-12} \text{ M} \pm 8.1 \times 10^{-13}$ . When the xtMC2REC1/hMC2R chimeric receptor was co-expressed with mMRAP1 there was a 10-fold shift in sensitivity to hACTH(1-24); the EC<sub>50</sub> value for this dose response curve (blue) was  $2.4 \times 10^{-11} \text{ M} \pm 3.1 \times 10^{-12}$ . A t-test calculated a p-value of 0.0002 indicating that there is statistical difference between the xtMC1R/hMC2R EC1 and the wild type hMC2R. These results indicate insertion of the xtMC1R domain into hMC2R resulted in a negative effect on activation of the

xtMC1RE $\mathbf{EC1}$ /hMC2R chimeric receptor as compared to the wild-type hMC2R control. The curve shown in green is the xtMC1RE $\mathbf{EC1}$ /hMC2R expressed in the absence of mMRAP1 which resulted in no activation. Therefore, exchanging the EC1 domain of hMC2R with the EC1 domain of xtMC1R did not make the chimeric receptor MRAP1 independent.

#### EC1 Cassette Alanine Mutants

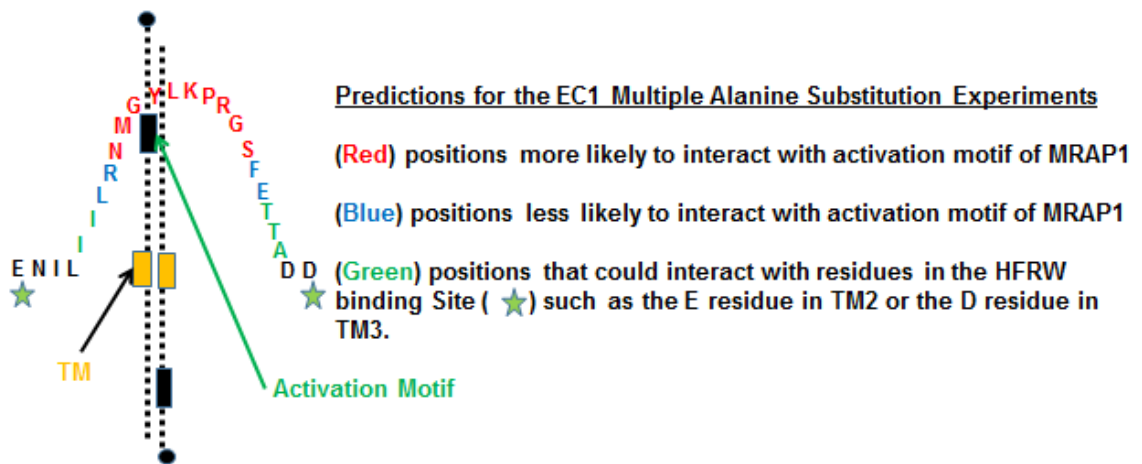
To determine which amino acid positions in the human EC1 have an effect on activation, an alanine cassette paradigm was used (Figure 14 & 15). The EC1 domain is 18 amino acids in length (Table 1). Therefore, six mutant receptors were made in the hMC2R EC1 domain where each mutant replaced three amino acid residues with three alanines; these mutants are presented in Table 1. By only replacing three amino acids at a time for each cassette mutant, it might decrease the chances of disrupting the tertiary structure of the receptor.

Wild type EC1 hMC2R	I I L R N M G Y L K P R G S F E T T A
Mutant 1 (AAA1)	<u>AAA</u> R N M G Y L K P R G S F E T T A
Mutant 2 (AAA2)	I L L <u>AAA</u> G Y L K P R G S F E T T A
Mutant 3 (AAA3)	I I L R N M <u>AAA</u> K P R G S F E T T A
Mutant 4 (AAA4)	I I L R N M G Y L <u>AAAG</u> S F E T T A
Mutant 5 (AAA5)	I I L R N M G Y L K P R <u>AAA</u> E T T A
Mutant 6 (AAA6)	I I L R N M G Y L K P R G S F <u>AAAA</u>

**Table 1: hMC2R EC1 Domain Cassette Mutants.** This table includes the six cassette mutants in the EC1 domain of hMC2R. The red underline denotes where three residues were replaced with three alanines.

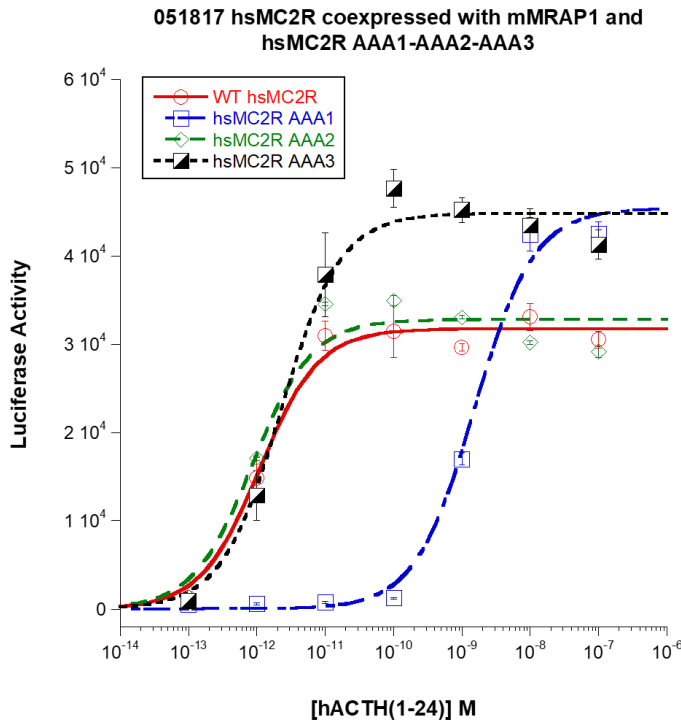
Predictions for possible outcomes of the cassette alanine substitution experiments are presented in Figure 14. Given the position of the activation motif in MRAP1, the amino acid positions in “red” appeared to be the mostly contacts with the activation motif of MRAP1.

After designing these mutant receptors, the EC1 domain cassette mutants were tested using an activation assay. The results for the hMC2R EC1 domain AAA1, AAA2, AAA3 cassette mutants are shown in Figure 15. For this assay, the mutant receptors were separately co-expressed with mMRAP1, and stimulated with hACTH(1-24). The curve shown in red is the wild type hMC2R receptor which had an  $EC_{50}$  value of  $1.1 \times 10^{-12}$  M  $\pm 2.3 \times 10^{-13}$ . The blue curve shows the hMC2R EC1 AAA1 receptor ( $EC_{50}$  value =  $1.5 \times 10^{-9}$  M  $\pm 2.3 \times 10^{-10}$ ).



**Figure 14: Predictions for the EC1 Multiple Alanine Substitution Experiment.** This figure shows the 19 residues in EC1 loop of hMC2R. Based on the position of the MRAP1 homodimer, the residues shown in red are most likely to interact with the activation motif of MRAP1. Shown in blue are the residues that are less likely to interact with the activation motif of MRAP1. Finally, the residues shown in green might interact with the HFRW binding site. The (★) indicates positions E<sup>80</sup> in the TM2 region and D<sup>104</sup> in the TM3 region that have been shown to affect activation of the MC2R (Chen et al. 2007 and Chung et al. 2005).

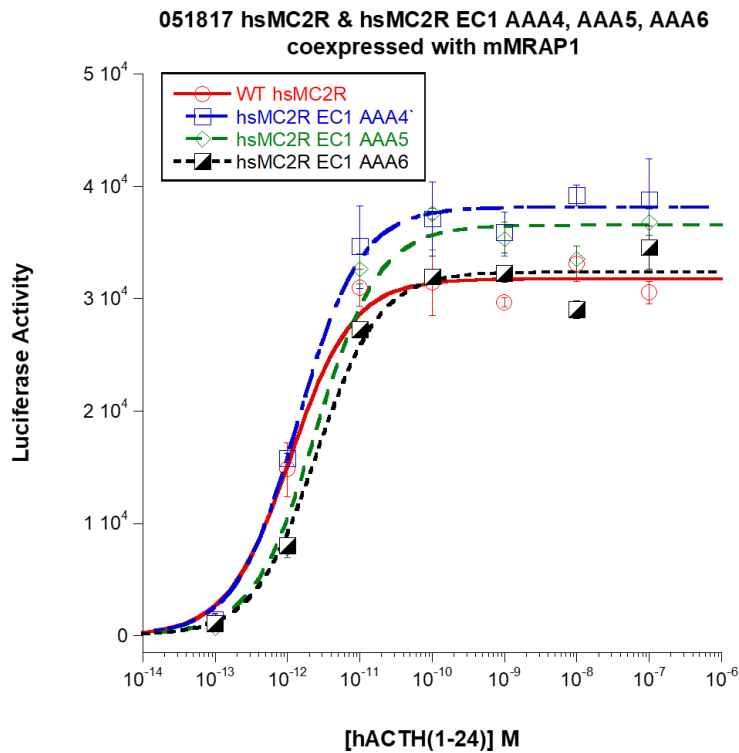
This mutant receptor had a 1000-fold drop in sensitivity to stimulation by hACTH(1-24) ( $p = 0.0001$ ; One-Way ANOVA). The green curve shows the hMC2R EC1 AAA2 receptor which had an EC<sub>50</sub> value of  $8.6 \times 10^{-13} \text{ M} \pm 3.2 \times 10^{-13}$  ( $p \geq 0.99$  relative to wild-type control; One-Way ANOVA). The black curve shows the hMC2R EC1 AAA3 receptor which had an EC<sub>50</sub> value of  $2.2 \times 10^{-12} \text{ M} \pm 5.2 \times 10^{-13}$  ( $p \geq 0.99$  relative to wild-type control; One-Way ANOVA).



**Figure 15: hMC2R EC1 Cassette Mutants AAA1, AAA2, and AAA3 co-expressed with mMRAP1 and stimulated with hACTH(1-24).** The extracellular 1 domain of hMC2R was replaced with three different triplicate cassettes of alanine substitutions to make the hMC2R AAA1, AAA2, AAA3 mutants (Figure 11). The wild type hMC2R (red dose response curve) was separately co-expressed with mMRAP1 in CHO cells as described in Methods. The hMC2R EC1 AAA1 (blue dose response curve), hMC2R EC1 AAA2 (green dose response curve), and hMC2R AAA3 (black dose response curve) were each co-expressed with mMRAP1 as described in Methods (n=3)

Next, Figure 16 summarizes the results of the activation assay of the hMC2R EC1 AAA4, AAA5, and AAA6 receptors. For this assay, each receptor was separately co-expressed with mMRAP1, and stimulated with hACTH(1-24). The curve shown in red is the wild type hMC2R receptor which had an EC<sub>50</sub> value of  $1.1 \times 10^{-12} \text{ M} \pm 2.3 \times 10^{-13}$ . The green curve shows the hMC2R EC1 AAA4 receptor. The EC<sub>50</sub> value for this mutant receptor was  $1.4 \times 10^{-12} \text{ M} \pm 2.3 \times 10^{-13}$  and when compared to the EC<sub>50</sub> value for the

wild-type hMC2R the p value was 0.8 (One-Way ANOVA). The curve shown in blue is the hMC2R EC1 AAA5 receptor which had an EC<sub>50</sub> value of  $2.5 \times 10^{-12}$  M +/-  $7.2 \times 10^{-13}$  (p = 0.02 as compared to wild-type hMC2R; One-Way ANOVA). The black curve shows the hMC2R EC1 AAA6 receptor with an EC<sub>50</sub>



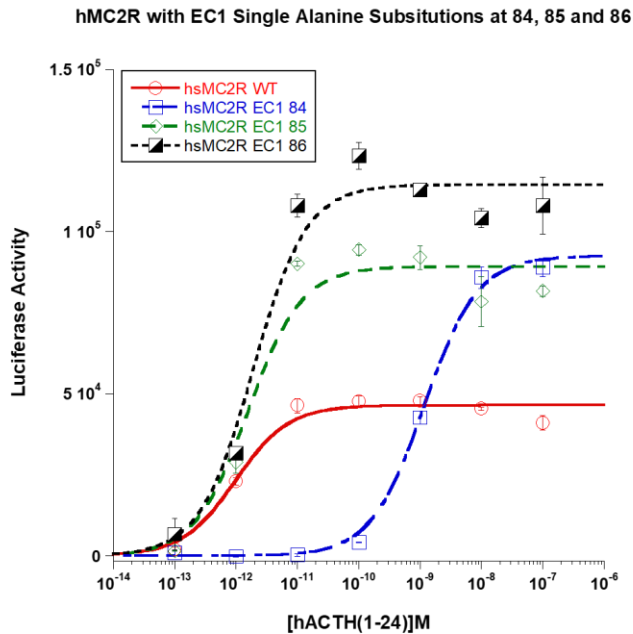
**Figure 16: hMC2R EC1 Cassette Mutants AAA4, AAA5, and AAA6 co-expressed with mMRAP1 and stimulated with hACTH(1-24).** The extracellular 1 domain of hMC2R was replaced with three different triplicate cassettes of alanine substitutions to make the hMC2R AAA4, AAA5, AAA6 mutants (Figure 11). The wild type hMC2R (red dose response curve) was separately co-expressed with mMRAP1 in CHO cells as described in Methods. The hMC2R EC1 AAA1 (blue dose response curve), hMC2R EC1 AAA2 (green dose response curve), and hMC2R AAA3 (black dose response curve) were each co-expressed with mMRAP1 as described in Methods (n=3).

value of  $2.5 \times 10^{-12} \text{ M} \pm 6.3 \times 10^{-13}$  ( $p = 0.01$  as compared to the wild-type MC2R; One-Way ANOVA). However, the differences in  $EC_{50}$  values for the positive control and the AAA5 and AAA6 mutants were less than 2-fold. While the precision of these assays resulted in what appears to be a statistically significant difference in activation, from a physiological perspective neither cassette alanine mutant had an effect on activation.

