ROLE OF RECEPTOR ACTIVITY MODIFYING PROTEIN-2 (RAMP2) IN ENDOCRINE PHYSIOLOGY OF FEMALE MICE

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ABSTRACT

MAHITA KADMIEL: Role of Receptor Activity Modifying Protein-2 (RAMP2) in endocrine physiology of female mice
(Under the direction of Dr. Kathleen Caron)

Receptor activity modifying proteins (RAMPs 1, 2, and 3) are single-pass transmembrane proteins that can regulate the trafficking, ligand-binding, and signaling of several G protein-coupled receptors (GPCRs). For example, biochemical studies have shown that RAMP2 interacts with calcitonin receptor-like receptor (CLR), parathyroid hormone receptor (PTHR1) and calcitonin receptor (CTR). However, the most well-characterized role of RAMP2 is in the regulation of adrenomedullin (AM; gene: *Adm*) binding to CLR (gene: *Calcrl*), and previous *in vivo* work from the Caron laboratory supports this canonical signaling paradigm. Loss of RAMP2 causes embryonic lethality associated with failed lymphatic vascular development, which is a precise phenocopy of the defects seen in $Adm^{-/-}$ and $Calcrl^{-/-}$ mice. However, $Ramp2^{+/-}$ mice survive, and here we present the phenotypic analyses of $Ramp2^{+/-}$ female mice and $Ramp2^{-/-}$ placentas, many of which are distinct from the phenotypes observed in *Adm* and *Calcrl* mice. Haploinsufficiency of *Ramp2* causes severe subfertility in female mice characterized by intrauterine growth restriction and postnatal lethality. Furthermore, $Ramp2^{+/-}$ female mice exhibit hyperprolactinemia, pituitary gland

hyperplasia, precocious mammary gland development, and skeletal abnormalities—phenotypes that are distinct from those observed in *Adm*$^{+/-}$ and *Calcrl*$^{+/-}$ mice. In addition, *Ramp2*$^{-/-}$ placentas have marked defects, including reduced labyrinth size, reduced cellularity and reduced proliferation of labyrinthine trophoblast cells. Interestingly, loss of *Ramp2* was also associated with reduced expression of the *Pthr1* gene and protein in placental lysates, which is consistent with the previously described function of PTHR1 signaling in regulating trophoblast cell proliferation. Taken together, this work provides the first *in vivo* evidence demonstrating an essential role for RAMP2 in the female endocrine system. The expanded range of phenotypes observed in the *Ramp2* gene targeted animals, compared to those seen in *Adm* and *Calcrl* animals, considerably extends the biological functions of RAMP2 beyond the canonical AM/CLR signaling pathway and supports an essential role for RAMP2 in PTHR1 signaling. Therefore, these studies provide novel physiological insights and potential pharmacological targets for the future development of RAMP2-based therapies for the treatment of female endocrine disorders.

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ABBREVATIONS

1AH	1α-hydroxylase
ACTH	adrenocorticotropic hormone
AM	adrenomedullin
AMY	amylin
BrdU	5-bromo-2'-deoxyuridine
Calb-D28K	calbindin D_{28k}
Calb-D9K	calbindin-D_{9k}
Calcrl (gene)	calcitonin receptor-like receptor
cAMP	cyclic adenosine monophosphate
CaSR	calcium sensing receptor
CGRP	calcitonin gene related peptide
CLR (protein)	calcitonin receptor-like receptor
CRF	corticotropin releasing factor
CTR	calcitonin receptor
DAPI	4’,6-diamidino-2-phenylindole
DEXA	dual-emission x-ray absorptiometry
ERK	extracellular signal-regulated kinases
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCGR	glucagon receptor
GH	growth hormone

GLP	glucagon-like peptide
GPCR	G-protein coupled receptors
LPS	lipopolysaccharide
LSH	luteinizing hormone
MAPK	microtubule-associated protein
Micro-CT	micro computed tomography
NCX1	Na-Ca exchanger
NHERF	Na^+/H^+ exchanger regulatory factor
NSF	N-ethylmaleimide-sensitive factor
PE	preeclampsia
PMCA1	plasma membrane Ca2+ ATPase 1
PRL	prolactin
PTH	parathyroid hormone
PTHR1	parathyroid hormone receptor1
PTHrP	parathyroid hormone related peptide
RAMP	receptor activity modifying proteins
SCTR	secretin receptor
TM	transmembrane domain
TRPV	transient receptor potential vanilloid channel
TSH	thyroid-stimulating hormone
uNK	uterine natural killer
VDR	vitamin D receptor

VPAC1	vasointestinal peptide/pituitary adenylate cyclase-activating peptide 1

CHAPTER 1

BACKGROUND AND INTRODUCTION

RAMPs

Receptor activity modifying proteins (RAMPs) belong to a class of single-pass transmembrane proteins that regulate the function of several G-protein coupled receptors. In mammals, there are three RAMPs, RAMP1, 2 and 3, identified to date. RAMPs were discovered in 1998, when McLatchie and colleagues ended the controversy among receptor biologists who studied the actions of calcitonin gene related peptide (CGRP) and adrenomedullin (AM) by showing that RAMP1 can translocate calcitonin receptor-like receptor (CLR) to the plasma membrane from the endoplasmic reticulum to form a functional receptor for CGRP (1). Later, sequence based analyses led to the discovery of 2 additional RAMPs, -2 and -3 that when bound to CLR, formed receptors AM1 and AM2 for adrenomedullin, respectively. Therefore, based on the RAMP expressed in a given cell type, CLR can form three functional receptors for two different ligands, indicating that RAMPs can dictate ligand-binding specificity of GPCRs. Not surprisingly, this novel range of diversity of GPCRs created a paradigm shift in our understanding of GPCR pharmacology.

GPCR-RAMP interactions

Although RAMPs were discovered in the context of CLR, additional biochemical studies have been employed to identify several novel GPCR partners for RAMPs. All RAMPs are shown to interact with calcitonin receptor (CTR) to form a functional receptor for Amylin (2). Besides CLR and CTR, RAMP2 also interacts with parathyroid hormone receptor1 (PTHR1), glucagon receptor (GCGR), and the receptor for the vasoactive intestinal peptide (VPAC1)

(3). RAMP3 also interacts with the PTHR2 and the secretin receptor (SCTR) (4) These receptors belong to Family B of GPCRs—receptors with unusually long extracellular domains (ECD) that may form interfaces with the ECD of RAMP proteins. However, not all family B GPCRs associate with RAMPs. Receptors such as the VPAC2, receptors for glucagon-like peptide, GLP1 and GLP2 and the receptor for growth hormone releasing hormone have been shown to not associate with any of the RAMPs (3). Moreover, RAMP interactions with GPCRs are not confined to the Family B GPCRs. Calcium sensing receptor (CaSR) has been shown to interact with RAMP1 and RAMP3, but not RAMP2 (5). Considering the range of GPCRs that associate with RAMPs and the wide expression pattern of RAMPs (57) in several tissues of the body, both in humans and in mice, it is highly likely that RAMPs have a much broader purpose than what is currently appreciated. Further studies are required to identify novel receptor partners for RAMPs. Importantly, future studies should address the physiological significance, if any, for both currently known and as-yet undiscovered GPCR-RAMP interactions.

GPCR regulation by RAMPs

RAMPs have been well-characterized in the context of the GPCRs- CLR and CTR. RAMPs have been shown to chaperone GPCRs from the endoplasmic reticulum to the plasma membrane. A classic example of this phenomenon is CLR, which is retained in the endoplasmic reticulum and cannot go to the cell surface on its own, but requires any one of the three RAMPs to do so (1). Another example is CaSR, which is chaperoned to the cell surface either by

RAMP1 or RAMP3, but not by RAMP2 (5). In contrast, certain GPCRs such as CTR can constitutively traffic to the plasma membrane without a RAMP, but the interaction with a RAMP changes the ligand binding affinity of the receptor. The paradigm of RAMPs altering ligand binding specificity is well-established and broadly applied. For example, CLR bound to RAMP1 forms a receptor for CGRP, while CLR bound to RAMP2 or RAMP3 form AM1 and AM2 receptors, respectively. Another example is CTR, which binds to calcitonin in the absence of a RAMP, but binds to amylin when associated with either RAMP1, 2 or 3. RAMPs are also involved in recycling of GPCRs back to the cell surface following agonist-mediated receptor internalization. In particular, RAMP3, containing a PDZ-domain, has been shown to interact with proteins such as NHERF-1 and NSF to mediate the recycling of AM2 receptors (6-7).

Finally, RAMPs can also alter GPCR signaling. The first observation of this phenomenon was seen with the VPAC1 receptor, which in the presence of RAMP2, enhanced phosphoinositide hydrolysis in COS-7 cells in response to the agonist, VIP (3). More recently, studies have shown that amylin receptors selectively modulated downstream signaling, including cAMP generation. Specifically, AMY1 (CTR/RAMP1) and AMY3 (CTR/RAMP3) receptors displayed robust induction of cAMP in response to AMY, compared to CTR alone, indicating that RAMPs can influence GPCR signaling (8). The numerous modes of regulation of GPCRs by RAMPs are summarized in **Fig1**.

Molecular basis of GPCR-RAMP interaction

Considering the drug targetable potential of RAMPs, it is important to understand the interactions between GPCRs and RAMPs at the molecular level. Most of what we know about RAMP/GPCR interactions comes from studies performed with RAMP/CLR complex, mostly RAMP1/CLR. Early studies using chimeras of RAMPs revealed that the N-terminus of RAMPs plays a predominant role in determining receptor pharmacology by dictating ligand-binding specificity as well as for the interaction with the receptor (9-10). Evidence suggests asymmetric arrangement of CLR/RAMP1, where a CGRP receptor comprises of 2 CLR molecules interacting with one RAMP1 molecule (11). Very little is known about the stoichiometry between GPCRs and RAMPs. However, since most of the RAMP-interacting GPCRs belong to the Secretin family of GPCRs, a general opinion about the nature of RAMP-GPCR interactions can be gathered from structural studies on other members of the Secretin family. Secretin family of GPCRs consists of 15 peptide binding receptors, all of which have an N-terminal domain ranging from 100-160 residues, a juxtamembrane domain with seven transmembrane α-helices forming intervening loops, and an intracellular C-terminal end. Intracellular signaling is initiated when the intracellular loops interact with G-proteins, predominantly through G_s coupled pathways for Secretin family GPCRs (12) . Studies that solved structures of receptors of GLP-1, PTH1, and CRF have shown that the ECD of GPCRs binds the C-terminus of the ligand. Moreover, the recently resolved crystal structures of a complex containing the ECD of hRAMP1 (13), a complex with ECD of human CLR/RAMP1 and a small

molecule antagonist (14), and the ECD of hRAMP2 (15) provide insight on GPCR-RAMP interaction and peptide binding. These structures revealed that the ECD of CLR was similar to the ECD of other members of the Secretin family of GPCRs. Of the three helices in RAMP1, αR2 and αR3 form an interface with the N-terminal helix of CLR by forming hydrophobic and electrostatic interactions (CLR residues: y49, Q45 and M42; RAMP1 residues: Y66, F93, H97, and F101). The findings from these structural studies are consistent with previous mutagenesis studies, where mutation of the key residues in RAMP1 resulted in poor cell surface expression of CLR/RAMP1 complex (16). The residues on RAMP1 that are crucial for interaction with CLR are also conserved in RAMP2 (residues Y66 and F101).

Based on the structural studies, it is speculated that the RAMPs bind to the C-terminal end of the peptide. In this aspect, the residues potentially responsible for peptide-binding are not conserved between all RAMPs, thereby determining ligand-binding affinity and/or specificity. For example, W74 of RAMP1 when substituted with its equivalent residue in RAMP2 (E101) and RAMP3 (E74) resulted in enhanced AM binding and potency. Contrastingly, an E101W substitution in RAMP2 and an E74W substitution in RAMP3 did not result in a CGRP receptor, but completely disrupted the function of AM1 receptors and attenuated the function of AM2 receptors.

Lastly, the structure of the ECD of the complex containing CLR, RAMP1 and an antagonist revealed three regions that interact with the ligand — a CLR binding region, an interface binding region, and a RAMP1 binding pocket.

Although the information obtained from these structures is specific to CLR/RAMP1 in complex with small molecules, this knowledge can be utilized to develop future therapeutic molecules. While challenging, future studies aimed at resolving GPCR-RAMP complex at the transmembrane (TM) region and the intracellular domain would provide insights on the role of TM of RAMPs in stabilization of its interaction with the receptor. In addition, understanding the role of the C-terminus of RAMPs would shed light on how RAMPs can influence trafficking and/or downstream signaling of GPCRs.

Mouse models of RAMPs

Since their initial discovery in 1998, the RAMP field has grown swiftly in terms of identifying the biochemical and pharmacological properties of RAMPs with a wide variety of GPCRs. However, what remains unclear, and is ultimately of high importance, is the physiological significance of RAMPs in normal and disease conditions. Therefore, our laboratory as well as other groups have generated genetically engineered mouse models of all three RAMPs, with the ultimate goal of deleting, reducing or overexpressing RAMP proteins (17-23). Comparative phenotyping of all the available mouse models has enabled researchers in the field to uncover the most pertinent physiological functions of the RAMPs. All the various mouse models of RAMPs are reviewed in detail in **Chapter 3** (24). Briefly, loss of RAMP1 and RAMP3 led to no overt phenotypes. However, loss of RAMP2 is incompatible with life, indicating that the function of this RAMP cannot be compensated by the other two RAMPs. More recently, our work has shown that dosage of RAMP2 is critical for normal development of the

female endocrine system in mice (25). Taken together, genetically engineered mouse models of RAMPs have shed light on the physiological as well as clinically relevant roles of RAMPs.

Disease relevance of RAMPs

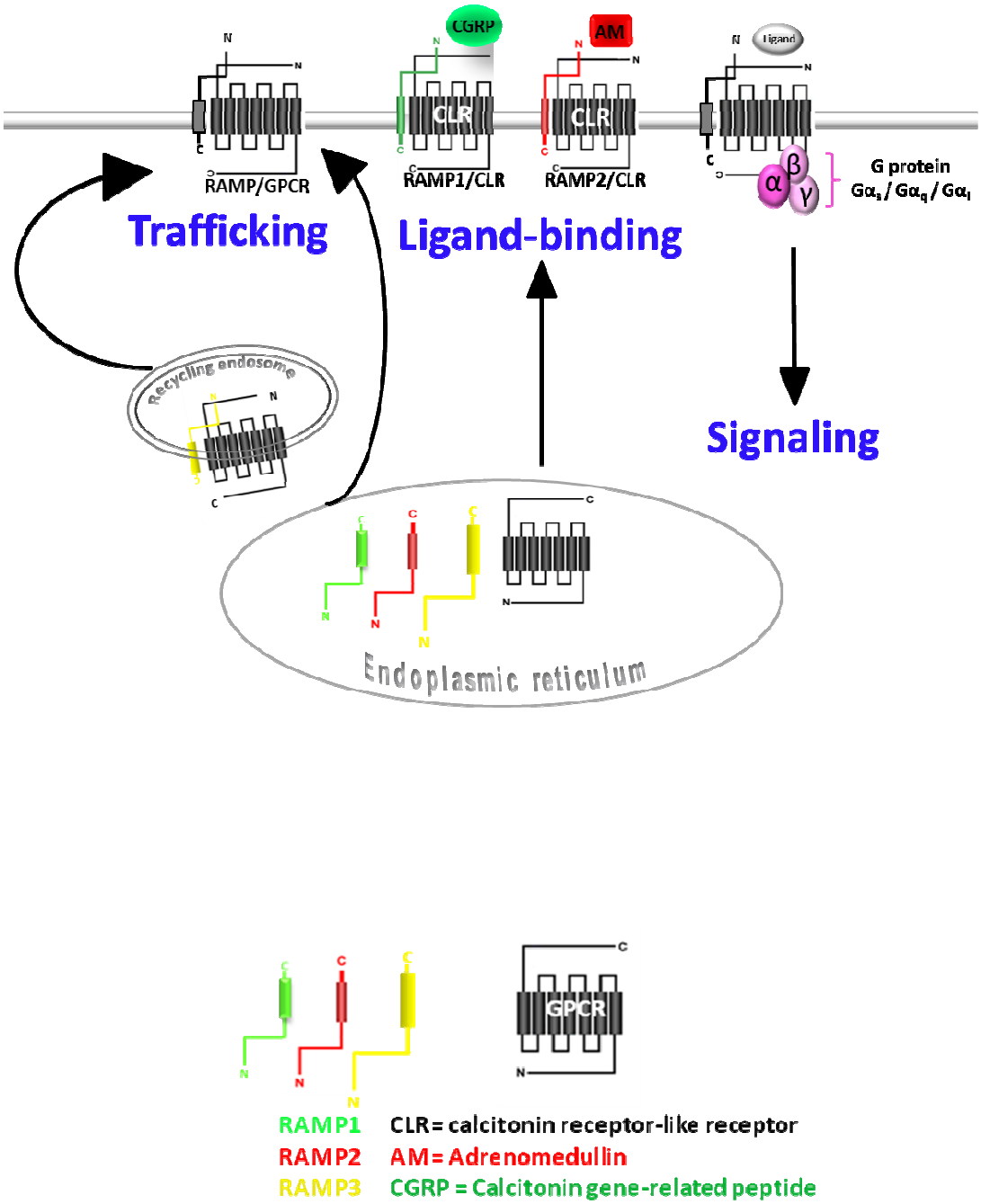
Ramp mRNA levels are differentially regulated in disease conditions such as heart failure (26-28), hypertension (29-30), kidney failure (31-32) and diabetes (32). Interestingly, RAMPs have been shown to be upregulated in normal pregnancy, suggesting a physiological function (33). Work from our laboratory, which is presented in **Chapter 4** of this dissertation shows that loss of RAMP2 in the placenta results in phenotypes that are also observed in preeclampsia. RAMPs are also upregulated in hypoxic conditions (34-35), suggesting relevance in tumor biology. Taken together, the disease implications of RAMPs make them attractive drug targets.

RAMPs as drug targets

RAMPs diversify the receptor repertoire of GPCRs; based on their availability, different RAMPs enable the same GPCR to bind to more than one ligand. As a result, the GPCR-RAMP interface can be exploited to develop drugs that confer a high level of specificity. Considering that more than 50% of the drugs on the market target GPCRs, it is not surprising that the pharmaceutical industry has taken advantage of the drug-targetable GPCR-RAMP interface. Several chemical antagonists targeting the CLR-RAMP1 interface have been developed for the treatment of migraine and are currently undergoing clinical trials (36-37). This is only the beginning for RAMP-based therapeutics and as we

expand our knowledge of the physiological and pathophysiological roles of RAMPs, the scope of novel GPCR-RAMP targets will only increase further. The work presented in this dissertation sheds light on the physiological roles of RAMP2 and aims to serve as a tool in the development of RAMP2-based therapeutics.