### EC1 Single Alanine Mutants

From the cassette mutant activation experiments, the only mutant to show any effect on activation in the EC1 domain of hMC2R that would be considered physiologically significant (Dores and Garcia, 2015) was the AAA1 mutant. Therefore, single alanine mutants were made at these three amino acid positions in the EC1 domain: I<sup>84</sup>, I<sup>85</sup>, and L<sup>86</sup> to determine whether one or more of these residues may interfere with ACTH activation. Figure 17 illustrates the activation assay results of the single alanine mutants at position I<sup>84</sup>, I<sup>85</sup>, and L<sup>86</sup> in the EC1 domain of hMC2R. For this assay, the wild type and single alanine mutant receptors were separately co-expressed with mMRAP1 as well as stimulated with hACTH(1-24). The curve shown in red is the wild type hMC2R receptor which had an  $EC_{50}$  value of  $9.7 \times 10^{-13} \text{ M} \pm 2.7 \times 10^{-13}$ . The blue curve shows the single alanine mutant at position I<sup>84</sup>. This mutant had an  $EC_{50}$  value of  $1.2 \times 10^{-9} \text{ M} \pm 1.3 \times 10^{-10}$  and yield a  $p$  value  $\leq 0.0001$  relative to the wild-type hMC2R (One-Way ANOVA). This mutant resulted in a 1000-fold shift in sensitivity to hACTH(1-24) when compared to the wild type hMC2R. The green curve shows the single alanine mutant at position I<sup>85</sup> which had an  $EC_{50}$  of  $1.5 \times 10^{-12} \text{ M} \pm 6.4 \times 10^{-13}$ . When compared to the  $EC_{50}$  value for the wild-type hMC2R, this mutation did not result in a statistically

significant change in sensitivity to stimulation by ACTH ( $p \geq 0.99$ ; One-Way ANOVA). The black curve shows the single alanine mutant at residue L<sup>86</sup> which had an EC<sub>50</sub> value of  $1.8 \times 10^{-12} \text{ M} \pm 6.4 \times 10^{-13}$ . Once again, substitution at this position did not result in a statistically significant shift in sensitivity to stimulation by ACTH ( $p \geq 0.99$ ; One-Way ANOVA). These results indicate that the only single alanine mutant that showed any effect on activation was the single alanine mutant at position I<sup>84</sup>.



**Figure 17: hMC2R EC1 single alanine mutants at positions 84,85, and 86 co-expressed with mMRAP1 and stimulated with hACTH(1-24).** Single alanine mutants at residue positions I<sup>84</sup>, I<sup>85</sup>, and L<sup>86</sup> were made in the EC1 domain of hMC2R (Figure 11). All receptors in this assay were separately co-expressed with mMRAP1 and stimulated with hACTH(1-24) as described in Methods. The curve shown in red is the wild type hMC2R. The curve shown in blue is the single alanine mutant at position I<sup>84</sup>. The curve shown in green is the single alanine mutant at position I<sup>85</sup>. The curve shown in black is the single alanine mutant at position L<sup>86</sup> (n=3).



## Conclusions

In an early study Hinkle et al. (2011) observed that substitution of the entire TM2/EC1/TM3 regions of hMC2R with corresponding domains of hMC4R resulted in a decrease in activation of the chimeric receptor co-expressed with mMRAP1 following stimulation with hACTH(1-24). However, this chimeric receptor/mMRAP1 did traffic to the plasma membrane. Since interaction with MRAP1 involves both activation and trafficking, the expectation was that trafficking should have been blocked by this radical substitution of both TM2 and TM3. To clarify the results of Hinkle et al (2011) study, a less drastic chimeric receptor was used in Figure 13. In this experiment, only the EC1 domain of the hMC2R was replaced with the corresponding domain of the xtMC1R. This chimeric receptor resulted in a 10-fold shift in sensitivity to ACTH(1-24), but did not completely block activation of the chimeric receptor. These results are in agreement with the Hinkle et al. (2011) study, and once again suggest that while disruption of EC1 domain can interfere with activation, this region of hMC2R is most likely not the contact site with the activation motif of MRAP1. The next set of experiments in this chapter were designed to explain the 10 fold drop in sensitivity to activation by hACTH(1-24) that is associated with perturbations of EC1.

As shown in Figure 14, the most likely sites for interaction between the activation motif on the N-terminal of MRAP1 and amino acid positions on EC1 would be between N<sup>88</sup> and S<sup>97</sup>. Surprisingly, the EC1 cassette alanine substitution activation assays did not support this hypothesis. The results showed that there was no significant shift in sensitivity when the hMC2R EC1 AAA3 (G<sup>01</sup>Y<sup>92</sup>L<sup>93</sup>) and AAA4 (K<sup>94</sup>P<sup>95</sup>R<sup>96</sup>) receptors

were stimulated with hACTH (1-24) (Figures 15 and 16). In fact, the only receptor that showed a shift in sensitivity to hACTH (1-24) stimulation was the EC1 AAA1 (I<sup>84</sup>I<sup>85</sup>L<sup>86</sup>) receptor. As shown in Table 1 and Figure 14, the hMC2R EC1 AAA1 receptor is positioned at the beginning of EC1 loop, and therefore, right at the surface of the plasma membrane of the cell. Since the AAA1 mutant is close to a portion of the HFRW binding site on hMC2R (Figure 14), it would appear that alanine substitution at this site has interfered with ability of hACTH(1-24) to enter the HFRW binding site. In Figure 18, a critical amino acid residue (E<sup>80</sup>) is shown in the TM2 region of the receptor that is needed for activation of the hMC2R (Chen et al. 2007). Because the hMC2R EC1 AAA1 is near this residue, it could have interfered with activation of the receptor. In fact single alanine substitution at I<sup>84</sup>I<sup>85</sup>L<sup>86</sup> indicate that only position I<sup>84</sup> interfered with stimulation by hACTH(1-24) (Figure 17).



**Figure 18: The hMC2R TM2, EC1 and TM3 Amino Acid Sequence.** The hMC2R EC1 domain is shown in its entirety. The hMC2R TM2 and TM3 domains are partially included because of the importance of two amino acid residues in these regions. The partial TM2 region starts at position I<sup>78</sup> while the partial TM3 region stops at residue S<sup>108</sup>. In the hMC2R TM2 region, the amino acid residue, E<sup>80</sup>, is critical for activation of the hMC2R. This amino acid residue is depicted by a yellow star. In the hMC2R TM3 region, the amino acid, D<sup>103</sup>, is critical for activation of the receptor as well. This amino acid residue is depicted by a green plus sign. Both of these amino acids are found in the HFRW binding pocket of the receptor (Chen et al., 2007).

Furthermore, Figure 18 shows another important amino acid, D<sup>103</sup>, which is part of the HFRW binding site as well (Chen et al., 2007). In the cassette mutant experiments,

the hMC2R EC1 AAA6 mutant is in close proximity to this amino acid residue. However, the hMC2R EC1 AAA6 mutant receptor resulted in only a 2-fold drop in activation. That said, activation of hMC2R can be adversely affected by substitution at D<sup>104</sup> (Chung et al. 2005). It would appear that substitutions to the C-terminal side of either E<sup>80</sup> or D<sup>103</sup> can have adverse effects on activation. In conclusion, these results provide evidence that the single alanine mutant I<sup>84</sup> is affecting the activation of the receptor, but contact between this residue and the activation motif of MRAP1 is highly unlikely. Hence collectively, these observations eliminate EC1 as the contact site for MRAP1.

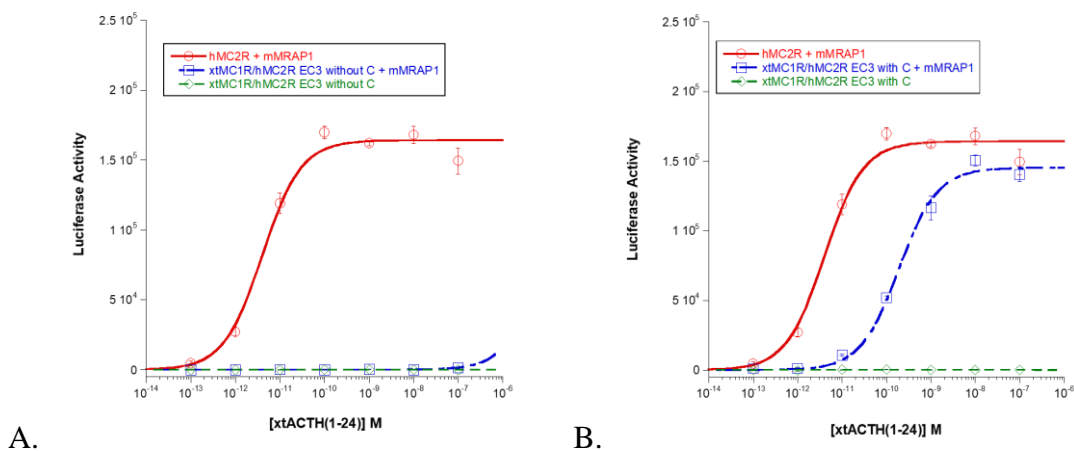
## CHAPTER 4: Evaluation of the EC3 Chimeric Receptor

Having eliminated the N-terminal domain and EC1 as the contact site for MRAP1, the next extracellular loop that was evaluated was EC3. In the next set of chimeric receptor experiments the EC3 domain of hMC2R was replaced with the EC3 domain of xtMC1R (Figure 11). Figure 17A and B illustrates the results of the chimeric xtMC1REC3/hMC2R activation assay. In Figure 17A, the curve shown in red is the wildtype hMC2R co-expressed with mMRAP1 (positive control) and the EC<sub>50</sub> value was  $4.0 \times 10^{-12} \text{ M} \pm 9.2 \times 10^{-13}$ . Next, the chimeric xtMC1REC3/hMC2R receptor was co-expressed with mMRAP1 and is shown as the blue curve. Following stimulation with hACTH(1-24), the results show that there was no activation of this chimeric receptor. These results appear to coincide with an earlier chimeric study where the TM6/EC3/TM7 domain of hMC2R was exchanged with the TM6/EC3/TM7 domain of hMC4R (Hinkle et al., 2010). In the Hinkle et al. study, this substitution caused complete loss of surface expression and activation of the hMC4R/hMC2R chimeric receptor. However, this drastic substitution of these three domains could have interfered with the HFRW binding site on hMC2R (Chen et al., 2007) as well as proper folding for the hMC2R to be trafficked to the plasma membrane. Shown in the green curve, the chimeric xtMC1REC3/hMC2R receptor when expressed alone resulted in no activation after stimulation with hACTH(1-24). Therefore, this negative control shows that swapping out the EC3 domain of hMC2R

with the EC3 domain of xtMC1R does not make this chimeric receptor MRAP1 independent.

Although these chimeric receptor results suggest that the EC3 domain plays an important role in activation of the receptor, past studies have shown that there are critical amino acid residues located in the EC3 domain that are required for proper protein folding of the hMC2R. In 2007, Yang et. al. showed that there are three cysteines that are involved in disulfide bridge formation at positions C<sup>246</sup>, C<sup>252</sup>, and C<sup>254</sup> (Figure 11). When looking at the EC3 domain of xtMC1R (Figure 11), this receptor contains only two of these critical residues which are located at positions C<sup>261</sup> and C<sup>267</sup> (Figure 11). Since the EC3 domain of xtMC1R lacks the third cysteine residue, a new chimeric receptor was designed in which the EC3 domain of the hMC2R was replaced with the a modified EC3 domain of xtMC1R in which position S<sup>270</sup> was replace with a cysteine residue. Figure 17B illustrates the results of the new chimeric xtMC1REC3/hMC2R receptor. The curve shown in red is the wild type hMC2R co-expressed with mMRAP1 and the EC<sub>50</sub> value was  $4.0 \times 10^{-12} \text{ M} \pm 9.2 \times 10^{-13}$  when stimulated with hACTH(1-24). The curve shown in blue is the new chimeric xtMC1REC3/hMC2R chimeric receptor with cysteine at position C<sup>270</sup> co-expressed with mMRAP1. Following stimulation of this chimeric receptor resulted in a 100-fold shift in sensitivity to hACTH(1-24) when compared to the wild type hMC2R control. The chimeric receptor had an EC<sub>50</sub> of  $1.9 \times 10^{-10} \text{ M} \pm 2.8 \times 10^{-11}$ , and a t-test showed this shift to be statistically significant ( $p = 0.001$ ). The curve shown in green is the chimeric xtMC1REC3/hMC2R with C<sup>270</sup> expressed alone and stimulated with hACTH(1-24). These results show that replacing the EC3 domain of

hMC2R with the corresponding domain of xtMC1R did not make the chimeric receptor MRAP independent.



**Figure 19: Analysis of the xtMC1REC3/hMC2R chimeric receptors.** A) The EC3 domain of hMC2R was replaced with the EC3 domain of xtMC1R (Figure 11) to make the xtMC1REC3/hMC2R chimeric receptor. Wild-type hMC2R (red dose response curve) and the chimeric xtMC1R/hMC2R (blue dose response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. The chimeric xtMC1REC3/hMC2R receptor was also expressed alone (green dose response curve)(n=3). B) A second chimeric xtMC1REC3/hMC2R receptor was made in which the S<sup>270</sup> (xtMC1R; Figure 11) was replaced with C<sup>270</sup>. Wild type hMC2R (red dose response curve) and the chimeric xtMC1REC3/hMC2R with C<sup>270</sup> (blue dose response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. The chimeric xtMC1REC3/hMC2R with C<sup>270</sup> was also expressed alone (green dose response curve) (n= 3).