Figure 1.1 RAMPs regulate GPCRs
RAMPs regulate trafficking, ligand-binding and signaling of GPCRs.



CHAPTER 2

UNDERSTANDING RAMPS THROUGH GENETICALLY ENGINEERED MOUSE MODELS (24)

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ABSTRACT

The family of receptor activity modifying proteins consists of three members, RAMP1, 2 and 3, which are each encoded by a separate gene and have diverse spatiotemporal expression patterns. Biochemical and pharmacological studies in cultured cells have shown that RAMPs can modulate several aspects of G protein-coupled receptor (GPCR) signaling, including receptor trafficking, ligand binding affinity, second messenger signaling and receptor desensitization. Moreover, these studies have shown that RAMPs can interact with several GPCRs other than the canonical calcitonin receptor-like receptor (CLR), with which they were first identified. Given these expanding roles for RAMPs, it becomes interesting to question how these biochemical and pharmacological properties bear significance in normal or disease physiology. To this end, several gene targeted knockout and transgenic models have been generated and characterized in recent years. Fortunately, they have each supported important roles for RAMPs during embryonic development and adulthood. This chapter provides a comprehensive overview of the most recent findings from gene targeted knockout mouse models and transgenic over-expression models, and gives special consideration to how comparative phenotyping approaches and conditional deletion strategies can be highly beneficial. In the future, these genetically engineered mouse models will provide both insights and tools for the exploitation of RAMP-based therapies for the treatment of human diseases.

Introduction

The family of mammalian receptor activity modifying proteins (RAMPs) offers an exciting opportunity to elucidate the pharmacological and biological complexities of G protein-coupled receptor (GPCR) signaling while also enabling the unique pharmacological manipulation of numerous GPCRs that are involved in a wide variety of physiological process and disease conditions. The wide tissue distribution of RAMP proteins and their evolutionary conservation suggests that they have much broader functions than just mediating the ligand binding specificity of the calcitonin receptor-like receptor, through which the RAMPs were originally identified by Foord and colleagues (1). In fact, numerous studies by several groups have demonstrated that RAMPs can functionally interact with at least 5 other receptors of the Secretin Family (38), the calcium sensing receptor (5) as well as the non-receptor cytoskeletal protein, alpha tubulin (39). Moreover, pharmacological and biochemical studies in cultured cell lines suggest that RAMPs can modify numerous aspects of GPCR signaling, including ligand binding, receptor desensitization, receptor trafficking and second messenger signaling and so they make attractive pharmacological targets (40).

These exciting and seemingly expanding functions for RAMP proteins also complicate our efforts to better understand the physiological significance of RAMPs in normal and disease conditions. Therefore, our laboratory has employed a gene targeting approach to generate mouse models with absent and/or reduced expression of each RAMP and then comparatively phenotype the models to uncover the most pertinent physiological functions of the RAMPs.

Several other groups have also independently generated individual RAMP knockout mice so that the comparative evaluation of different mouse lines, genetic backgrounds and phenotypes can be extremely valuable. Finally, the in vivo over-expression of RAMP proteins in specific cell types using conventional transgenic approaches has also been utilized to uncover new insights into RAMP biology. Results and interpretations from these genetic animal models are summarized below.

However, we must remain cognizant of several confounding variables when trying to infer the function of RAMPs from genetic animal model phenotypes. First, if the loss of a RAMP gene is incompatible with life, for example with RAMP2, then the assessment of loss-of-function effects during adulthood is precluded. To overcome this barrier, sophisticated gene targeting approaches which can conditionally inactivate a gene either in time or in a specific tissue or cell type can be used, but this typically requires generation of additional mouse models and complex breeding schemes. Alternatively, the surviving haploinsufficient mice can be evaluated for phenotypes, but the phenotypes must be robust enough to be detected on a heterozygous background. Secondly, because the gene expression of RAMPs is dynamically regulated in a spatio-temporal manner by a variety of stimuli and conditions, the physiological effects of loss or reduction in *Ramp* gene expression may not be obvious under basal conditions. Therefore, challenging the animal models so that they are under appropriate physiological conditions which mimic the spatio-temporal regulation of *Ramp* gene expression may be desirable. Thirdly, as we

have learned from *in vitro* pharmacological studies, the RAMPs can interact with numerous GPCRs in a manner which is not always straightforward. For example, association of RAMPs 1, 2 and 3 with the calcitonin receptor dynamically changes the relative affinity of the receptor for the amylin ligand (8), so that a functional knockout of one RAMP may be partially compensated for by the expression of other RAMPs with respect to amylin signaling. As another example, association of RAMPs and receptors from different species can lead to marked differences in pharmacologcial profiles (41), so that the lessons learned from *in vitro* studies using combinations of reconstituted human, rat or other species receptors and RAMPs should be considered carefully when interpreting *in vivo* phenotypes of mouse models. Finally, as is the case with most genetically engineered mouse models, the influence of genetic background on the observed phenotype plays an important role. In our own studies, we have found drastic changes in the gene expression levels of RAMPs between different genetic backgrounds which directly translates to a different presentation of phenotype for the disrupted allele on different genetic backgrounds (Kadmiel, Fritz-Six & Caron; unpublished observation (25)).

Nevertheless, it is clear that genetically engineered animal models can provide useful and clinically-relevant insights into the broad functions of the RAMP family of proteins. As we begin to exploit RAMPs for pharmacological manipulation of GPCRs, these models, as well as those generated in the future, will provide useful *in vivo* tools for the pre-clinical testing of relevant compounds.

RAMP1

Gene targeted deletion of RAMP1

The CLR-RAMP1 heterodimer makes a functional receptor for CGRP, a neuropeptide which plays important roles in the regulation of cardiovascular and immune systems. A mouse line lacking the *Ramp1* gene ubiquitously was generated utilizing the Cre-loxP strategy (42). Although *Ramp1*$^{-/-}$ mice had no obvious abnormalities in their appearance, they had slightly elevated basal blood pressure with normal heart rate compared to *wild type* mice, as measured by carotid catheters under anesthesia. Experiments measuring the activity of the vasodilators αCGRP, acetylcholine and sodium nitroprusside were performed in *Ramp1*$^{-/-}$ and *wild type* mice to address the function of RAMP1 in mediating vasodilation. *Ramp1*$^{-/-}$ and *wild type* mice exhibited similar responses to acetylcholine and sodium nitroprusside, but *Ramp1*$^{-/-}$ mice failed to respond to the vasodilatory effects of αCGRP. These data demonstrate that the lack of a response to αCGRP in *Ramp1*$^{-/-}$ mice is not due to any abnormalities in the vascular smooth muscle cells or endothelial cells and confirm that the vasodilatory action of αCGRP is dependent on the availability of CLR-RAMP1 receptor complex. Interestingly, *Ramp1*$^{-/-}$ mice had elevated levels of serum CGRP, which further confirms that in spite of the availability of the ligand, the lack of the functional receptor leads to dysregulation of vasodilation. Although CLR-RAMP1 receptor complex is defined as a CGRP receptor, little is known about the differential effects of the two isoforms of CGRP, αCGRP and ßCGRP, on this receptor. Responses to αCGRP and ßCGRP on the relaxation of aortic rings from *Ramp1*$^{-/-}$ and *wild type* mice demonstrated that CLR-

RAMP1 serves as a receptor for both isoforms, but that the α-isoform elicits a stronger effect than the ß- isoform of CGRP. In support of the promiscuous nature of RAMP-receptor pharmacology, differential responses to relaxation of the aortic rings to adrenomedullin in *Ramp1*$^{-/-}$ and *wild type* mice suggested that adrenomedullin may partially transduce signaling via CLR-RAMP1 receptor.

Administration of lipopolysaccharide (LPS) in *Ramp1*$^{-/-}$ and *wild type* mice helped to elucidate an important function for CGRP in regulating inflammation (42). Interestingly, LPS-induced cytokine production and inflammation caused a remarkable increase in serum CGRP levels of *Ramp1*$^{-/-}$ mice compared to *wild type* mice. These data suggest a mechanism where CGRP, via the CLR-RAMP1 receptor, carries out an anti-inflammatory role by suppressing the production of proinflammatory cytokines.

Altogether, findings from the characterization of *Ramp1*$^{-/-}$ mice have confirmed the crucial role of RAMP1 in the CGRP signaling pathway, particularly in the cardiovascular and inflammatory processes.

Transgenic overexpression of RAMP1

A transgenic mouse line that expresses *hRAMP1* primarily in the neurons and glia has been generated by Zhang et al (22) Nestin/hRAMP1 mice express *hRAMP1* RNA in the brain, trigeminal ganglion, spinal cord and dorsal root ganglion. Quantitative gene expression showed that the mRNA levels of *hRAMP1* in the brain were 50% of the endogenous mouse *Ramp1* expressed in neuronal tissues. Therefore, the overall increase in *RAMP1* mRNA expression is modest, but importantly not supra-physiological, in the brain and the trigeminal

ganglion of nestin/hRAMP1 transgenic mice. As a consequence, increased production of hRAMP1 in the trigeminal ganglia enhanced CGRP-induced release of substance P from these neurons, leading to plasma extravasation and inflammation in subcutaneous tissues (such as paws and whisker pads). The effect of CGRP-triggered neurogenic inflammation could be blocked by the CGRP antagonist, CGRP8-37; further indicating that trigeminal RAMP1 is involved in CGRP-induced inflammation. Importantly, the expression of *hRAMP1* mRNA exclusively in neuronal tissues, but not in subcutaneous tissues, confirms the involvement of trigeminal hRAMP1 in CGRP-evoked inflammation. Therefore, the finding that the availability of RAMP1 is rate-limiting for the actions of CGRP in the trigeminal ganglion opens a new dimension on understanding trigeminal pathologies, such as migraine, by the regulation of CGRP and its receptor, CLR/RAMP1.

More recently, Chrissobolis et al characterized the protective effects of RAMP1 in the vasculature using a transgenic mouse that ubiquitously expresses *hRAMP1* (20). Quantitative PCR in several tissues showed ubiquitous expression of *hRAMP1* in these transgenic mice. The transgene did not affect the endogenous levels of mouse *RAMP1* because the gene expression levels were not different when compared to the controls. *In vitro* studies involving carotid and basilar arteries of the transgenic mice exhibited a robust response to CGRP-mediated vasodilation, when compared to other vasodilatory agents such as adrenomedullin or acetylcholine, confirming the selective response of the hRAMP1 rich endothelium to CGRP. Additionally, *in vivo* studies exhibited

vasodilation of the cerebral arteries in a CGRP-specific manner in hRAMP1 transgenic mice compared to controls. In the same transgenic hRAMP1 mice, Sabharwal et al (21) have shown that these mice display an attenuated response to Ang II-induced hypertension, suggesting that increased expression of RAMP1 is vasoprotective. More interestingly, when the carotid arteries of mice were treated with acetylcholine in the presence or absence of Ang II to test AngII-mediated vascular dysfunction, hRAMP1 expression in transgenic mice abrogated the effects of Ang II on the vasculature. This is a novel finding attributing the functional role of RAMP1 in Ang II mediated vascular dysfunction. As shown by Zhang et al, Chrissobolis and group also show that increased expression of RAMP1 displays selective and enhanced vascular response to CGRP, but not Adrenomedullin, thereby making the effect of CGRP RAMP1-limited.

RAMP2

Gene targeted deletion of RAMP2

Unlike *Ramp1* and *Ramp3* null mouse models which survive to adulthood, *Ramp2*$^{-/-}$ mice are embryonic lethal at mid gestation (17-18, 23). These findings demonstrate that the endogenous expression of *Ramp1* and *Ramp3* are unable to compensate for the loss-of-function of *Ramp2 in vivo*. Amazingly, comparative phenotyping on similar isogenic genetic backgrounds revealed that gene knockout mice for AM (43), Calcrl (44) and RAMP2 (17, 45) share a conserved phenotype consisting of mid-gestation embryonic lethality characterized by generalized edema. The conservation of phenotypes between the AM, Calcrl

and Ramp2 knockout lines not only highlights the importance of AM signaling for embryonic survival but also provides the first genetic evidence to substantiate the RAMP-GPCR signaling paradigm, and specifically the function of the CLR-RAMP2 complex, *in vivo*.

Generalized edema has been reported in other knockout mice that encode for genes crucial for lymphangiogenesis (46). Characterization of *AM*$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ mice, which were all generated and maintained on an isogenic 129/S6-SvEv-TC1 background, revealed that the principal cause of the edema was due to defects in lymphatic vascular development (18). The jugular lymph sacs of the *Ramp2*$^{-/-}$ mice were significantly smaller than those of their control littermates. *In vivo* BrdU incorporation assays further demonstrated a reduced rate of lymphatic endothelial cell proliferation compared to blood endothelial cells in all mutant lines tested. Electron microscopy studies showed that the junctional barrier of blood and lymphatic vessels remained intact, but that the lymphatic endothelial cells appeared thin and often necrotic in the *Ramp2*$^{-/-}$ mice. *In vitro* studies showed that AM signaling, mediated through RAMP2-CLR receptors, causes an enhanced activation of the MAPK/ERK signaling cascade, which is essential for endothelial cell survival and driving normal developmental lymphangiogenesis. Because these studies, and findings from other groups, (47-49) show that the expression of the *Calcrl* and *Ramp2* genes is regulated by the lymphatic-specific transcription factor, Prox1 (47, 50), their expression is preferentially higher in lymphatic endothelial cells compared to blood endothelial cells. Other cardiovascular defects in the *Ramp2*$^{-/-}$ embryos, which are also

present in the *Adm* (43) and *Calcrl* (44) null models, include thin vascular smooth muscle walls and small hearts with thin compact zones and disorganized ventricular trabeculae. Together, these data identify a previously unrecognized role for RAMP2-mediated AM signaling in the development and function of the cardiovascular system and highlight the importance of CLR-RAMP2 signaling as a pharmacologically-tractable regulator of lymphatic proliferation.

Ichikawa-Shindo et al. have also reported an independent line of *Ramp2* null embryos which were generated by global CAG-Cre driven excision of a floxed *Ramp2* allele (23). These animals also demonstrated extensive generalized edema and pericardial effusion. Ultrastructural analysis revealed defects in blood endothelial and vascular smooth muscle structure resulting in the presence of occasional hemorrhagic plaques. Using RNA lysates isolated from whole embryo extracts, significant reductions in the expression of endothelial adhesive genes was shown in *Ramp2*$^{-/-}$ mice compared to *wild type* controls, suggesting that the expression of *Ramp2* is required for maintaining the blood vessel barrier. The subtle phenotypic differences between the two independent *Ramp2* null mouse strains could be influenced by the different genetic backgrounds. Importantly, the lymphatic and blood vascular defects are not mutually exclusive and actually shed greater insights into the complexity of and interplay between the blood and lymphatic vascular systems in maintaining tissue fluid balance (51).

The embryonic lethality of *Ramp2* global knockout mice precludes the study of RAMP2 loss-of-function in adult animals, but heterozygote animals

expressing half the normal levels of *Ramp2* have been useful to study. *Ramp2* heterozygous females on an SvEv129/S6 genetic background have severely reduced fertility with litter sizes approximately one third of *wild type* mice and other isogenic RAMP models (17). While reduced fertility is also a hallmark feature of the $AM^{+/-}$ female mice, (52-53) the fertility defects of the $Ramp2^{+/-}$ females is much more prominent and severe, and in fact contributes to difficulties in maintaining the strain. Our most recent studies suggest that the fertility defects can be attributed to marked endocrine imbalances in the hypothalamic-pituitary axis which are not observed in the $AM^{+/-}$ model (M. Kadmiel & K. Fritz-Six, unpublished observations, (25)). Therefore, a divergence in phenotypes between the $Ramp2^{+/-}$ and $AM^{+/-}$ mice (all maintained on an identical genetic background), suggests that RAMP2 may have broader *in vivo* roles beyond its requirement for generating an AM receptor with CLR. Consistent with our previous findings, a modest genetic reduction in *Ramp2* had no effect on basal blood pressures or heart rates of conscious male or female mice, as measured by the tail cuff method (17).

The *Ramp2* heterozygote mice reported by Ichikawa-Shindo and colleagues also survived to adulthood, but unlike the Dackor et al $Ramp2^{+/-}$ mice these animals showed modest increases in basal systolic blood pressure, as measured in anesthetized animals using carotid artery catheters (23). Consistent with the canonical paradigm of RAMPs regulating CLR's ligand binding specificity, the $Ramp2^{+/-}$ mice showed a markedly reduced vasodilatory response to AM treatment, but not to calcitonin gene related peptide (CGRP). In a series

of elegant *in vivo* angiogenesis assays, the *Ramp2*$^{+/-}$ mice also revealed a reduced angiogenic response to VEGF, decreased neovascularization and increased *in vivo* vascular permeability in the footpad, skin and brain. These studies, which are consistent with the discoveries made in the global *Ramp2* knockout embryos, highlight the importance of RAMP2-mediated signaling in regulating pathological angiogenesis and vascular permeability.

Transgenic overexpression of RAMP2

The effects of overexpression of RAMP2 *in vivo* have been investigated using a transgenic approach in which *Ramp2* was overexpressed in smooth muscle, under the control of an α-actin promoter (54). Consistent with a modest role for RAMP2 in regulating basal blood pressures, the *Ramp2* transgenic mice had mean arterial blood pressures and heart rates that were indistinguishable from their *wild type* littermates. As expected, the *Ramp2* transgenic mice exhibited potent and selective responsiveness to vasodilatory peptides. For example, while AM treatment of *Ramp2* transgenic mice enhanced vasodilation leading to increased stroke volume and reduced end-systolic pressure compared to similarly treated *wild type* animals, the administration of CGRP did not result in appreciable differences between the *Ramp2* transgenic mice and *wild type* animals. These data support a principal physiological function of RAMP2 in mediating the vasodilatory effects of AM in vascular smooth muscle cells. The *Ramp2* transgenic animals also showed increased inflammatory fluid extravasation after subcutaneous injection of substance P with co-treatment of

AM, but not with CGRP co-treatment, again supporting an important role for CLR-RAMP2 mediated regulation of tissue fluid balance.

RAMP3

Gene targeted deletion of Ramp3

Ramp3 null mice survive to adulthood without any obvious developmental problems (17). Basal blood pressures and heart rates are unaffected by the loss of *Ramp3*, and both male and female mice reproduce normally compared to their *wild type* littermates. Despite the fact that global *Ramp3* null mice exhibit normal food and water intake, they suffer from markedly reduced body weights after approximately 6 months of age. Although the mechanisms underlying this phenotype are not yet understood, the age-dependent lean phenotype did not affect health or longevity up to 18 months of age.