#### hMC2R EC3 Single and Double Alanine Mutants

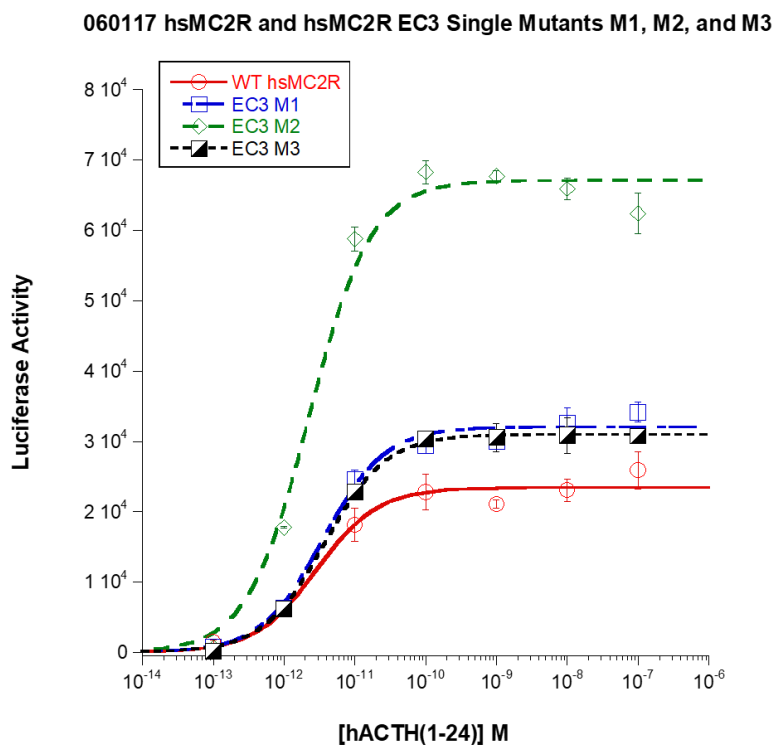
To determine which amino acid positions effect activation in the EC3 domain of hMC2R, a single alanine substitution approach was utilized in the next set of experiments. There are a few observations that led to the use of single site directed mutagenesis. As noted previously, there are three cysteine residues in EC3 that are

critical for successful disulfide bridge formation as reported by Yang et al. (2007). A concern for this portion of the study was that breaking any of these three disulfide bridges would result in a change in the shape of the receptor that would disrupt activation. Hence, the objective in this set of experiments was to minimize interference with the cysteine residues in EC3 to hopefully avoid disruption of disulfide bond formation. One approach would be to use a single alanine substitution paradigm to hopefully minimize disruption of disulfide bond formation. In addition, the operating assumption was that the positions to investigate in the EC3 domain should be positions that differ between xtMC1R EC3 and hMC2R EC3. As shown in Figure 11, position P<sup>262</sup> and position Y<sup>270</sup> are identical in xtMC1R and hMC2R. Excluding substitution at the three cysteine residues, there are six positions in the EC3 domain that would be appropriate for single alanine substitution: S<sup>248</sup>, N<sup>249</sup>, P<sup>250</sup>, Y<sup>251</sup>, M<sup>256</sup>, and S<sup>257</sup> (Table 2).

Wild type hMC2R EC3 Domain	<b>CPSNPYCACYMS</b>
Mutant 1	<b>C</b> <u>P</u> <b>A</b> <b>N</b> <b>P</b> <b>Y</b> <b>C</b> <b>A</b> <b>C</b> <b>Y</b> <b>M</b> <b>S</b>
Mutant 2	<b>C</b> <b>P</b> <b>S</b> <u><b>A</b></u> <b>P</b> <b>Y</b> <b>C</b> <b>A</b> <b>C</b> <b>Y</b> <b>M</b> <b>S</b>
Mutant 3	<b>C</b> <b>P</b> <b>S</b> <b>N</b> <u><b>A</b></u> <b>Y</b> <b>C</b> <b>A</b> <b>C</b> <b>Y</b> <b>M</b> <b>S</b>
Mutant 4	<b>C</b> <b>P</b> <b>S</b> <b>N</b> <b>P</b> <u><b>A</b></u> <b>C</b> <b>A</b> <b>C</b> <b>Y</b> <b>M</b> <b>S</b>
Mutant 5	<b>C</b> <b>P</b> <b>S</b> <b>N</b> <b>P</b> <b>Y</b> <b>C</b> <b>A</b> <b>C</b> <u><b>Y</b></u> <b>A</b> <b>S</b>
Mutant 6	<b>C</b> <b>P</b> <b>S</b> <b>N</b> <b>P</b> <b>Y</b> <b>C</b> <b>A</b> <b>C</b> <b>Y</b> <b>M</b> <u><b>A</b></u>

**Table 2: hMC2R EC3 Domain Single Alanine Mutants.** This table includes the six single alanine mutants in the EC3 domain of hMC2R. The important cysteine residues are denoted in red while the positions that were substituted with alanines are denoted in green and underline.

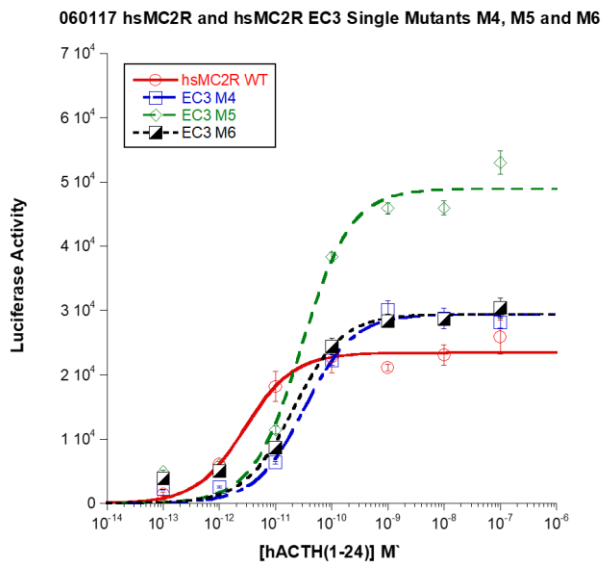
Figure 18 illustrates the activation assay of the first three single alanine mutants in which positions S<sup>248</sup>, N<sup>249</sup>, and P<sup>250</sup> were individually replaced with an alanine residue (Table 2). The wild type hMC2R receptor and each hMC2R single-alanine mutant receptor was separately co-expressed with mouse MRAP1 and stimulated with hACTH(1-24).



**Figure 20: hMC2R EC3 single alanine mutants at residues S<sup>248</sup>, N<sup>249</sup>, and P<sup>250</sup> co-expressed with mMRAP1 and stimulated with hACTH(1-24).** The wild type hMC2R and the three mutant receptors were separately co-expressed with mMRAP1 and stimulated with hACTH(1-24) as described in Methods. The curve shown in red is the wild type hMC2R. The curve shown in blue is the single alanine mutant at position S<sup>248</sup> (M1 = Mutant 1) The curve shown in green is the single alanine mutant at position N<sup>249</sup> (M2 = Mutant 2) The curve shown in black is the single alanine mutant at position P<sup>250</sup> (M3 = Mutants 3)(n=3).



The wild type hMC2R receptor is shown in red and the  $EC_{50}$  value was  $2.8 \times 10^{-12}$  M  $\pm 7.6 \times 10^{-13}$ . The curve shown in blue is the first single-alanine mutant ( $S^{248}/A^{248}$ ). For this mutant receptor the  $EC_{50}$  value was  $3.5 \times 10^{-12}$  M  $\pm 7.2 \times 10^{-13}$  ( $p = 0.99$ ; One-Way ANOVA). The curve shown in green is the second single alanine mutant ( $N^{249}/A^{249}$ ). This mutant receptor had an  $EC_{50}$  value of  $2.3 \times 10^{-12}$  M  $\pm 4.7 \times 10^{-13}$  ( $p = 0.42$ ; One-Way ANOVA). The curve shown in black is the third single alanine mutant ( $P^{250}/A^{250}$ ). This mutant receptor had an  $EC_{50}$  value of  $3.7 \times 10^{-12}$  M  $\pm 2.1 \times 10^{-13}$

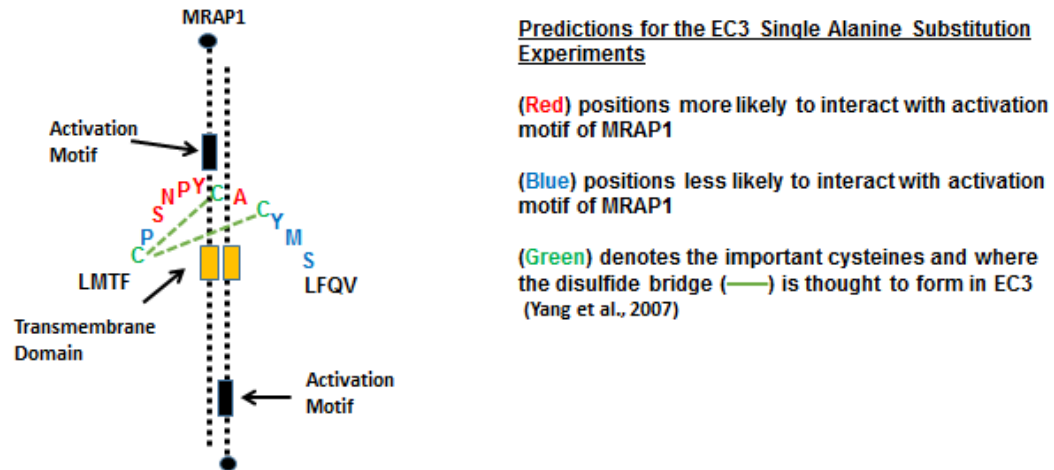


**Figure 21: hMC2R EC3 single alanine mutants at residues  $Y^{251}$ ,  $M^{256}$ , and  $S^{257}$  co-expressed with mMRAP1 and stimulated with hACTH(1-24).** The wild type hMC2R and the three mutant receptors were separately co-expressed with mMRAP1 and stimulated with hACTH(1-24) as described in Methods. The curve shown in red is the wild type hMC2R.

In summary, the single alanine mutants at residues S<sup>248</sup>, N<sup>249</sup>, and P<sup>250</sup> in the EC3 domain of the hMC2R had no effect, either positive or negative on activation of hMC2R following stimulation with hACTH(1-24).

Figure 19 illustrates the activation assay of the next three single alanine mutants in the EC3 domain of hMC2R at positions Y<sup>251</sup>, M<sup>256</sup>, and S<sup>257</sup> (Table 2). The wild type hMC2R and each mutant receptor was separately co-expressed with mMRAP1 and stimulated with hACTH(1-24). The curve shown in red is the wild type hMC2R receptor that had an EC<sub>50</sub> value of  $2.8 \times 10^{-12} \text{ M} \pm 7.6 \times 10^{-13}$ . The curve shown in blue is the fourth mutant where an alanine replaced residue Y<sup>251</sup> in the EC3 domain of hMC2R. This mutant had an EC<sub>50</sub> value of  $3.2 \times 10^{-11} \text{ M} \pm 7.2 \times 10^{-12}$  ( $p = 0.98$ ; One-Way ANOVA). The curve shown in blue is the fifth mutant where an alanine replaced residue M<sup>256</sup> in the EC3 domain of hMC2R. This mutant had an EC<sub>50</sub> value of  $2.9 \times 10^{-11} \text{ M} \pm 9.5 \times 10^{-12}$  ( $p = 0.93$ ; One-Way ANOVA). The curve shown in black is the sixth mutant where an alanine replaced residue S<sup>257</sup> in the EC3 domain of hMC2R. This mutant had EC<sub>50</sub> value of  $2.1 \times 10^{-11} \text{ M} \pm 7.2 \times 10^{-12}$  ( $p = 0.98$ ; One-Way ANOVA). When compared to the wild type hMC2R, each of the hMC2R EC3 single alanine mutants at positions Y<sup>251</sup>, M<sup>256</sup>, and S<sup>257</sup> resulted in little to no difference in activation. These results were perplexing. The operating hypothesis was that residues in the extracellular loop near the apex of the loop would interact with the activation motif of MRAP1 (Figure 20). Hence, it was assumed that one or more of the single-alanine mutants would interfere with activation. While care was taken to presumably preserve the disulfide bridges formed in

EC3, none of these single-alanine mutations appear to be involved with the contact site for MRAP1.



**Figure 22: Predictions for the EC3 Single and Double Alanine Substitution Experiment.** This figure shows the 12 residues in the EC3 extracellular loop of hMC2R. Based on the position of the MRAP1 homodimer, the residues shown in red are most likely to interact with the activation motif of MRAP1. Shown in blue are the residues that are less likely to interact with the activation motif of MRAP1. Finally, the residues shown in green denote the important cysteines. Note the internal disulfide bridge. The third cysteine in EC3 forms a disulfide bridge with the cysteine residue located in the N-terminal of hMC2R (Yang et al., 2007).

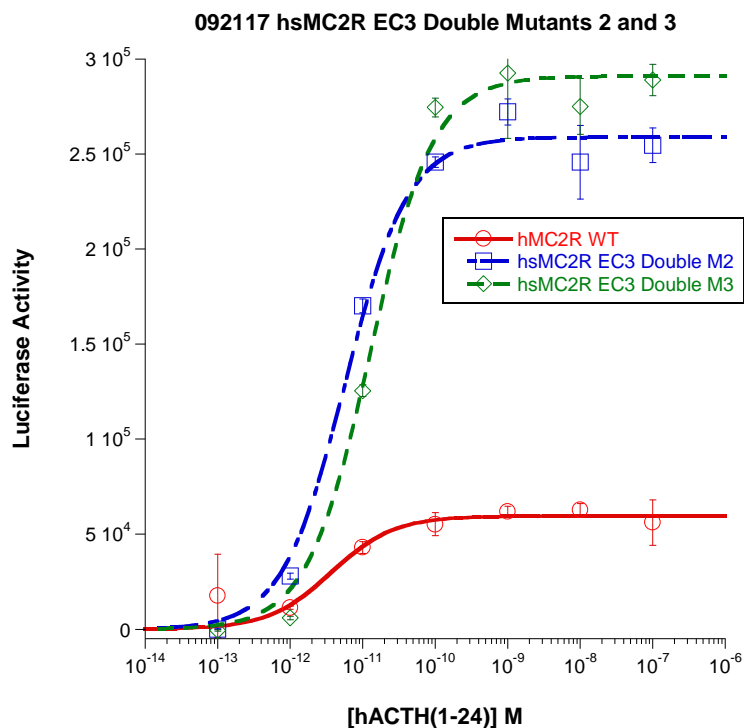
Perhaps by inserting multiple alanine substitutions in EC3, it might be possible to interfere with activation. To achieve this end, a double alanine substitution experiments were done (Figure 21). In this experiment, two separate double alanine mutants were made to see if an increase in the number of alanines might adversely affect activation by hACTH(1-24)). A double alanine mutant was made at residues S<sup>248</sup> and N<sup>249</sup> (Mutant 2) while another double alanine mutant was made at residues P<sup>250</sup> and Y<sup>251</sup> (Mutant 3). Note

that these double alanine substitution mutants were made without replacing any of the cysteines in an effort to avoid disrupting disulfide bridge formation (Table 3).

Wild type hMC2R EC3 Domain	CPSNPY <b>CAC</b> YMS
Double Alanine Mutant 2 (M2)	C <b>PAA</b> PY <b>CAC</b> YMS
Double Alanine Mutant 3 (M3)	CPSN <b>AA</b> C <b>CAC</b> YMS

**Table 3: hMC2R EC3 Domain Double Alanine Mutants.** This table includes the two double alanine mutants in the EC3 domain of the hMC2R. The important cysteine residues are denoted in red while the positions that were substituted with alanine are denoted in blue and underline.

Figure 21 illustrates the hMC2R EC3 domain double alanine substitution activation assay (Table 3). The wild type hMC2R and each double mutant receptor was separately co-expressed with mMRAP1 and stimulated with hACTH(1-24). The curve show in red is the wild type hMC2R and the EC<sub>50</sub> value was 3.6 x 10<sup>-12</sup> M +/- 9.5 x 10<sup>-12</sup>. The curve shown in blue is the first double mutant, Mutant 2 (S<sup>248</sup> N<sup>249</sup>/A<sup>248</sup>A<sup>249</sup>). This double mutant had an EC<sub>50</sub> value of 5.7 x 10<sup>-12</sup> M +/- 9.5 x 10<sup>-13</sup> (p= 0.99; One-Way ANOVA). The curve shown in green is the second double mutant, Mutant 3 (P<sup>250</sup> Y<sup>251</sup>/A<sup>250</sup>A<sup>251</sup>). This double mutant had an EC<sub>50</sub> of 1.2 x 10<sup>-12</sup> M +/- 2.2 x 10<sup>-12</sup> (p = 0.67; One-Way ANOVA). Neither double mutant had a negative effect on activation.



**Figure 23: hMC2R EC3 double alanine mutants M2 and M3 co-expressed with mMRAP1 and stimulated with hACTH(1-24).** The wild type receptor hMC2R receptor as well as double alanine mutants M2 and M3 were separately co-expressed with mMRAP1 and stimulated with hACTH(1-24) as described in Methods. The curve shown in red is the wild type hMC2R. The curve shown in blue is the double alanine mutant, Mutant 2 (M2), at positions S<sup>249</sup> and N<sup>250</sup>. The curve shown in green is the EC3 double alanine mutant, Mutant 3 (M3), at positions P<sup>250</sup> and Y<sup>251</sup> (n = 3)

### Conclusions

Overall, the operating premise of this thesis is based on the study by Malik et al. (2015); this study's results indicated that one of the EC domains of hMC2R must be making contact with the activation motif of MRAP1 that is positioned on the extracellular side of the plasma membrane. Since the N-terminal domain and the EC1 domain were

eliminated from consideration in Chapters 2 and 3, this chapter has focused on the EC3 domain. However, the single and double alanine mutants of the hMC2R EC3 domain did not disrupt activation of these mutant receptors when co-expressed with mMRAP1. Hence, it was not possible to show that MRAP1 is interacting with the EC3 domain of hMC2R. As a result, it would appear that the chimeric xtMC1RE $\text{EC3}$ /hMC2R receptor (Figure 19) must have misfolded in some way which caused a drop in sensitivity to hACTH(1-24) rather than an interference with an interaction with MRAP1. That said, the chimeric hMC2R/hMC4R experiments conducted by Hinkle et al. (2011) in which the TM6/EC3/TM7 region of hMC2R was replaced with the corresponding region of hMC4R must have also caused an unexpected distortion of the 3-D shape of the chimeric receptor. Since disrupting the disulfide bridges associated with the EC3 appears to be the only way to block activation of hMC2R (Yang et al., 2007), there is not sufficient evidence to support the assumption that the EC3 domain is not the contact site for MRAP1.

In retrospect, interaction between the TM6/EC3/TM7 domain of hMC2R and MRAP1 seems rather unlikely. Yang et al., (2007) had already shown that the proper disulfide bridge formation between EC3 and the N-terminal of hMC2R was essential for activation. In the current study this point was made very clear in Figure 12A (N-terminal chimeric receptor that lacked a cysteine residue), and Figure 19A (EC3 chimeric receptor that lacked a cysteine residue at position 265). In addition the single alanine substitution experiments for the EC3 domain of hMC2R indicated no adverse effect on activation (Figures 20 and 21). The rationale for the chimeric receptor experiments using xtMC1R and the alanine substitution experiments for EC3 are all based on the premise that

difference in amino acid positions between xtMC1R and hMC2R should help identify the possible sites in hMC2R that could be interacting with the activation motif of MRAP1 (activation function) and the TM of MRAP1 (trafficking function). However as shown in Figure 24, there is considerable primary sequence identity with the TM6/EC3/TM7 domains of xtMC1R and hMC2R. For the TM6 domain, the primary sequence identity is 76% (positions highlighted in red). However, when neutral substitutions are considered, the sequence similarity is 88%. Hence, TM6, a portion of the HFRW binding site, would appear to be an unlikely contact site for the TM of MRAP1. The single-alanine substitution experiments of EC3 domain eliminated the possibility that the activation motif of MRAP1 is interacting with this domain. Likewise, the sequence identity between xtMC1R and hMC2R for the EC3 domain is 64%, and the sequence similarity between these two domains is 86%. Hence, interaction between hMC2R and MRAP1 at these domains seems highly unlikely.

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[-----TM6-----]      EC3      [-----TM7-----]

hMC2  KGAITLTILLGVFIFCWAPFVLHVLLMTFCPSNPYCACYMSLFQVNGMLIMCNAVIDPFIYAFR
xtMC1  KGAITLTLLLVGFFLCWGPLFLHLTLFVSCPGHHICNSYFYFNTIYLLLVICNSVIDPLIYAFR

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**Figure 24: Alignment of the TM6/EC3/TM7 domain of hMC2R and xtMC1R.** The TM<sup>h</sup>/EC3/TM7 domains of xtMC1R and hMC2R were aligned in Figure 11. Positions highlighted in red were identical. Positions highlighted in green represent neutral substitution (Betts and Russell, 2003).

## CHAPTER 5: Evaluation of the EC2 Chimeric Receptor – Testing Hypothesis 2

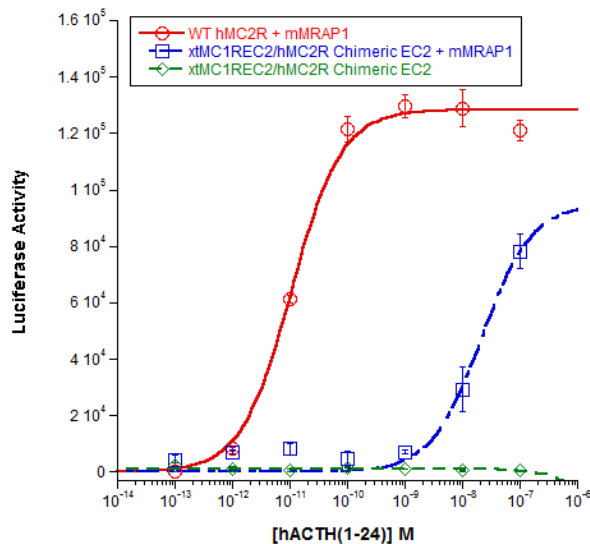
Human MC2R has by far the most complicated activation mechanism of the five human melanocortin receptors (Dores, 2018). As discussed in the **INTRODUCTION**, Schwyzer (1977) observed that the mammalian “ACTH” receptor (aka MC2R) could only be activated by ACTH(1-39), but not by  $\alpha$ -MSH [N-acetyl-ACTH(1-13)NH<sub>2</sub>], and within the sequence of ACTH(1-39) there were two amino acid motifs (H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> & K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>) that are required for activation of the “ACTH” receptor. The cloning and sequencing of hMC2R confirmed the unique ligand selectivity of the “ACTH” receptor (Mountjoy et al., 1992). The studies of Pogosheva et al. (2005) on hMC4R and Chen et al. (2007) on hMC2R identified critical amino acid positions in TM2, TM3, TM6, and TM7 located close to the surface of the plasma membrane as the binding site for the HFRW motif. Analysis of the amino acid sequences of melanocortin receptors from bony fishes, a cartilaginous fish, an amphibian, a reptile, a bird, and mammals indicated that these amino acid positions in TM2, TM3, TM6, and TM7 have been rigorously conserved during the radiation of the gnathostomes (Dores, 2009; Baron et al., 2009). In addition, the Chen et al. (2007) study indicated that F<sup>168</sup> in EC2 may be at the KKRR binding site of hMC2R, and F<sup>178</sup> in TM5 is required for the activation of the receptor, and this conclusion was confirmed by the single alanine substitution paradigm used our lab (Dores et al., 2016). Furthermore, Chung et al. (2008) identified a naturally occurring



human mutation at H<sup>170</sup> in EC2 that resulted in Familial Glucocorticoid Deficiency Type I (FGD-1). The H<sup>170</sup> mutation while inhibiting activation of MC2R did not interfere with trafficking of the mutant receptor to the plasma membrane (Chung et al., 2008). Finally, the Malik et al (2015) study revealed that the activation motif in the N-terminal of MRAP1 made contact with one of the extracellular domains of MC2R to facilitate activation of the receptor. The objective, then, of this study has been to identify that extracellular domain. Chapters 2, 3, and 4, tested Hypothesis 1 that MRAP1 was making contact with hMC2R at either the N-terminal domain, the EC1 domain, or the EC3 domain to facilitate activation. The operating premise was that contact with any of these three extracellular domains would place the KKRR binding site (EC2) of the receptor in a conformation such that ACTH could bind to the receptor and initiate the two-step activation process presented in Figure 8 (Dores, 2018). However, the results of the chimeric receptor and alanine-substitution experiments presented in Chapters 2, 3, and 4 do not support Hypothesis 1. Hence, this chapter of the thesis will address Hypothesis 2: the contact site between MRAP1 and hMC2R occurs at extracellular loop 2 (EC2).

As indicated in Figure 11, the EC2 domain of xtMC1R and hMC2R had not primary sequence identity, hence a chimeric receptor paradigm was a reasonable approach to initially test Hypothesis 2. Analysis of the xtMC1REC2/hMC2 chimeric receptor is shown in Figure 25. The dose response curve shown in red is the wild-type hMC2R co-expressed with mMRAP1 which had an EC<sub>50</sub> value of  $1.1 \times 10^{-11} \text{ M} \pm 1.5 \times 10^{-12}$ . The curve shown in blue is the xtMC1REC2/hMC2R chimeric receptor co-expressed with mMRAP1 which had an EC<sub>50</sub> value of  $2.2 \times 10^{-8} \text{ M} \pm 8.4 \times 10^{-9}$ . The

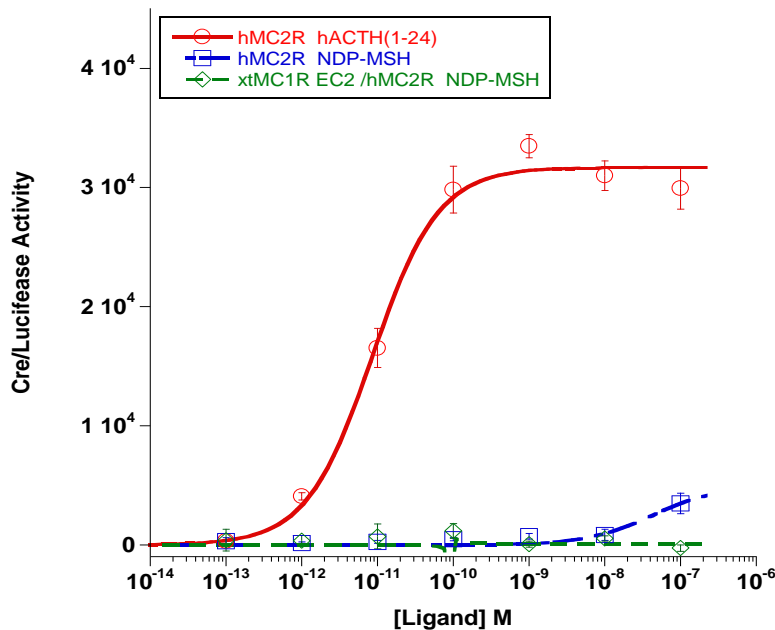
chimeric EC2 receptor was nearly 1000 less sensitive to stimulation by hACTH(1-24) as compared to the wild-type hMC2R ( $p < 0.001$ ). In addition, this level of suppression of activation for the EC2 chimeric receptor far exceeded the results observed for the N-terminal chimeric receptor (Figure 12), the EC1 chimeric receptor (Figure 15), or the EC3 chimeric receptor (Figure 17). The negative control for this experiment was to express the xtMC1REC2/hMC2R EC2 chimeric receptor alone, and this curve is shown in green. The negative control resulted in no activation; clearly showing that the xtMC1REC2/hMC2R chimeric receptor is not MRAP1 independent.



**Figure 25: xtMC1REC2/hMC2R co-expressed with mMRAP1 and simulated with hACTH(1-24).** The extracellular 2 domain (EC2) of xtMC1R was replaced with the EC2 domain of xtMC1R (Figure 11) to make the xtMC1REC2/hMC2R chimeric receptor. Wild type hMC2R (red dose response curve) and the xtMC1R/hMC2R EC2 chimeric receptor (blue does response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. Also, the xtMC1REC2/hMC2R chimeric receptor was expressed alone (green dose response curve) (n=3).