Lessons learned from comparative phenotyping

A summary of the most well-characterized phenotypes discovered in genetic RAMP mouse models is provided in Table 1. Characterization of any individual RAMP mouse model reveals important information about RAMP biological functions *in vivo*. Comparing agonist activity in RAMP mice has provided direct *in vivo* evidence that RAMPs covey specificity for different ligands, such as AM and CGRP under physiological conditions. For example, AM treatment, but not CGRP, shows potent effects on vasodilation and inflammation in RAMP2 animals, while CGRP treatment, but not AM, reveals physiological effects in Ramp1 transgenic mice.

A comparative approach to phenotyping between models can also provide powerful information, as long as the comparisons are performed on similar or identical genetic backgrounds. Conservation of phenotypes between genetic RAMP models and genetic models of their putative ligands (for example, the conserved phenotypes of *Adm*, *Calcrl* and *Ramp2* mice) can reveal physiologically important signaling paradigms that can be exploited for disease treatment or therapies. On the other hand, highly divergent or unexpected phenotypes in the genetic RAMP models can reveal previously unrecognized roles for RAMPs in mediating the signaling of other ligands and receptors.

It is also this direct comparison of RAMP models that allows us to better understand compensatory effects of the RAMPs for one another. For example, embryonic lethality of the Ramp2 null mice demonstrates that loss of RAMP2 cannot be compensated for by other RAMP family members, either because they are not expressed in the appropriate place and/or time or because RAMP1 and RAMP3 have non-redundant functions with RAMP2. In contrast, global loss of either RAMP1 or RAMP3 does not affect survival, perhaps because the expression of other RAMPs compensates for their absence. More definitive answers to these compensatory paradigms can come from careful evaluation of homeostatic responses in gene expression and protein expression of RAMPs in specific cells and tissues (57), but we are currently hindered by the lack of effective, commercially available murine antibodies for RAMP proteins.

Finally, much can be gained from directly comparing phenotypes between gain-of-function and loss-of-function alleles for each RAMP model. The

prediction is that altering the genetic dosage of a RAMP will result in a range of phenotypes. In fact, studies on the angiogenic and permeability effects of RAMP2 in both transgenic and gene targeted models provides an elegant example of the strength of this comparative phenotyping approach. In the future, additional animal models which expand our repertoire of both spatial and temporal manipulation of RAMP gene expression will continue to shed new insights in the physiological functions of RAMP proteins in normal and pathological conditions and potentially elucidate processes in which the pharmacological manipulation of RAMPs may be beneficial for treating human disease.

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Table 1.1 Comparative phenotyping of RAMP mouse models

Comparative Phenotyping of *RAMP* Mouse Models

	RAMP1 -/-	RAMP1 TG	RAMP2 -/-	RAMP2 +/-	RAMP2 TG	RAMP3 -/-
Survive to Adulthood	Yes	Yes	No	Yes	Yes	Yes
Altered Blood Pressure	Yes	N/A	N/A	Yes/No	Yes	No
CGRP Relaxation Response	α=Enhanced β=Normal	N/A	N/A	Normal	Normal	N/A
AM Relaxation Response	Reduced	N/A	N/A	Reduced	Enhanced	N/A
Inflammation/Fluid Extravasation basally #	Enhanced (LPS)	N/A	N/A	Enhanced (histamine)	Normal (Substance P)	N/A
Inflammation/Fluid Extravasation with CGRP Tx	N/A	Enhanced	N/A	N/A	Reduced*	N/A
Inflammation/Fluid Extravasation with AM Tx	N/A	N/A	N/A	N/A	Enhanced*	N/A
Fertility Defects	No	No	N/A	Yes/No	No	No
Body Weight Defects	No	No	N/A	No	No	Yes

Inflammatory treatment indicated in parentheses

*coinjected with SP

CHAPTER 3

HAPLOINSUFFICIENCY OF RECEPTOR ACTIVITY-MODIFYING PROTEIN-2 (*RAMP2*) CAUSES REDUCED FERTILITY, HYPERPROLACTINEMIA, SKELETAL ABNORMALITIES AND ENDOCRINE DYSFUNCTION IN MICE (25)

Kadmiel M, Fritz-Six K, Pacharne S, Richards GO, Li M, Skerry TM, Caron KM. Haploinsufficiency of receptor activity-modifying protein-2 (RAMP2) causes reduced fertility, hyperprolactinemia, skeletal abnormalities, and endocrine dysfunction in mice. Molecular Endocrinology 2011 Jul;25(7):1244-53.

ABSTRACT

Receptor activity-modifying protein-2 (RAMP2) is a single-pass transmembrane protein that can regulate the trafficking, ligand binding, and signaling of several G protein-coupled receptors (GPCR). The most well-characterized role of RAMP2 is in the regulation of adrenomedullin (AM) binding to calcitonin receptor-like receptor (CLR), and our previous studies using knockout mouse models support this canonical signaling paradigm. For example, *Ramp2*$^{-/-}$ mice die at midgestation with a precise phenocopy of the *AM*$^{-/-}$ and *Calcrl*$^{-/-}$ mice. In contrast, *Ramp2*$^{+/-}$ mice are viable and exhibit an expanded variety of phenotypes that are distinct from those of *Calcrl*$^{+/-}$ mice. Using *Ramp2*$^{+/-}$ female mice, we demonstrate that a modest decrease in *Ramp2* expression causes severe reproductive defects characterized by fetal growth restriction, fetal demise, and postnatal lethality that is independent of the genotype and gender of the offspring. *Ramp2*$^{+/-}$ female mice also exhibit hyperprolactinemia during pregnancy and in basal conditions. Consistent with hyperprolactinemia, *Ramp2*$^{+/-}$ female mice have enlarged pituitary glands, accelerated mammary gland development, and skeletal abnormalities including delayed bone development and decreased bone mineral density. Because RAMP2 has been shown to associate with numerous GPCRs, it is likely that signaling of one or more of these GPCRs is compromised in *Ramp2*$^{+/-}$ mice, yet the precise identification of these receptors remains to be elucidated. Taken together, this work reveals an essential role for RAMP2 in endocrine physiology

and provides the first *in vivo* evidence for a physiological role of RAMP2 beyond that of AM/CLR signaling.

Introduction

Receptor activity modifying proteins (RAMP) are single pass transmembrane proteins that can influence G protein-coupled receptor (GPCR) pharmacology by either altering the cell-surface trafficking of receptors, dictating ligand-binding specificity, regulating receptor desensitization, for modulating second messenger signaling (38). There are three mammalian RAMPs (RAMP1, -2, and -3), each encoded by a separate gene. The RAMPs were originally identified and characterized because they could potentiate the translocation of the calcitonin receptor-like receptor, (CLR) from the endoplasmic reticulum to the plasma membrane (1). In addition, each RAMP was shown to associate with CLR and change its ligand-binding affinity for different peptide ligands (1). For example, if CLR associates with RAMP1, then a functional receptor for calcitonin gene related peptide is formed, but if either RAMP2 or RAMP3 associates with CLR, then a functional receptor for Adrenomedullin (AM) is made. Therefore, the spatial and temporal expression of RAMP determines the cell surface expression and receptor pharmacology of RAMP-interacting GPCRs.

Importantly, this added level of receptor complexity offers unique opportunities for the design of small-molecule compounds and drugs that are specifically targeted to the RAMP-receptor interface. For example, BIBN4096BS (olcegepant), MK-0974 (telcagepant), and MK-3207 are nonpeptide, small-molecule antagonists that have clinical efficacy in targeting the CLR-RAMP1

interface to inhibit the functions of calcitonin gene-related peptide in migraine pain (55-58). Whether the RAMP-receptor paradigm can be exploited for pharmacological drug targeting of other GPCRs remains to be determined, but is currently hindered by our lack of knowledge regarding other physiologically relevant GPCRs that functionally associate with RAMPs.

The broader cell and tissue expression pattern of RAMPs compared with that of CLR and its ligands suggests that RAMPs are likely to interact with other GPCRs and modulate their properties. Indeed, numerous studies have demonstrated that the calcitonin receptor preferentially binds amylin, rather than calcitonin, when the receptor is associated with any of the three RAMPs. Moreover, *in vitro* biochemical studies in which the subcellular trafficking of overexpressed, fluorescently labeled RAMP is changed by receptor overexpression suggest that many other GPCRs functionally interact with RAMPs. These include several members of the class II GPCR: the PTH receptors (PTHR) 1 and 2, the vasointestinal peptide/pituitary adenylate cyclase-activating peptide 1 (VIP/VPAC1) receptor, the glucagon receptor (3), and more recently the secretin receptor (4). Bouschet and colleagues also showed that a class III GPCR, the calcium-sensing receptor (CaSR), requires RAMP for efficient cell surface expression in transfected clonal cells (5). However, it remains largely unclear whether these *in vitro* interactions have any physiological relevance either in normal or pathological conditions.

To address this gap in our knowledge, we have undertaken a phenotype-driven approach to identify the physiologically relevant functions of RAMPs, and

ultimately their GPCR partners, by generating and characterizing gene knockout mice for each of the three mammalian *Ramp* genes. The overt phenotypes of various mouse models of RAMPs, including those generated by others, has recently been reviewed (24). Here, we describe a constellation of endocrine-related phenotypes that are present in *Ramp2*$^{+/-}$ mice, but are notably absent in mice heterozygous for the gene encoding CLR. Therefore, these data provide *in vivo* evidence for an important role of RAMP2 in endocrine physiology and extend the physiologically relevant functions of RAMP2 beyond the canonical CLR paradigm.