The operating assumption for the results observed for Figure 25 was that substitution of the xtMC1R EC2 domain has disrupted the KKRR binding site of hMC2R, thus inhibiting activation of the chimeric receptor by hACTH(1-24). Since xtMC1R can be activated by NDP-MSH, it is conceivable that the chimeric xtMC1REC2/hMC2R receptor might respond to stimulation by NDP-MSH. To test this hypothesis, the xtMC1REC2/hMC2R chimeric receptor was co-expressed with mMRAP1 and stimulated with NDP-MSH (Figure 26). For this experiment, two controls were used. The wild-type hMC2R receptor was co-expressed with mMRAP1 and stimulated with hACTH(1-24) to show that the cAMP/reporter gene assay worked in this experiment. The second control involved expressing the wild-type hMC2R co-expressed with mMRAP1, and to stimulate with NDP-MSH. As shown in Figure 26, following stimulation with hACTH(1-24), the wild type receptor (red curve) was activated by hACTH(1-24) and had an EC<sub>50</sub> value of  $8.5 \times 10^{-12} \text{ M} \pm 1.4 \times 10^{-12}$ . The blue curve shows the wild-type hMC2R co-expressed with mMRAP1 stimulated with NDP-MSH. No activation was observed at concentrations of NDP-MSH from  $10^{-12} \text{ M}$  to  $10^{-8} \text{ M}$ . There was a minimal increase in stimulation at  $10^{-7} \text{ M}$ . When compared to the positive control, there is an 84.5% decrease in activation when hMC2R is stimulated with  $10^{-7} \text{ M}$  NDP-MSH as compared to the corresponding concentration of hACTH(1-24). This level of stimulation is marginally above background. Finally, the green curve shows the xtMC1REC2/hMC2R chimeric receptor co-expressed with mMRAP1 and stimulated with NDP-MSH. There was no stimulation of the chimeric receptor at any of the concentrations of NDP-MSH tested. Therefore, replacing the EC2 domain of the hMC2R

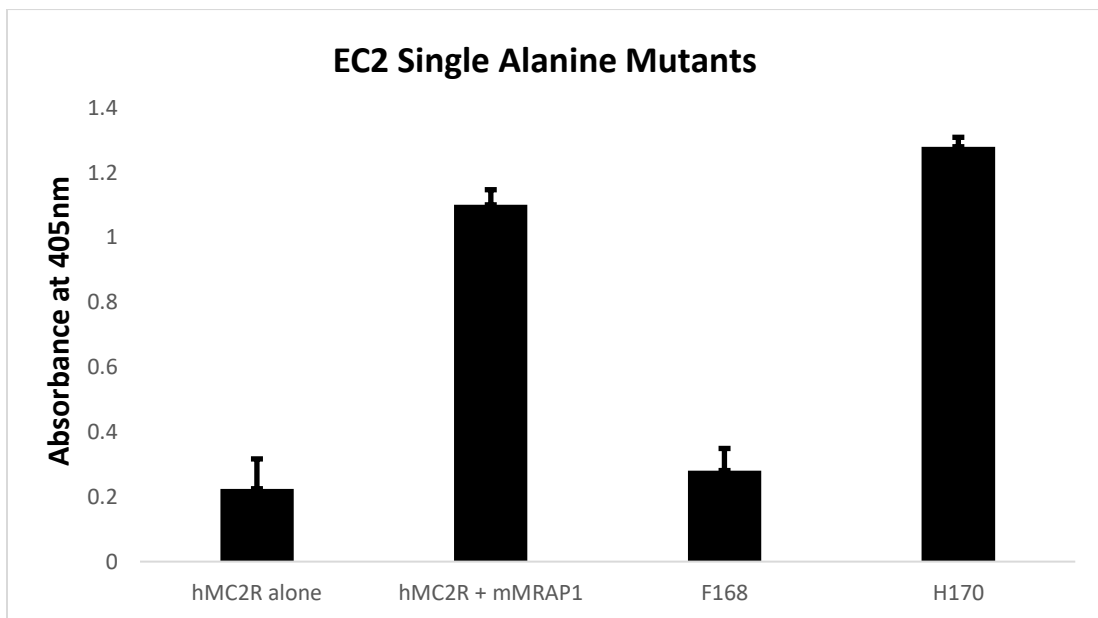
with the corresponding EC2 domain of xtMC1R did not alter the structure of the chimeric receptor in a way such that the chimeric receptor could be activated by NDP-MSH.



**Figure 26: xtMC1REC2/hMC2R co-expressed with mMRAP1 and stimulated with hACTH(1-24) or NDP-MSH.** The extracellular 2 domain (EC2) of xtMC1R was replaced with the EC2 domain of xtMC1R (Figure 11) to make the xtMC1REC2/hMC2R chimeric receptor. Wild type hMC2R (red/blue response curves) and the xtMC1REC2/hMC2R chimeric receptor (green does response curve) were separately co-expressed with mMRAP1 in CHO cells as described in Methods. In this activation assay, the wild type hMC2R (red/blue dose response curves) was stimulated with hACTH(1-24) or NDP-MSH. The xtMC1REC2/hMC2R chimeric receptor (green dose response curve) was stimulated with NDP-MSH. (n=3).

Earlier studies had shown that alanine substitution at F<sup>168</sup> in the EC2 domain (Chen et al., 2007) or at H<sup>170</sup> in the EC2 domain (Dores et al., 2016) interfered with the activation of the alanine substituted hMC2R cDNAs. To determine whether either of

these sites played a role in trafficking, the F<sup>168</sup>/A hMC2R mutant receptor, and the H<sup>170</sup>/A<sup>170</sup> hMC2R mutant receptor were analyzed using a cell surface ELISA assay. In this experiment the wild-type hMC2R, and the two alanine-substituted mutants of hMC2R were co-expressed with mMRAP1. As shown in Figure 27, alanine substitution at the H<sup>170</sup> position did not decrease the trafficking of the mutant receptor to the plasma membrane as compared to the wild-type hMC2R control (p = 0.27). However, alanine substitution at F<sup>168</sup> did lower trafficking of the mutant receptor to the plasma membrane as compared to the wild-type hMC2R (p < 0.001).



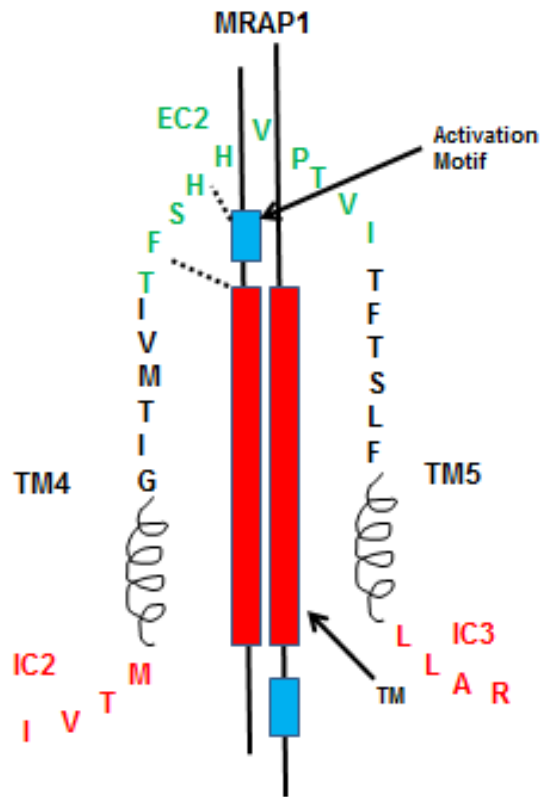
**Figure 27: Cell Surface ELISA analysis of F<sup>168</sup>/A<sup>168</sup> hMC2R and the H<sup>170</sup>/A<sup>170</sup>hMC2R.** This figure illustrates the surface expression of two single alanine mutants in the EC2 domain of hMC2R. These mutants include alanine substitution at F<sup>168</sup>/A<sup>168</sup> and H<sup>170</sup>/A<sup>170</sup>. Each of these mutants was co-expressed with mMRAP1. The positive control is the wild type hMC2R co-expressed with mMRAP1 while the negative control is the wild type hMC2R expressed alone. One-way ANOVA analysis for positive control vs. negative control was p < 0.001 (n=3).

## Conclusions

The primary objective of this study was to identify the extracellular loop on hMC2R that is making contact with the activation motif in the N-terminal of MRAP1. The results from Figure 25 point to extracellular loop 2 (EC2) as that contact point. Substituting the EC2 domain of xtMC1R into hMC2R resulted in a significant drop in sensitivity of the receptor for stimulation by hACTH(1-24) which far exceeded the chimeric experiments for the N-terminal domain, EC1 domain or EC3 domain (Figures 12, 15, and 17). This result is in agreement with the decline in activation observed when residues in the EC2 domain were replaced with alanines (Chen et al., 2007; Dores et al., 2016), and the spontaneous mutation observed in a human patient at H<sup>170</sup> in the EC2 domain (Chung et al., 2008). When the observations from Figure 25 and 27 are combined, the most parsimonious explanation for the MC2R/MRAP1 interaction is that the N-terminal domain of the MRAP1 homodimer that faces the extracellular space, together with H<sup>170</sup> position in EC2 form a binding pocket for the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup> motif of hACTH(1-24). This conclusion is shown graphically in Figure 28. In addition, the results of the cell surface ELISA analysis (Figure 27) indicate that residue F<sup>168</sup> in the EC2 domain plays a role in the trafficking of hMC2R by presumably interacting with the transmembrane domain of MRAP1 (Sebag and Hinkle, 2009).

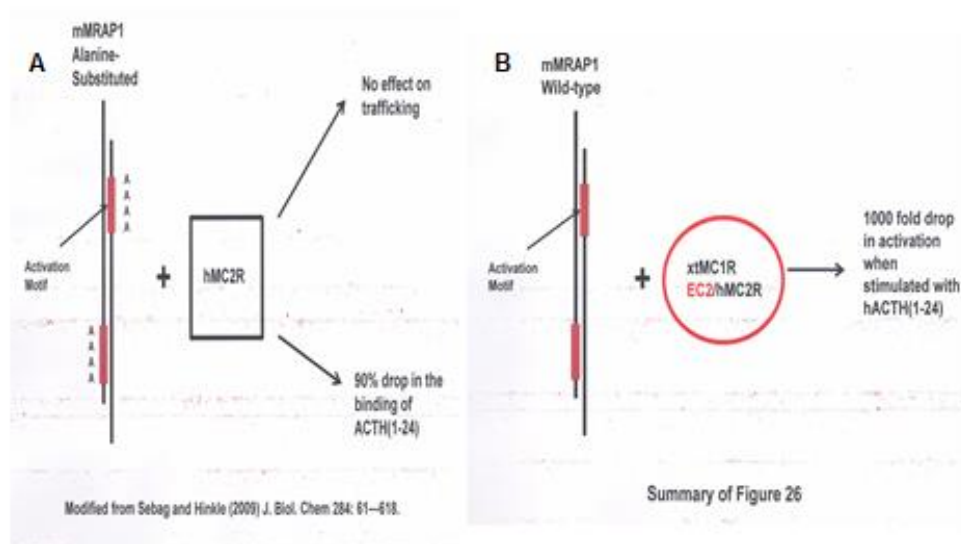
Partial support for the preceding conclusions comes from a previous study by Sebag and Hinkle (2009). The results of that study are summarized in Figure 29A. Sebag and Hinkle observed that when the four amino acid activation motif in the N-terminal of mMRAP1 was replaced with alanine residues, there was a dramatic decline in the binding

of <sup>125</sup>I-ACTH(1-39) to hMC2R. The outcome of the Sebag and Hinkle (2009) study could be explained by two mutually exclusive hypotheses; either the MRAP1 homodimer makes contact with hMC2R at the KKRR binding site, or the MRAP1 homodimer make contact at some other site on hMC2R and the outcome of that interaction placed the KKRR contact site on hMC2R in the proper conformation to allow ACTH to binding to hMC2R. The Sebag and Hinkle (2009) experiment could not distinguish between these two possibilities. However, the EC2 chimeric receptor experiment summarized in Figure 29B is the reciprocal experiment to the Sebag and Hinkle (2009) study. When the wild-type mMRAP1 was co-expressed with the EC2 Chimeric hMC2R there was a significant drop in activation of the EC2 chimeric receptor. Collectively, these two experiments point to an intimate interaction between MRAP1 and hMC2R. The interaction is most likely creating a binding site for the KKRR motif of ACTH the would be required to initiate the predicted two-step activation mechanism of hMC2R, and this interaction with EC2 also appears to contribute to the trafficking function associated with MRAP1.



**Figure 28: Modeling the interaction between the EC2 domain of hMC2R and the activation motif of MRAP1.** As mentioned in the text, the N-terminal domain of the MRAP1 homodimer that faces the extracellular space contains the activation motif that may interact with position H<sup>170</sup> in the EC2 domain of hMC2R to create binding pocket for the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup> motif of hACTH(1-24). Note (----) that indicated interaction between EC2 amino acid residue and activation motif of MRAP1. Also, this model includes the position, F<sup>168</sup>, located in the EC2 domain. Cell Surface ELISA results in Figure 27 suggest that residue F<sup>168</sup> may play a role in trafficking due to a significant drop in surface expression. Therefore, this residue may interact with the transmembrane domain of the hMC2R. Note the (----) that indicates an interaction between the EC2 amino acid residue and the transmembrane domain of MRAP1 (Sebag and Hinkle, 2009).

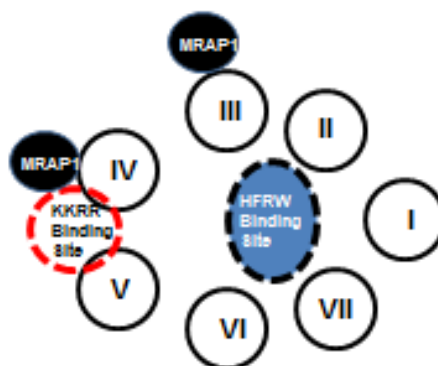




**Figure 29: A) This figure summarizes the study of Sebag and Hinkle (2009).** In brief, co-expression of mutant form of mMRAP1 (alanine substitution at the activation motif) and wild-type hMC2R resulted in a significant drop in the binding of  $^{125}\text{I}$ -ACTH(1-39). **B) This figure summarizes the outcome of Figure 25.** Co-expression of wild-type mMRAP1 with the xtMC1REC2/hMC2R chimeric receptor resulted in a significant drop in activation of the receptor following stimulation with hACTH(1-24).

A survey of the literature indicates that at least one other group proposed that the EC2 domain and MRAP1 form the binding site for the KKRR motif of ACTH (Fridmanis et al., 2010). In this study a confocal imaging procedure was used to evaluate interactions between mMRAP1 and various chimeric receptors of hMC2R and hMC4R. This novel procedure was combined with hormone/receptor binding studies, and the results of that study are summarized in Figure 30. However, the Fridmanis et al (2009) does raise some concerns with respect to the conclusions that were drawn for this study, as indicated in the comments in the figure legend.

### hMC2R/MRAP1 Interaction



Fridmanis et al., (2010) Mol. Cell. Endo. 321: 175-183

**Figure 30: Summary of the conclusions presented in Fridmanis et al., 2010.** In this study the authors propose that two MRAP1 homodimers interact with hMC2R. The authors propose that MRAP1 contact at TMIII interferes with the HFRW binding domain in the receptor. In addition, MRAP1 also makes contact with TM4/EC2 domain to form the KKRR binding site. The stoichiometry presented in this figure does not agree with the study of Cooray et al., 2010 which used FRET imaging analysis to show that one hMC2R monomer and one MRAP1 monomer form a heterodimer.

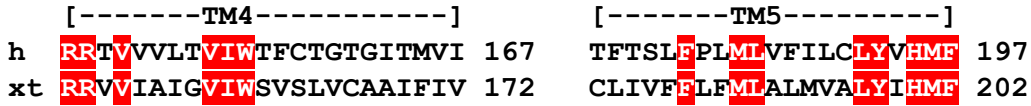
Although the experimental data to support the Fridmanis et al (2010) conclusions is not strong, the current study in combination with the Sebag and Hinkle (2009), the Chen et al (2007) study, and the Chung et al (2008) all point to the N-terminal terminal of MRAP1 making contact with the EC2 domain of hMC2R, and that interaction appears to create the KKRR binding pocket on hMC2R.

Given these conclusions, the next issue to resolve was the role that F<sup>178</sup> in the TM5 domain plays in the activation process. Alanine substitution at this amino acid position greatly reduced activation (Dores et al., 2016). However, given the location of this residue relative to F<sup>168</sup> and H<sup>170</sup>, it would be difficult to imagine that F<sup>178</sup> is also in the KKRR binding site. However, F<sup>178</sup> may have a role in the trafficking of hMC2R to the plasma membrane. The next experiments set out to evaluate this possibility.

## CHAPTER 6: Evaluating the role of TM4 and TM5 in the trafficking of hMC2R

Since aligning xtMC1R and hMC2R to identify regions within domains that are clearly distinct between the two receptors proved useful in identifying the EC2 domain of hMC2R as the putative contact site for the N-terminal of MRAP1, a similar strategy was employed to evaluate residues in the TM4 and TM5 domains of hMC2R that may be interacting with the TM of MRAP1 to facilitate trafficking. As noted in Figure 10B, mMRAP1 did not affect the trafficking of xtMC1R in either a positive or negative manner. Hence, the operating assumption for designing the next set of experiments was that regions in either TM4 or TM5 of xtMC1R and hMC2R that are conserved, are less likely to be the contact site for the TM of mMRAP1 than regions that are variable in the two receptors. As shown in Figure 31, for TM4 the primary sequence identity between R<sup>146</sup> and T<sup>162</sup> is 35% and the primary sequence similarity was 66%. Between G<sup>163</sup> and I<sup>167</sup> there was no primary sequence identity, but the sequence similarity was 60%. For TM5 the primary sequence identity between L<sup>184</sup> and F<sup>197</sup> was 50%, and the primary sequence similarity was 93%; whereas between T<sup>178</sup> and P<sup>183</sup> the primary sequence identity was 14% and the primary sequence similarity was 71%. Based on these observations, single alanine mutants of hMC2R in the TM4 domain (G<sup>163</sup> to I<sup>167</sup>) and in the TM5 domain (T<sup>178</sup>

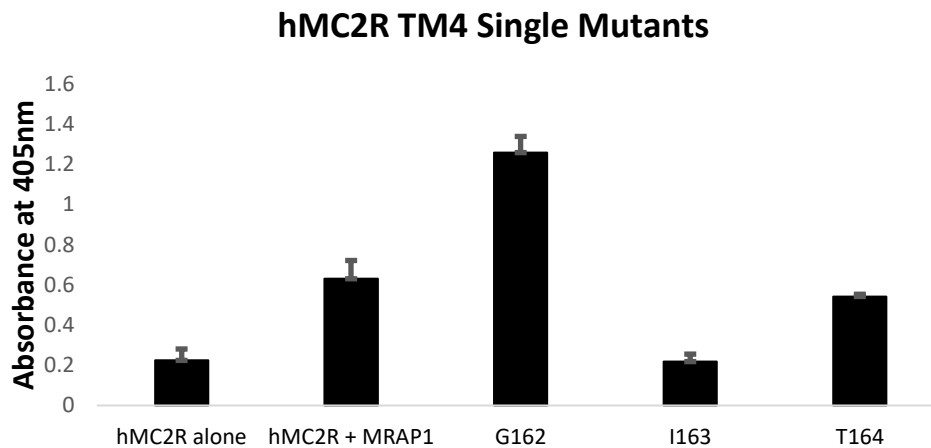
to P<sup>183</sup>) were made and analyzed using a cell surface ELISA protocol to determine whether TM4 or TM5 is involved in the trafficking of hMC2R to the plasma membrane.



**Figure 31: Alignment of xtMC1R (xt) and hMC2R (h) TM4 and TM5.** Positions that were identical in these two domains are highlighted in red.

As shown in Figure 32, alanine-substitution at G<sup>162</sup> and T<sup>164</sup> had no effect on the trafficking of the alanine-substituted G<sup>162</sup>/A<sup>162</sup> or the T<sup>164</sup>/A<sup>164</sup> hMC2R receptors.

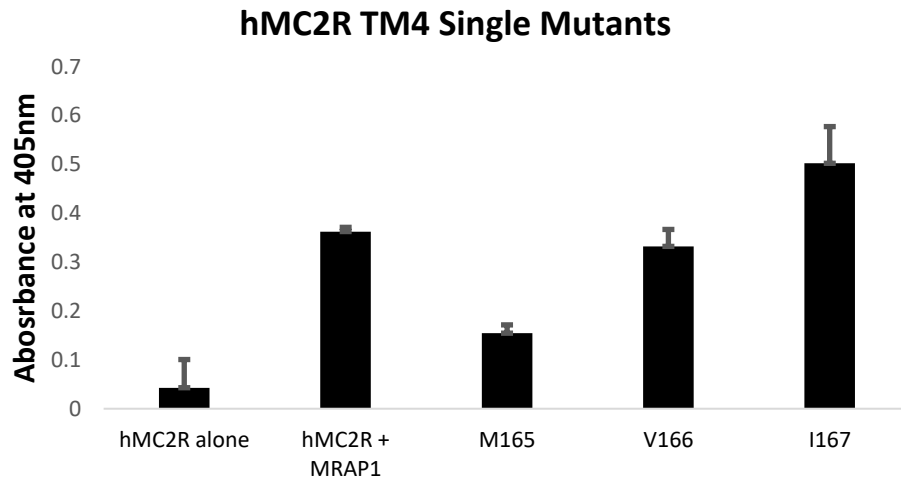
However, alanine-substitution at I<sup>163</sup> resulted in a statistically significant decline in the trafficking of the I<sup>163</sup>/A<sup>163</sup> hMC2R to the plasma membrane (p = 0.01).



**Figure 32: Cell Surface ELISA of TM4 Single Alanine Mutants of hMC2R.** This figure illustrates the results of surface expression of the single-alanine mutants at positions G<sup>162</sup>/A<sup>162</sup>, I<sup>163</sup>/A<sup>163</sup>, and T<sup>164</sup>/A<sup>164</sup>. All TM4 alanine mutants were co-expressed with mMRAP1. The positive control is wild type hMC2R co-expressed with mMRAP1. The negative control is hMC2R alone. One-way ANOVA analysis for positive control vs. negative control was p = 0.007. (n = 3).

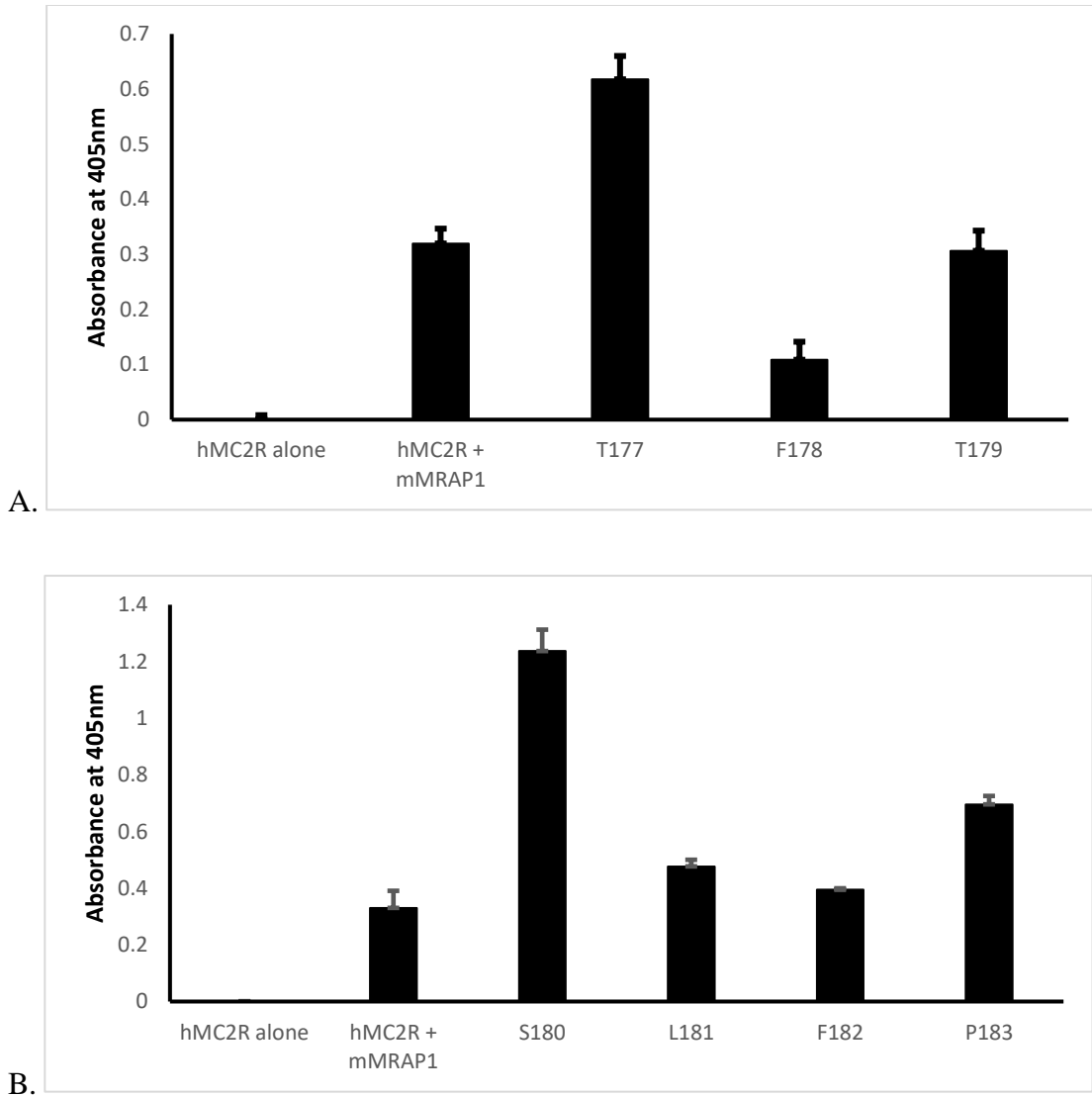
The remainder of the residues in TM4 is analyzed in Figure 33. Note that alanine substitution at V<sup>166</sup> and I<sup>167</sup> did not have a negative effect on the trafficking of the

alanine-substituted hMC2 receptors to the plasma membrane. However, trafficking to the plasma membrane of the M<sup>165</sup>/A<sup>165</sup> hMC2R mutant was less relative to the positive control (p = 0.05).



**Figure 33: Cell Surface ELISA of TM4 Single Alanine Mutants of hMC2R.** This figure illustrates the results of surface expression of the single-alanine mutants at positions M<sup>165</sup>/A<sup>165</sup>, V<sup>166</sup>/A<sup>166</sup>, and I<sup>167</sup>/A<sup>167</sup>. All TM4 alanine mutants were co-expressed with mMRAP1. The positive control is wild type hMC2R co-expressed with mMRAP1. The negative control is the hMC2R expressed alone. One-way ANOVA analysis for positive control vs. negative control was p = 0.004. (n = 3).

The same analysis was done for the TM5 domain (T<sup>178</sup> to P<sup>183</sup>) of hMC2R (Figure 34). The T<sup>177</sup>/A<sup>177</sup>, T<sup>179</sup>/A<sup>179</sup>, S<sup>180</sup>/A<sup>180</sup>, and P<sup>183</sup>/A<sup>183</sup> mutant forms of hMC2R trafficked to the plasma membrane as well as the wild-type hMC2R control. However, there was a significant decline in the trafficking of the F<sup>178</sup>/A<sup>178</sup> mutant (p = 0.002), the L<sup>181</sup>/A<sup>181</sup> mutant (p < 0.001), and the F<sup>182</sup>/A<sup>182</sup> mutant (p = 0.004) to the plasma membrane.

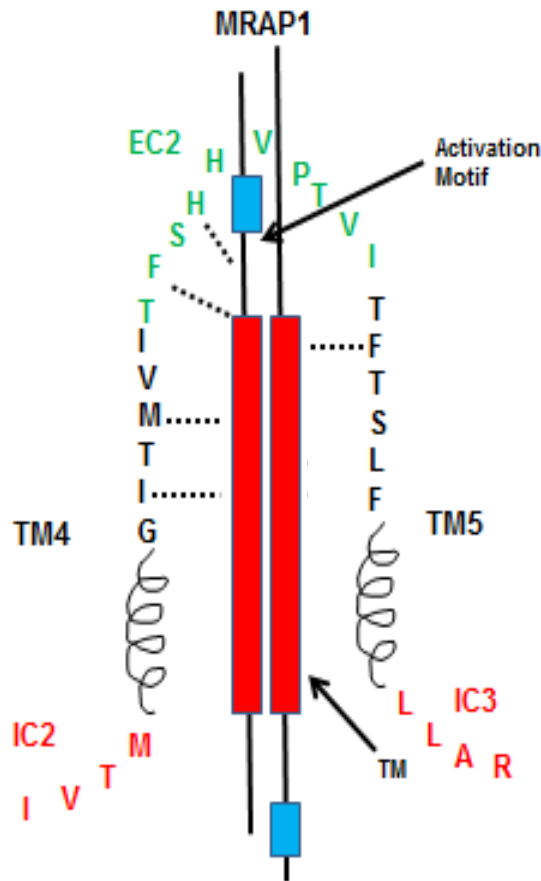


**Figure 34: Cell Surface ELISA of TM5 Single Alanine Mutants of hMC2R.** This figure illustrates the results of surface expression of the single-alanine mutants at positions  $T^{177}/A^{177}$ ,  $F^{178}/A^{178}$ , and  $T^{179}/A^{179}$ ,  $S^{180}/A^{180}$ ,  $L^{181}/A^{181}$ ,  $F^{182}/A^{182}$ , and  $P^{183}/A^{183}$ . All TM5 alanine mutants were co-expressed with mMRAP1. The positive control is wild type hMC2R co-expressed with mMRAP1. The negative control is the hMC2R expressed alone. One-way ANOVA analysis for positive control vs. negative control was  $p = 0.001$ . ( $n = 3$ ).