Materials and Methods

Animals

Mice with a targeted deletion of *Ramp2* were generated and characterized as described previously (17). Briefly, chimeric mice with *Ramp2* gene targeted were generated using targeting vectors and embryonic stem cells of the SvEV129/6-TC1 background. Isogenic colonies were established by breeding chimeric male mice with SvEV129/6-TC1 female mice to generate SvEv-*Ramp2*$^{+/-}$ mice. We previously published that subfertility seen in SvEv-*Ramp2*$^{+/-}$ mice is completely rescued by backcrossing to the C57BL6 background (18). To obtain F2-*Ramp2*$^{+/-}$ mice, we bred SvEv-*Ramp2*$^{+/-}$ mice to C57BL6 wild-type mice to generate F1-hybrid offspring. F1 siblings heterozygous for *Ramp2* were intercrossed to yield F2-*Ramp2*$^{+/-}$. Genotyping was performed by PCR using a three-primer-based approach: primer 1, 5'-TCTGTCTGGATGCTGCCTTGC-3'; primer 2, 5'- GAAGTCAGGCAGTCAGGGTTG-3'; and primer 3, 5'-

GACGAGTTCTTCTGAGGGGA-3’. Primers 1 and 2 amplified a 900-bp wild-type fragment, whereas primers 1 and 3 amplified a 650-bp targeted fragment. For timed matings, crosses were established, and the morning of vaginal plug detection was considered as E0.5day. Control animals were wild type with similar age, gender, and genetic background. All experiments performed on animals were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Serum hormone measurements

Serum was collected by mandibular vein bleed at 0600 h where the light cycle was from 0700–1900 h. Prolactin and GH were assayed from serum by RIA by A. F. Parlow at the National Hormone and Peptide Program (Torrance, CA). Serum for calcium measurement was collected at 1000 h and was processed at the Animal Clinical Chemistry and Gene Expression Laboratories core facility at the University of North Carolina at Chapel Hill.

Antibodies

The antibodies used in this study were obtained from National Institute of Diabetes and Digestive and Kidney Diseases and are the following: mouse prolactin (AFP-131078), mouse GH (AFP5672099), rat β-TSH (AFP-1274789), rat ACTH (AFP-156102789), rat β-LH (AFP-C697071P), and rat β-FSH (AFP7798-1289). Corresponding fluorescent secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Nuclei were stained with Hoechst 33258 dye (Sigma-Aldrich, St. Louis, MO).

Histology and immunofluorescence microscopy

Tissues were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT (Tissue-Tek), and cryosectioned at 8–10 μm. For immunostaining, sections were rehydrated in PBS, permeabilized in 0.2% Triton X-100, blocked in 4% BSA, and incubated in primary antibody overnight. After washing, sections were incubated in secondary antibody for 1–2h, washed, and mounted for imaging. Images were acquired on a Nikon E800 microscope with a Hamamatsu ORCA-ER charge-coupled device camera with Metamorph software (Molecular Devices Corp., Sunnyvale, CA) and processed with Photoshop.

Pituitary gland volume and density measurements

Pituitary volume was calculated from two-dimensional images by making the assumption that the parameter of depth is proportional to the parameter of height for an ellipsoid shape. For measurement of pituitary cell density, cells were manually counted in 4',6-diamidino-2-phenylindole-stained sections and represented as number of cells per square millimeter.

RNA extraction, cDNA synthesis, and quantitative RT-PCR analyses

RNA was extracted using the Trizol method following the manufacturer's protocol (Ambion, Austin, TX). Isolated RNA was deoxyribonuclease treated and reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), and gene expression was measured by MX3000 Q-PCR machine from Stratagene (Santa Clara, CA). All the primer-probe sets used in this study were TaqMan probes purchased from Applied

Biosystems (Carlsbad, CA). *mGAPDH* was used as a normalizing gene, and the $\Delta\Delta C_T$ method was used to analyze the relative levels of gene expression (63).

Mammary gland whole mounts

Mouse mammary glands were collected for whole-mount analysis. Fourth inguinal glands were carefully dissected, placed on a glass slide, air dried for 5 min, and fixed overnight in Carnoy's solution (three parts absolute ethanol and one part glacial acetic acid) and stained in Carmine alum (0.2% carmine dye and 0.5% aluminum potassium sulfate) overnight. Slides were destained in 70% ethanol containing 2% HCl until the background was minimal and then dehydrated in increasing concentrations of ethanol. Toluene was used to defat, and the slides were mounted with Permount. Mammary gland branch points were manually scored in two images per animal along three major ducts that surrounded the lymph node within a 30-mm^2 area. Terminal end buds located along a 5-mm length of a major peripheral duct were counted.

DEXA densitometry

DEXA (Lunar PIXImus densitometer) was used to measure bone mineral density and content of right femurs. *In vivo* DEXA scans were performed by anesthetizing animals with tribromoethanol and laying the animal flat on the DEXA machine.

Micro-CT

Micro-CT was performed to analyze femurs and vertebrae as described previously (59). Briefly, femurs were fixed, dehydrated, and scanned using a

high-resolution x-ray source to generate three-dimensional (3D) reconstructions. Virtual transverse sections at the mid-diaphysis of the femur 3D reconstructions were made that revealed the cortical and trabecular bone architecture.

Statistical analyses

All quantitative analyses were performed using unpaired, two-tailed Student's *t* test, unless otherwise specified. Data are represented as SEM unless indicated otherwise. Differences were considered significant when the *P* value was <0.05.

Results

Genetic reduction of maternal Ramp2 causes fetal loss throughout pregnancy

We have previously shown that *Ramp2-null* embryos exhibit interstitial edema due to defective lymphangiogenesis and die at midgestation [embryonic d 14 (E14.5)] on either a 129SvEV/S6 or an F2-129SvEv/S6-C57BL6 genetic background (18). We have also noticed that haploinsufficient $Ramp2^{+/-}$ mice on a 129SvEv/S6 background survive to adulthood, but have extremely small litter sizes, worse than what would be predicted from the Mendelian loss of $Ramp2^{-/-}$ embryos (17). To determine the gestational age of fetal loss, we established natural timed matings between *SvEv-*$Ramp2^{+/-}$ parents and scored the following: 1) number of viable embryos at various gestational ages beyond implantation, 2) number of live pups at birth, and 3) number of live pups at weaning. These data were then compared with data obtained from crosses between wild-type SvEv parents. As shown in the gray dashed line of **Fig.**3.1A, wild-type SvEv

intercrosses maintained within our animal colony resulted in average litter sizes that are consistent with our previously published observations and those reported for the 129SvEv/S6-Taconic line (14) (www.taconic.com/wmspage.cfm?parm1_426), with no appreciable decline in litter size throughout gestation, at birth, or at weaning. In contrast, SvEv-*Ramp2*$^{+/-}$ intercrosses (*black solid line*, **Fig.**3.1.A) showed a gradual and continuous decrease in litter size from implantation to weaning.

Morphological evaluation of embryos from SvEv-*Ramp2*$^{+/-}$ intercrosses revealed a high incidence of fetal growth restriction compared with embryos from *wild type* SvEv crosses. Fig.3.1.B shows representative images of litters from E9.5 wild-type SvEV and SvEv-*Ramp2*$^{+/-}$ intercrosses. Although we noticed occasional fetal growth restriction in wild-type SvEV crosses, SvEv-*Ramp2*$^{+/-}$ intercrosses exhibited a high proportion of severe fetal growth restriction. In the representative example shown, three of eight embryos are severely growth restricted (marked by *arrows*). Moreover, the fetal growth restriction was independent of the genotype of the embryo, because two of the three growth-restricted embryos were wild type. Consistent with these morphological observations, crown to rump length of E9.5 embryos from SvEv-*Ramp2*$^{+/-}$ intercrosses was significantly reduced compared with that of embryos from wild-type SvEv intercrosses (**Fig.**3.1.C). Therefore, the dramatic and progressive decline in litter size in SvEv-*Ramp2*$^{+/-}$ intercrosses is not solely due to the embryonic lethality of *Ramp2*$^{-/-}$ embryos but is also caused by a high incidence of fetal growth restriction that affects both wild-type and *Ramp2*$^{+/-}$ embryos.

Genetic reduction of maternal *Ramp2* causes postnatal pup and litter lethality

In addition to fetal loss during gestation, a large percentage of individual pups, as well as entire litters, born to SvEv-*Ramp2*$^{+/-}$ females died within 1–2 d after birth. Within the 140 born litters we characterized from SvEv-*Ramp2*$^{+/-}$ intercrosses, we observed a 12% postnatal lethality of individual pups, double that of the 6% postnatal lethality rate observed for SvEV wild-type crosses (**Fig.**3.1.A). Most striking was our observation that nearly one quarter, 24%, of litters born to *Ramp2*$^{+/-}$ females lost all of the pups (12 complete litters died among the 50 litters examined). This unusually high rate of postnatal litter loss was not observed in wild-type SvEv mice maintained in our colony (one litter died among 30 litters examined, 3.3%) or in heterozygote intercrosses of *Calcrl*$^{+/-}$ or *Adm*$^{+/-}$ mice that are maintained on an identical isogenic 129SvEv/S6 background in our colony. Moreover, our observations of average litter size at birth, at weaning, and percentage of entire litter loss from wild-type SvEv intercrosses are similar to the breeding characteristics established by Taconic Farms for the 129S6/SvEvTac strain (www.taconic.com/wmspage.cfm?parm1_426). Therefore, compared with control *wild type* SvEV intercrosses, these data reveal an unusually high incidence of postnatal lethality for individual pups, as well as entire litters, born to SvEv-*Ramp2*$^{+/-}$ intercrosses.