## Conclusions

An earlier study indicated that alanine substitution in the G<sup>163</sup> to I<sup>167</sup> domain of TM4 had a minimal effect on activation of the receptor following stimulation with hACTH(1-24); whereas alanine substitution in the T<sup>178</sup> to P<sup>183</sup> domain of TM5 significantly interfered with activation of the alanine-substituted hMC2 receptors (Dores et al., 2016). Hence the operating assumption prior to attempting the cell surface ELISA analysis was that the TM4 domain may not be involved in trafficking, while the TM5 domain should be playing a prominent role in the trafficking of hMC2R to the plasma membrane as a result of the interaction with mMRAP1. As shown in Figures 32, 33, and 34, it appears the interactions with the TM of mMRAP1 are more complex than anticipated. First, it is important to point out that the single-alanine substitution experiments assume that the cDNAs for all of the mutant hMC2Rs are expressed at the same level in CHO cells. While this assumption is made for all of the experiments presented in this thesis, confirmation by real-time PCR analysis or Western Blot analysis would resolve this issue. This discussion will proceed based on the assumption that all mutant forms of hMC2R express in CHO cells at the same level as the wild-type mMC2R. Given this caveat, Figure 35 summarizes the current view on the interaction between hMC2R and mMRAP1.





**Figure 35: Modeling the complex interaction between the TM4/EC2/TM5 region of hMC2R with the activation motif and TM region of MRAP1.** This figure illustrates plausible interactions between the TM4/EC2/TM5 domain of the hMC2R with the activation motif positioned on the extracellular side as well as the transmembrane domain of MRAP1. In Figure 28, it was discussed that due to the interaction with MRAP1, positions F<sup>168</sup> and H<sup>170</sup> of hMC2R may be important for trafficking and activation. Furthermore, Cell Surface ELISA results have shown that there may be more residues located in the TM4 and TM5 of hMC2R that are interacting with MRAP1 for successful trafficking of the receptor. Due to the drop in trafficking, the single alanine mutants at positions I<sup>163</sup> and M<sup>165</sup> in the TM4 region of the hMC2R are thought to interact with the transmembrane domain of MRAP1. Also, single alanine mutants at positions F<sup>178</sup>, L<sup>181</sup>, and F<sup>182</sup> in the TM5 domain of the receptor showed a drop in trafficking. Therefore, these positions are thought to interact with the TM domain of MRAP1 as well. These interactions between the amino acid residues of the TM4/EC2/TM5 regions of hMC2R and the respective domains of MRAP1 are denoted by (----) in the figure.

In the model presented in Figure 35, the activation motif of mMRAP1 (LDYL) may directly interact with H<sup>170</sup>. It is conceivable that the positively charged R-group of H<sup>170</sup> may ionically interact with the negatively charged R-group of the D residue in the activation motif of mMRAP1. In this scenario, F<sup>168</sup> at the bottom of EC2, M<sup>165</sup> and I<sup>163</sup> in TM4 either through h-bonding or hydrophobic interactions interact with corresponding residues on the TM of mMRAP1 along with F<sup>178</sup>, L<sup>181</sup>, and F<sup>182</sup> on TM5 to facilitate the trafficking of hMC2R to the plasma membrane. Partial support for this scenario comes from the cAMP-reporter activation experiments done on TM5 (Dores et al., 2016) which indicated that alanine substitution at F<sup>178</sup> and L<sup>181</sup> significantly impairs activation of hMC2R following stimulation with hACTH(1-24). The absence of an apparent effect by alanine mutants in the TM4 domain (Dores et al., 2016) as compared to the results presented in Figures 32 and 33, would indicate that re-evaluation of the cAMP reporter analysis for the TM4 domain of hMC2R is required.

Finally, the importance of the proposed interaction between hMC2R and mMRAP1 as summarized in Figure 35 cannot be overstated. Because of this interaction, hMC2R is delivered to the plasma membrane. Without this interaction hMC2R would be stranded in the endoplasmic reticulum. Without this interaction, hMC2R once at the plasma membrane would not be in the proper conformation to facilitate the proposed two-step activation mechanism that is proposed to begin with the binding of the KKRR motif of hACTH(1-24) to the EC2 domain of hMC2R and leads to the proposed opening of the HFRW binding site on hMC2R and the docking of the HFRW motif of hACTH(1-24); an event that leads to a conformation change in the receptor that activates the G-protein on

the cytoplasmic side of the plasma membrane and in turn activated adenylyl cyclase to begin the cascade of events that results in a biological response in the target cell.

## CHAPTER 7: Insights on the MC2R/MRAP1 Interaction

This thesis has used a chimeric receptor paradigm and an alanine-substitution paradigm to tease apart the interaction between hMC2R and mMRAP1. While it was known that the N-terminal region of mMRAP1 is required for activating the receptor. The results of this thesis point to the possibility that the N-terminal of MRAP1 and the EC2 domain of the receptor are forming the primary binding site for ACTH. However, the actual structure of this proposed binding site cannot be deduced from the approaches used in this thesis. Molecular modeling of the MC2R/MRAP1 heterodimer is a logical next step in understanding the activation of MC2R in bony vertebrates. Since previous and current studies on bony vertebrate MC2R orthologs all point to a common mechanism for activating the receptor, perhaps by taking a comparative view of the N-terminal of MRAP1 and the EC2 domain of MC2R a perception of this binding domain may emerge.

In 2009, studies on the N-terminal of mouse MRAP1 identified an important motif for activation (L<sup>18</sup>D<sup>19</sup>Y<sup>20</sup>I<sup>21</sup>) (Sebag and Hinkle, 2009). In Figure 36, the activation motif is identified in red. In this study, a cell surface expression assay and an cAMP activation assay showed significant results that lead to this conclusion. Using cell surface ELISA analysis this study showed that co-expression of the hMC2R and mouse MRAP1 resulted in trafficking of the receptor to the plasma membrane. However, substitution of

the TM of mouse MRAP1 with the TM of RAMP3 (the accessory protein for the calcitonin receptor) completely blocked trafficking. Therefore, the TM of MRAP1 is responsible for trafficking. What role does the N-terminal domain of mouse MRAP1 play in activation of human MC2R?

In a cAMP activation assay, co-expression of hMC2R with an alanine substituted mutant of mouse MRAP1 in which the L<sup>18</sup>D<sup>19</sup>Y<sup>20</sup>I<sup>21</sup> motif was replaced with alanines completely blocked activation. To clarify the relative importance of each amino acid position in the L<sup>18</sup>D<sup>19</sup>Y<sup>20</sup>I<sup>21</sup> motif, single alanine substituted mutants were made, and in this experiment substitution of an alanine at Y<sup>20</sup> resulted in a 50% drop in activation. However, alanine substitution at L<sup>18</sup>, D<sup>19</sup>, or I<sup>21</sup> had no negative effect on activation. While Y<sup>20</sup> is clearly critical for activation, L<sup>18</sup>, D<sup>19</sup>, and I<sup>21</sup> may contribute collectively to the secondary structure of the activation motif, and substitution at all four positions may have disrupted this critical secondary structure. Hence the A<sup>18</sup>A<sup>19</sup>A<sup>20</sup>A<sup>21</sup> mutant resulted in no activation because of the loss of this critical secondary structure. To complement the activation analysis, a binding assay was done and alanine substitution at the L<sup>18</sup>D<sup>19</sup>Y<sup>20</sup>I<sup>21</sup> motif completely blocked binding of hACTH(1-24). Prior to this thesis, the binding experiment with the A<sup>18</sup>A<sup>19</sup>A<sup>20</sup>A<sup>21</sup> analog of mMRAP1 could be explained by assuming that the N-terminal of mMRAP1 makes contact with either the N-terminal of hMC2R, or the EC1 domain of hMC2R, or the EC3 domain of hMC2R, to cause a conformational change that exposes the KKRR binding site on hMC2R (i.e., the EC2 domain). An alternative hypothesis was that the N-terminal of MRAP1 and the EC2 domain form the KKRR

binding site for ACTH as a result of the forming MC2R/MRAP1 heterodimer. The results of this thesis are consistent with the second hypothesis.

More recent experiments may indicate that other motifs in the N-terminal of mMRAP1 play a role in the activation mechanism (Malik et al. 2015). In this study, phenylalanines were substituted at positions Y<sup>14</sup>, Y<sup>16</sup>, Y<sup>17</sup>, and Y<sup>20</sup> to make a mutant form of mMRAP1. When this mutant form of MRAP1 formed a heterodimer with hMC2R there was a 60% drop in activation relative to the positive control (wild-type hMC2R/mMRAP1). These results suggest that Y<sup>14</sup>, Y<sup>16</sup>, and Y<sup>17</sup> may be involved in the activation mechanism. However, their earlier study (Sebag and Hinkle, 2009) observed that alanine substitution at Y<sup>20</sup> caused a 50% drop in activation when co-expressed with hMC2R, hence the role of Y<sup>14</sup>, Y<sup>16</sup>, and Y<sup>17</sup> in the activation process is still unclear. Future experiments to resolve the role played by each of the tyrosine residues should be done using a single alanine substitution paradigm. Finally, as shown in Figure 36 the avian MRAP1 ortholog has nearly all of the critical amino acids found in the activation motif of mouse MRAP1 (i.e., Y<sup>14</sup>, Y<sup>16</sup>, Y<sup>17</sup>, D<sup>19</sup>, Y<sup>20</sup>, I<sup>21</sup>). Hence, amniote MRAP1 orthologs may activate amniote MC2R orthologs through a common mechanism.

```

[-----N-terminal-----]
mMRAP1 MANGTDASVPLTSYEYYLDYIDLIPVDEKCLKANKH 36
cMRAP1 MANRTNSSEYFWSYEYYWDYIDPI PVDGRKCLKVNKY

```

**Figure 36: Alignment of the N-terminal of MRAP1 orthologs** Figure 36 illustrates the N-terminal sequence of the mouse and chicken MRAP1. The areas of primary sequence identity outside the activation motif are shown in grey. The aspartic acid (D), the tyrosine (Y), and the isoleucine (I) are important residues within the activation motif of MRAP1. These three residues are found in cMRAP1 and mMRAP1. These residues are shown in red. Abbreviations: m(mouse: *Mus musculus*), c(chicken: *Gallus gallus*).

Alanine substitution studies have also been done for the MRAP1 orthologs of two bony fishes: rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Dores et al, 2016). As shown in Figure 37, rainbow trout MRAP1 and zebrafish MRAP1 have activation motifs very similar to mouse MRAP1. The common amino acids are aspartic acid (D) and tyrosine (Y). Alanine substitution experiments revealed that both the aspartic acid (D) and tyrosine (Y) are required for activation. Note that these same amino acid positions are found in the MRAP1 ortholog of the fugu fish (*Takifugu rubripes*) and gar (*Lepisosteus oculatus*). Perhaps for the bony fishes, the aspartic acid (D) and tyrosine (Y) positions work together to facilitate activation. The zebrafish and rainbow trout study revealed the importance of the D residue in the activation motif. As the results of this thesis indicate, the N-terminal of MRAP1 and EC2 together appear to form the binding site for the KKRR motif in ACTH. In this regard, the D residue in the activation motif may ionically interact with an R-group of one of the basic amino acids in the KKRR motif of ACTH. Given this conclusion, perhaps the role of the D<sup>19</sup> residue in the activation motif of mMRAP1 should be re-evaluated.

```

      [-----N-terminal-----]
mMRAP1 MANGTDASVPLTSYEYYLDYIDLIPVDEKCLKANKH 36
rtMRAP1 M-----DSWRYEWE-YYDYLDPIPVDERKCLKYNKY
zfMRAP1 MKNSS---EYVWGYEYYDYVDPVLVNAATLKYSRY
fMRAP1  MENST--GTYEW--EYYYDYIEPVIVDESCLKYNKY
gMRAP1  MANTS---FYVW--EYYDYLDPIVIVDEKQLKFNKY

```

**Figure 37: Alignment of the N-terminal of Mouse, Zebrafish, and Rainbow trout MRAP1 orthologs** Figure 37 illustrates the N-terminal sequence of mouse, rainbow trout, zebrafish, fugu fish, and gar MRAP1 orthologs. The areas of primary sequence identity are shown in grey. The aspartic acid (D) and the tyrosine (Y) are important residues within the activation motif of MRAP1. These residues are shown to be universally conserved between all MRAP1 orthologs. These residues are shown in red. Abbreviations: m(mouse: *Mus musculus*), rt(rainbow trout: *Oncorhynchus mykiss*), zf(zebrafish: *Danio rerio*), f(fugu fish: *Takifugu rubripes*), and g(gar: *Lepisosteus occulatus*).

While alignment of the N-terminal of MRAP1 orthologs and the identification of the activation motif as well as additional amino acid positions that may be involved with activation of MC2R orthologs was fairly straightforward, identifying amino acid motifs in the EC2 domain is more challenging. When focusing on the amniote species there appears to be a relatively high degree of primary sequence identity in the EC2 domain as shown in Figure 38.

```

      [-----TM4-----]   EC2   [-----TM5-----]
h  RRTVVVLTVIWTFCTGTGITMVI FSHHVPVIT FTSLEPLMLVFILCLYVHMF 196
c  QRALVILAIWTFCAAGSSIAIAL FSHHVAIVIP FTILEPLMMIFILCLYIHMF

```

**Figure 38: Alignment of the TM4/EC2/TM5 domain of MC2R orthologs** Figure 38 illustrates the TM4/EC2/TM5 domain of the human and chicken MC2R orthologs. The areas of primary sequence identity within these domains are shown in grey. The positions shown in black are residues that are conserved within the EC2 domain of hMC2R and cMC2R orthologs. The positions shown in blue (F<sup>168</sup>, H<sup>170</sup>, and F<sup>178</sup>) are important residues within the EC2 and TM5 domain of hMC2R. Previous studies have shown that these residues either effect trafficking or activation of the hMC2R. These residues are conserved within the EC2 and TM5 domain the cMC2R ortholog as well. Abbreviations: h(human: *Homo sapiens*), c(chicken: *Gallus gallus*).