To determine whether this postnatal lethality was associated with the genotype or gender of the offspring, we tabulated the genotype and sex of both the surviving and dead pups from the 140 litters generated from SvEv-*Ramp2*$^{+/-}$ intercrosses. As shown in **Fig.**3.1.D (*left* pie charts), the genotypes of pups, both

surviving and dead, fit the expected Mendelian ratio of 1:2 (or 33:66%) for SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$, respectively, and do not show any association with lethality. Dead pups in which the quality of genomic DNA was not suitable for genotyping by PCR were not included in our analysis. Furthermore, we found no correlation between the sex of the pups and their lethality (**Fig.**3.1.D, *right* pie charts). These data reveal an unusually high incidence of postnatal pup lethality in SvEv-*Ramp2*$^{+/-}$ intercrosses that is independent of the genotype or gender of the offspring, suggesting that female *Ramp2*$^{+/-}$ mice have either abnormal rearing behavior and/or problems with lactation. Consistent with this hypothesis, we noticed that most of the postnatal dead pups had no milk in their stomachs.

To eliminate the confounding effects of *Ramp2*$^{-/-}$ embryos generated from heterozygote intercrosses and to determine whether the pup loss was dependent on the gender of either parent, we established and analyzed genetic reciprocal crosses in which only one parent was heterozygote for the *Ramp2* gene. As shown in the *black bars* of **Fig.**3.1E, fetal loss throughout pregnancy and small litter sizes at birth were evident only when the dam was heterozygote for *Ramp2*. In contrast, litter sizes throughout pregnancy and at birth were not significantly affected when the male parent was heterozygote for *Ramp2* (*gray bars*, **Fig.**3.1.E). Thus, genetic reduction of maternal *Ramp2*, but not of paternal *Ramp2*, causes fetal loss throughout pregnancy. Taken together, these data demonstrate that a modest genetic reduction in the maternal *Ramp2* gene causes remarkable fetal growth restriction and fetal loss throughout pregnancy that is independent of fetal genotype.

Ramp2$^{+/-}$ female mice exhibit hyperprolactinemia and anterior pituitary gland hyperplasia

The absence of milk in the stomachs of postnatal dead pups born to SvEv-*Ramp2*$^{-/-}$ female mice prompted us to measure serum prolactin in female mice during late gestation when mammary glands prepare for lactation (60). As expected, serum prolactin gradually increased with advancing gestation (from E14.5 to E18.5) in SvEv-*Ramp2*$^{+/+}$ females bred to SvEv-*Ramp2*$^{+/+}$ males (**Fig.**3.2.A), with an average level of 3.8 ng/ml. However, SvEv-*Ramp2*$^{+/-}$ females bred with SvEv-*Ramp2*$^{+/-}$ males had a significantly more robust increase in serum prolactin, averaging 20.5 ng/ml from E14.5 to E18.5, compared with controls (P < 0.01, **Fig.** 3.2A). Moreover, **Fig.**3.2.B shows that basal levels of serum prolactin were 2-fold higher in nonpregnant SvEv-*Ramp2*$^{+/-}$ females compared with nonpregnant wild-type females, although this trend failed to reach statistical significance (11.7 ± 3.0 ng/ml SvEv-*Ramp2*$^{+/+}$ *vs.* 25.8 ± 6.3 ng/ml SvEv-*Ramp2*$^{+/-}$; P = 0.06). The expression of *prolactin* mRNA was also significantly elevated 1.4-fold in the pituitary glands of 6-month old, virgin SvEv-*Ramp2*$^{+/-}$ female mice compared with SvEv-*Ramp2*$^{+/+}$ female mice (**Fig.**3.2.C).

Immunohistochemistry on pituitary gland sections of 18-wk-old, virgin SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$ female mice confirmed the basal hyperprolactinemia phenotype. As shown in **Fig.**3.2.D, the lactotropes of the anterior pituitary gland of SvEv-*Ramp2*$^{+/-}$ female mice showed intense staining with an anti-prolactin antibody compared with moderate staining intensity in wild-type control pituitaries. GH expression appeared somewhat elevated in the SvEv-

Ramp2$^{+/-}$ mice compared with controls, a finding that may not be unexpected because lactotropes and somatotropes share a common lineage pre-cursor during embryogenesis. Therefore, we also evaluated relative levels of pituitary GH by quantitative real time PCR (SvEv-*Ramp2*$^{+/+}$ = 1.04 ± 0.14, n = 9, *vs.* SvEv-*Ramp2*$^{+/-}$ = 0.76 ± 0.07, n = 8) and serum levels of GH by RIA (SvEv-*Ramp2*$^{+/+}$ = 3.49 ng/ml ± 0.69, n = 10, *vs.* SvEv-*Ramp2*$^{-/-}$ = 3.98 ng/ml ± 0.33, n = 8) and found no significant differences between genotypes. In addition, **Fig.**3.2.D shows that there were no remarkable differences in staining intensity for any of the other hormone-producing cell types of the anterior pituitary (anti-TSH, -ACTH, -LH, and -FSH).

Because increased prolactin gene expression, synthesis, and secretion are often associated with lactotrope hyperplasia, we evaluated the size of the pituitary glands from 18-wk-old, virgin SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$ female mice. As shown in **Fig.**3.2.E, the pituitary gland of SvEv-*Ramp2*$^{+/-}$ mice appeared larger and was appreciably more dense and firm upon dissection compared with pituitaries from SvEv-*Ramp2*$^{+/+}$ mice. Consistent with these morphological observations, SvEv-*Ramp2*$^{+/-}$ female mice had significant increases in pituitary gland volume and cell density compared with the pituitaries of *wild type* females (**Fig.** 3.2, F and G, respectively). The increase in pituitary cell density in SvEv-*Ramp2*$^{+/-}$ mice was also readily appreciated in 4',6-diamidino-2-phenylindole-stained sections of pituitaries, shown in **Fig.** 3.2.D. Taken together, these results demonstrate that genetic reduction of *Ramp2* in female mice leads to mild hyperprolactinemia, both basally and during pregnancy, which is associated with

enlarged and hyperplastic pituitary glands producing increased levels of prolactin. In this regard, when we used the Fisher method (61) to test for the combined significance of these related but independent variables, we found a highly significant ($P < 0.0001$) overall difference in prolactin measures between SvEv-*Ramp2*$^{+/-}$ mice and controls.

Accelerated mammary gland development in *Ramp2*$^{+/-}$ mice

To determine whether the mild hyperprolactinemia of SvEv-*Ramp2*$^{+/-}$ female mice might contribute to abnormalities in mammary gland development and ultimately lactation defects, we performed whole-mount analyses of mammary glands from 18-wk-old, virgin SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$ female mice. We chose to evaluate mammary gland development of virgin mice rather than pregnant or lactating mice because variable litter sizes and suckling stimulus are known to influence mammary gland development and would confound the results for SvEv-*Ramp2*$^{+/-}$ mice. As shown in **Fig.**3.3.A, SvEv-*Ramp2*$^{+/-}$ mice had accelerated branching of secondary and tertiary ducts (low magnification) as well as remarkably increased number of terminal end buds (high magnification) compared with SvEv-*Ramp2*$^{+/+}$ female mice. Quantitation of mammary gland ductal branch points as well as terminal end buds showed a marked and significant increase in SvEv-*Ramp2*$^{+/-}$ mice compared with control animals (**Fig.**3.3, B and C, respectively). This precocious mammary gland development in SvEv-*Ramp2*$^{+/-}$ females was further supported by an increase in *prolactin* gene expression in the mammary glands (data not shown).

Ramp2$^{+/-}$ mice have delayed mineralization during development and decreased bone mineral content and density

To determine whether the mild hyperprolactinemia of SvEv-*Ramp2*$^{+/-}$ mice contributed to other endocrine phenotypes, we evaluated the bones of SvEv-*Ramp2*$^{+/-}$ mice. Dual-energy x-ray absorptiometry (DEXA) analysis of excised femurs of 18-wk-old, virgin female mice revealed significantly reduced bone mineral content and bone mineral density in SvEv-*Ramp2*$^{+/-}$ mice compared with wild-type controls (**Fig.**3.4, A and B). Total bone area did not change significantly between the two groups of mice (**Fig.**3.4.C). Radiographic images of tibiae and femurs of SvEv-*Ramp2*$^{+/-}$ mice showed significantly longer bones compared with their wild-type controls (**Fig.**3.4, D and E). The tibiae of *Ramp2*$^{+/-}$ mice were significantly longer than wild-type controls (14.7 ± 0.3 mm for wild type and 15.89 ± 0.24 mm for SvEv-*Ramp2*$^{+/-}$, $P < 0.04$), and the femurs generally appeared longer, although the difference did not reach statistical significance (11.9 ± 0.3 mm for wild type and 12.5 ± 0.06 mm for SvEv-*Ramp2*$^{+/-}$). Consistent with the DEXA analysis, microcomputed tomography (micro-CT) analysis of femurs from 18-wk-old virgin mice showed that the reduced bone volume and density of SvEv-*Ramp2*$^{+/-}$ mice were associated with reduced cortical thickness and fewer, thinner trabecular structures compared with SvEv-*Ramp2*$^{+/+}$ controls (**Fig.**3.4.F). The lumbar vertebrae of SvEv-*Ramp2*$^{+/-}$ mice were similar in length to SvEv-*Ramp2*$^{+/+}$ mice but appeared underdeveloped with wider growth plates, wider intervertebral discs, and reduced mineralization (**Fig.** 3.4.G).