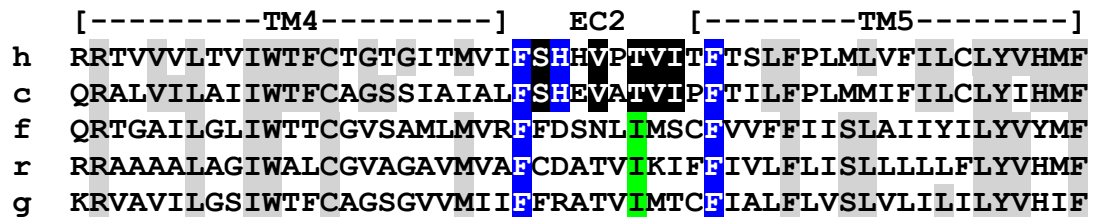
An alignment of the human EC2 and the EC2 domain of the chicken (*Gallus gallus*) indicates that there is 78% primary sequence identity in these two domains. Since



previous work on the EC2 domain of hMC2R had demonstrated the importance of F<sup>168</sup>, H<sup>170</sup>, and F<sup>178</sup> (Chen et al., 2007, Chung et al., 2008, Dores et al., 2015), it would be reasonable to conclude that these corresponding positions in the EC2 domain of the chicken should be required for activation of the chicken MC2 receptor. Alanine substitution experiments of the three conserved amino acids can confirm or refute this hypothesis.

That said, attempting to draw conclusions with regard to bony fish MC2R orthologs is more challenging. Alignment of the ray-finned fish TM4/EC2/TM5 domains with the aminote TM4/EC2/TM5 domains indicates minimal primary sequence identity for the EC2 domain (Figure 39). Within the EC2 domain of the ray-finned fishes only the phenylalanine (F) is universally conserved (Figure 39). Studies by Liang et al. (2018) indicated that the phenylalanine in the EC2 domain of rainbow trout does play a role in the activation mechanism. Alanine substitution at this site resulted in a statistically significant drop in activation. However, the position that is far more important for activation of rainbow trout MC2R is V<sup>166</sup> in the TM4 domain (Liang et al., 2018). It appears that there may be a shift in the proposed docking site for the KKRR motif of ACTH(1-24) in the ray-finned fishes. However, the Liang et al. 2018 study did not resolve whether alanine substitution at V<sup>166</sup> interfered with trafficking. Additional analysis of the ray-finned fish sequences also shows that F<sup>182</sup> is conserved in the TM5 domain, and alanine substitution at this position did have a significant negative effect on activation of the alanine substituted rainbow trout MC2 receptor (Liang et al., 2018). In addition, the study on rainbow trout MC2R also found that alanine substitution at F<sup>182</sup> had an even

bigger negative impact on activation as compared to alanine substitution at F<sup>178</sup> (Liang et al. 2018). This amino acid position is also conserved in the other ray-finned fish MC2R orthologs. Since F<sup>182</sup> is located in TM5, this position is most likely playing a role in trafficking. This hypothesis can also be tested by cell surface ELISA analysis for alanine substitution at position F<sup>182</sup> for other ray-finned fish orthologs presented in Figure 39. However, these experiments on the role that amino acid positions in TM4 and TM5 may play in trafficking does not resolve the role of the EC2 domain of the three ray-finned fish MC2R ortholog in the activation mechanism.



**Figure 39: Alignment of the TM4/EC2/TM5 domain of MC2R orthologs** Figure 39 illustrates the TM4/EC2/TM5 domain of the human, chicken, fugu fish, rainbow trout, and gar MC2R orthologs. The areas of primary sequence identity within these domains are shown in grey. The positions shown in black are residues that are conserved within the EC2 domain of hMC2R and cMC2R orthologs. Also, the phenylalanines in the EC2 and TM5 (shown in blue) are universally conserved in the MC2R orthologs. Positions in green are conserved in the ray-finned MC2R orthologs. Abbreviations: h(human: *Homo sapiens*), c(chicken: *Gallus gallus*), f(fugu fish: *Takifugu rubripes*), rt(rainbow trout: *Oncorhynchus mykiss*), and g(gar: *Lepisosteus oculatus*).

At present, based on primary sequence identity for the EC2 domain it is difficult to explain how this domain serves as the binding site for the KKRR motif of ACTH(1-24). It is assumed that the N-terminal domain of the respective MRAP1 is positioned with the EC2 domain of the three ray-finned fish sequences presented in Figure 39. The aspartic acid (D) in the activation motif of the ray-finned fish MRAP1 orthologs (Figure 37) could certainly form an ionic interaction with the basic amino acids in the KKRR motif ACTH(1-24). Perhaps the binding site created by MRAP1 and the EC2 domain relies on

the secondary structure of the amino acids in the EC2 domain rather than the primary sequence of amino acids in the EC2 domain. In other words, perhaps the EC2 domains of the ray-finned fish MC2 receptors while having different amino acid sequences actually have similar secondary structures to create the binding pocket. This issue can only be resolved through molecular modeling of the heterodimer made up of the MC2 receptor and MRAP1. To date, there are no x-ray crystallographic analyses to evaluate this hypothesis.

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## APPENDIX

### MATERIALS AND METHODS

#### Tissue Culture Procedure

Experiments were done utilizing Chinese Hamster Ovary (CHO) cells (ATCC, Manassas, VA). The cells were grown in Kaighn's Modification of Ham's F12K media supplied by ATCC. Media was supplemented with 10% fetal bovine serum, 10 unit/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml normacin (Complete CHO media) The cells were grown in a 25 cm<sup>3</sup> tissue culture flask with vent cap by CELLTREAT™ (Pepperell, MA), and maintained in an incubator with 95% air, 5% CO<sub>2</sub> at 37°C, and exposed to humidity. When the CHO cells reached 70-80% confluence, cells were split into new culture flasks using 0.05% trypsin/0.53 mM EDTA produced by CORNING cellgro™ (Corning, NY).

#### DNA Constructs

The human MC2R (hMC2R; Accession #: AA067714.1) cDNA construct was synthesized by GenScript (Piscataway, NJ). For activation assays, this receptor was tagged at the N-terminus with a FLAG epitope, and inserted into a pcDNA3.1+ vector. In addition, the mammalian, *Mus musculus* (mouse), MRAP1 (mMRAP1; Accession #: NM\_029844) was synthesized by GenScript, and inserted into a pcDNA3.1+ vector as well. The mMRAP1 cDNA was not tagged. This cDNA was used for both activation assays and cell surface ELISA assays. The cAMP reporter, CRE-Luc (Chepurny and Holz, 2007), was provided by Dr. Patricia Hinkle (University of Rochester, NY). A set of

chimeric mutants were made utilizing the human MC2R and *Xenopus tropicalis* MC1R (xtMC1R; Accession#: XP 012817790) where the N-terminal, EC1, EC2, and EC3 of the human MC2R were swapped out with the corresponding domains of xtMC1R. These chimeric mutants were tagged with a V-5 epitope tag at the N-terminus, and inserted into a pcDNA3.1+ vector. Furthermore, cassette and single alanine-substituted mutants of the wild type human MC2R were made at the EC1, and EC3 domains. Each of these alanine mutants were tagged with the V-5 epitope. For the cell surface ELISA assays, a set of TM4, EC2, and TM5 single alanine mutants were along with a wild-type hMC2R cDNA. Each of these cDNAs had a V-5 epitope tag, and were inserted individually into a pcDNA3.1+ vector (GenScript).

#### ACTH Peptide

The melanocortin peptide used in these experiments was human ACTH(1-24), and this synthetic hormone was purchased from Sigma-Aldrich Inc. located in Saint Louis, MO. The amino acid sequence for human ACTH(1-24) is shown in Figure 7.

#### cAMP Reporter Assay (Luciferase Assay)

In the cAMP Reporter Assay,  $3.0 \times 10^6$  cells/reaction were used (24 wells of a white 96 wells plate = per one reaction). It should be noted that 4 reactions could be performed on a white 96 well plate. Cells were co-transfected with the following cDNA constructs: hMC2R or chimeric xtMC1R/hMC2R or alanine-substituted hMC2R (10 nm/rxn), mMRAP1 (30 nm/rxn), and CRE-Luciferase (83 nm/rxn) (Chepurny & Holz, 2007). Transfections were done utilizing the Amaxa Cell Line Nucleofector II system



(Lonza Group, LTD, MD), 100  $\mu$ l Solution T/reaction, and program U-23. After a 10 minute period of recovery in 500  $\mu$ l of CHO media, the transfected cells were diluted in 7.5 ml of CHO media. It should be noted that the 600 $\mu$ l of reaction mixture should be mixed well in the dilution media, so that the mixture is homogenous and plated  $1 \times 10^5$  cells per well (300 $\mu$ L per well). After about 24 hours after the transfection, cells were fed with 300  $\mu$ l of fresh CHO media.

On the third day of the cAMP reporter luciferase assay, the transfected cells were stimulated with hACTH(1-24) in serum-free CHO Media (does not contain FBS). Serial dilutions were carried out with hACTH(1-24) concentrations ranging from  $10^{-7}$  to  $10^{-13}$  M. In addition, each dose was tested in triplicate. Then, the stimulated plate was incubated at 37°C incubator for 4 hours. After the 4 hour stimulation period, the plate was allowed to cool to room temperature, and the stimulation solution was removed. Next, a 1:1 ratio of serum free CHO media and Luciferase substrate reagent Bright GLO (Promega, WI) was gently mixed in a 15 mL conical tube. The Luciferase substrate solution was applied to each well (100  $\mu$ L/well), and allowed to incubate at room temperature for 5 minutes. Finally, luminescence of each well was measured using the Bio-Tek Synergy HTX plate reader (Winooski, VT).

Additionally, basal levels of cAMP production needed to be determined to produce an accurate activation curve. Therefore, a negative control was included in each assay where the transfected CHO cells were not stimulated with wild type ACTH(1-24) peptide used in the experiment. The negative control was subtracted from each data point, and the corrected data points for each dose response curve were fit to the Michaelis-

Menton equation to produce an EC<sub>50</sub> value for each activation curve. These activation curves were analyzed by using Kaleidograph software ([www.syngery.com](http://www.syngery.com)).

#### Cell Surface ELISA

First, a 24-well plate is treated with 500µl of fibronectin in each well to provide matrix for the CHO cells to adhere to bottom of the plate. Each reaction contains 6 wells. Hence, 4 reactions can be completed per 24-well plate. The fibronectin came from bovine plasma, and was reconstituted in 50 ml H<sub>2</sub>O to provide a final concentration of 1mg/ml. It was ordered from Sigma-Aldrich Inc. located in St. Louis, MO. The untransfected CHO cells were split using the method stated in section 2a. Each well of the 24 well plate needed to contain  $0.75 \times 10^5$  cells. The cells were left to grow over night in 500µl of CHO Complete Media (media recipe found in section 2a).

The second day of this protocol included the transfection of the cells. The Complete CHO media is replaced with new Complete CHO Media (500 µl/each well). Next, 1.6 µl of each plasmid (hMC2R-V5, mMRAP1- No Tag, or hMC2R alanine-substituted mutants-V5) is mixed with 325 µl of JetPrime buffer in a 1.7ml microcentrifuge tube. The equation for how much plasmid used in each reaction was  $0.25\mu\text{g}/\text{well}/1.0\mu\text{g}/\mu\text{l} \times 6.5$  wells. The plasmid/JetPrime Buffer was vortexed and spun down. Next, the JetPrime reagent is added to the plasmid/JetPrime Buffer mixture (2:1 ratio; 3.2 µl if only one plasmid/rxn or 6.5µl if two plasmids/rxn mixed with JetPrime Buffer) and incubated at room temperature for 10 minutes. Then, 50 µl of plasmid/JetPrime mixture was added to each well, and incubated over night at 37°C in the incubator. The JetPrime transfection

reagent (Polyplus-transfection S.A, Illkirch, France) is a lipid-based transfection reagent ordered from VWR (Randor, PA).

The third day is where the transfected cells undergo immunostaining using a primary antibody and secondary antibody conjugated to HRP. The transfected CHO cells were pre-treated with the DMEM + BSA media for 30 minutes in a 37°C incubator. This media is made up of 500 ml of D-MEM media, 5ml of penicillin/streptomycin (final concentration 1mM), 10ml of HEPES (stock solution of 1M and final concentration at 20Mm), and 500mg of BSA (final concentration of 1 mg/ml). Next, the primary antibody (mouse anti-V5) is diluted in the primary antibody in transfection media (1:1000) in a 15ml centrifuge tube. While making up the primary antibody, the plate is incubated on ice for 10 minutes at room temperature. After primary antibody is made up, replace media with the DMEM + BSA media + primary antibody mixture and incubate on ice for 1 hour at room temperature. Note that only half of the plate will be treated with the primary antibody.

After 1 hour, the cells need to be washed four times with 500µl/well of ice cold 1 x PBS. Next, the transfected wells need to be fixed with 300µl of 4% PFA on ice for 5 minutes. After the cells are fixed, the wells need to be washed 2 times with 500µl/well of ice cold 1 x PBS. The secondary antibody (goat anti-rabbit-HRP or goat anti-mouse-HRP) is prepared in the DMEM + BSA media and diluted to a concentration of 1:1000. All reactions are treated with the secondary antibody at 300µl/well, and incubated at room temperature for 1 hour. While incubating with the secondary antibody, an aliquot of ABTS 1-step solution is removed from fridge to warm to room temperature. The ABTS

1-step solution is a water-soluble peroxidase substrate that turns green when exposed to secondary antibody that is conjugated to HRP. The ABTS 1-step solution was ordered from Thermo-Scientific (Waltham, MA). After the 1 hour incubation period, the secondary antibody needs to be removed, and washed 4 times with 1 x PBS. Next, the wells are treated with 300  $\mu$ l of ABTS 1-step, and incubate at room temperature for 25 minutes. If there was presence of receptor on the surface of the cells, the ABTS 1-step substrate yields a green end product. Each reaction well is transferred into 96-well plate (100ul/well), and the absorbance is read at 405nm Bio-Tek Synergy HTX plate reader (Winooski, VT). For all cell surface ELISA assays the following controls were used: non-transfected CHO cells incubated with primary and secondary antibodies, CHO cells transfected with only hMC2R cDNA and incubated with primary and secondary antibodies, and CHO cells transfected with hMC2R + mMRAP1 cDNAs and incubated with primary and secondary antibodies (positive control).

#### Statistical Analysis

Data points were calculated as a mean with standard error values that were obtained from experiments performed in a triplicate. To determine statistical significance between experimental treatments and their corresponding controls, an unpaired two-tailed Student's t-test for equal variance was calculated; significance was set at  $P \leq 0.05$  for Figures 10B, 12, 13, 19A, 19B, 25. In addition, the data sets for Figures 10A, 15, 16, 17, 20, 21, 23, 27, 32, 33, 34. were analyzed using a one-way ANOVA, and then by Tukey's multi-comparison test to compare 3 or more dose response curves. The F-test was

calculated using the GraphPad Prism 2 software (GraphPad Software Inc., La Jolla, CA, USA). Significance was set at  $p \leq 0.05$ .