To distinguish whether these skeletal abnormalities were caused by the modest hyperprolactinemia or whether they arose independently of prolactin

dysregulation, we evaluated the hind limb bones and lumbar spines of SvEv-*Ramp2*$^{+/-}$ pups 2 d postnatally. The femurs of the hind legs of SvEv-*Ramp2*$^{+/-}$ pups exhibited modest, yet statistically significant increases in bone volume compared with the wild-type pups, whereas the tibia volumes also trended to be greater in SvEv-*Ramp2*$^{+/-}$ pups (**Fig.**3.4.H). In the spine, there was also delayed development of the lumbar vertebrae as indicated by reduced mineralization of the epiphyseal plates of the vertebrae and delayed development of lumbar transverse processes (**Fig.**3.4.I).

Despite these skeletal changes, SvEv-*Ramp2*$^{+/-}$ mice had no significant differences in circulating levels of calcium when compared with SvEv-*Ramp2*$^{+/+}$ controls (SvEv-*Ramp2*$^{+/-}$ = 11.88 ± 0.96 mg/dl, and SvEv-*Ramp2*$^{+/+}$ = 13.55 ± 0.11mg/dl). Nevertheless, these results clearly demonstrate that haploinsufficiency for *Ramp2* results in significant defects in bone homeostasis and development.

Endocrine phenotypes of Ramp2$^{+/-}$ mice are strain dependent

We have previously shown that the reduced fertility phenotype of *Ramp2*$^{+/-}$ mice was not present in *Ramp2*$^{+/-}$ intercrosses on an F2 129SvEv/S6-C57BL6 genetic background (18). Consistent with this observation, we also found no significant increase in serum prolactin levels in nonpregnant F2-*Ramp2*$^{+/-}$ mice (data not shown). We also evaluated mammary gland development in F2-*Ramp2*$^{+/-}$ females and found no significant differences compared with strain-matched controls (data not shown). Therefore, the endocrine-related phenotypes of reduced fertility, postnatal pup loss, hyperprolactinemia, and accelerated

mammary gland development in SvEv-*Ramp2*$^{+/-}$ mice can be rescued by crossing one generation onto a C57BL6 genetic background. These findings imply that the *Ramp2* gene must have potent genetic modifiers that vary between common laboratory mouse strains.

Discussion

We have previously shown that global knockout mice for either *Ramp2*, *Calcrl* (gene name for CLR), and *Adm* (gene name for AM peptide) share a strikingly similar phenotype of embryonic lethality associated with impaired lymphatic vascular development and interstitial edema (18, 43, 44). This striking similarity in phenotypes allowed us to conclude that the CLR/RAMP2/AM signaling complex is an essential mediator of lymphatic vascular development during embryogenesis. However, because the global knockouts die at midgestation, we have been limited in our ability to extend our phenotypic comparison of these mice to other phenotypes, particularly those that might be present in *Ramp2*$^{+/-}$ mice but not in *Calcrl*$^{+/-}$ or *Adm*$^{+/-}$ mice.

In this study, we have addressed this caveat by taking advantage of the viability of *Ramp2*$^{+/-}$ mice and characterized a constellation of endocrine-related phenotypes that include fetal loss throughout gestation, postnatal lethality of offspring, maternal hyperprolactinemia, accelerated mammary gland development, and altered skeletal properties and development. If these phenotypes were due solely to a reduction in signaling of the canonical CLR, then the logical expectation would be that female SvEv-*Calcrl*$^{+/-}$ mice should exhibit the same, or possibly exacerbated, phenotypes. However, SvEv-*Calcrl*$^{+/-}$

mice (which we generated and maintained on the identical genetic background as SvEv-*Ramp2*$^{+/-}$ mice) do not exhibit postnatal pup lethality, do not have elevated serum prolactin, do not have hyperplastic pituitaries, and do not have accelerated mammary gland development (data not shown). Therefore, this divergence in phenotypes allows us to conclude that in adults, RAMP2 must have physiologically relevant functions that extend beyond its well-established role as a modulator of CLR function. This conclusion is entirely consistent with *in vitro* biochemical and pharmacological studies that show associations between RAMP2 and several other GPCRs involved in endocrine physiology, like PTHR1, CTR, and GCGR (Fig.3.5).

Determining exactly which receptors, or more likely which combination of receptors, ultimately underlies the endocrine phenotypes of SvEv-*Ramp2*$^{+/-}$ mice remains a challenging task. Once again, a comparison of phenotypes from genetic mouse models of RAMP2-interacting GPCRs and their ligands may provide the best clues. For example, PTHrP, via the receptor PTHR1, plays essential roles in fetal and placental development (62-63) and mammary gland development (64-65) and in the endochondral ossification process of bone development (66-69). Glucagon signaling through GCGR also plays a critical role in fertility and fetal development because *Gcgr*$^{-/-}$ female mice exhibit reduced fertility with embryonic death and perinatal lethality (70). Mice deficient for amylin, a peptide ligand for the calcitonin-RAMP2 receptor complex, display bone phenotypes similar to *Ramp2*$^{+/-}$ mice (83). Last but not least, AM, the ligand for the canonical CLR-RAMP2 complex, can induce osteoblast proliferation *in vitro*

and increase bone formation *in vivo* (71) and is fundamentally required for normal female reproduction. And so, considered all together, it is most likely that the endocrine phenotypes of *Ramp2*-heterozygote mice are the combined result of reduced signaling for a cohort of previously identified, and yet to be identified, RAMP2-interacting GPCRs (Fig.3.5).

Additional genetic lines in which the compound haploinsufficient loss of RAMP2 with either CLR or other RAMP2-associating GPCRs could potentially shed light on which endocrine organ system is predominantly affected by a 50% reduction in RAMP2. For example, a compound RAMP2/CLR heterozygote mouse may show predominant phenotypes in the female reproductive system, whereas a compound RAMP2/PTHR1 heterozygote mouse may exhibit predominant phenotypes in the skeletal system.

Another important aspect of these findings, which further implies the involvement of multiple RAMP2-interacting GPCRs, is that the SvEv-*Ramp2*$^{+/-}$ phenotype is manifested on a haploinsufficient background. Although robust endocrine phenotypes are revealed in global knockouts and transgenic overexpression mouse models for many individual RAMP2-interacting GPCR and their ligands, it is remarkable that a modest 50% reduction in RAMP2 can result in similarly robust phenotypes. This constellation of phenotypes is also likely to be influenced by the very broad spatiotemporal expression pattern of RAMP2 compared with individual GPCR. Therefore, these results demonstrate that the appropriate genetic dosage of *Ramp2* is required for normal female reproduction

and endocrine homeostasis and that this requirement is likely to involve numerous GPCR.

In summary, we have demonstrated that a modest genetic reduction in the GPCR accessory protein *Ramp2* causes severe female subfertility, mild hyperprolactinemia, and endocrine-related defects in mammary gland and bone target organs. Although we cannot solely attribute these phenotypes to hyperprolactinemia, the presence of bone phenotypes as early as postnatal d 2 argues for a direct role of RAMP2 in bone development that is independent of hyperprolactinemia. In a broader context, the constellation of endocrine phenotypes demonstrates that RAMP2 has physiological functions that extend well beyond its interaction with the canonical CLR. The elucidation of the specific receptors (or combination of receptors) that underlie the *Ramp2*$^{+/-}$ phenotype in different tissues will ultimately require more sophisticated mouse modeling approaches. However, these phenotypic data provide a valuable research resource from which to begin exploring novel pharmacological and biochemical GPCR-RAMP2 interactions. Given the pharmacological tractability of GPCR-RAMP2 interfaces, these and future studies have the potential to identify receptor targets for the therapeutic treatment of numerous endocrine-related conditions such as infertility, osteoporosis, and hyperprolactinemia.

Acknowledgements

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Figure 3.1 Reduced fertility in SvEv-*Ramp2*$^{+/-}$ females and high incidence of postnatal lethality in SvEv-*Ramp2*$^{+/-}$ litters.

A, Average number of pups per litter after implantation to weaning (SvEv-*Ramp2*$^{+/+}$ intercrosses R2 value = 0.74; SvEv-*Ramp2*$^{+/-}$ intercrosses R2 value = 0.97). A total of 140 litters were scored for SvEv-*Ramp2*$^{+/-}$ intercrosses at birth and weaning, and 55 litters were scored for SvEv-*Ramp2*$^{+/+}$ intercrosses. Unless otherwise mentioned, three to 17 litters per gestational time point were scored. The *black arrows* indicate the gestational age at which *Ramp2*$^{-/-}$ embryos die from lymphatic vascular defects and at which postnatal pup loss is observed in SvEv-*Ramp2*$^{+/-}$ intercrosses. **B**, Representative litters from wild-type SvEv and SvEv-*Ramp2*$^{+/-}$ intercrosses at E9.5 of pregnancy, showing a high incidence of fetal growth restriction that is independent of fetuses' genotype in SvEv-*Ramp2*$^{+/-}$ intercrosses. *Arrows* point to severely growth-restricted embryos. Embryos were imaged at the same magnification and later grouped digitally based on their genotype. **C**, Crown to rump length (millimeters) of E9.5 embryos from SvEv-*Ramp2*$^{+/+}$ intercrosses and SvEv-*Ramp2*$^{+/-}$ intercrosses; *, $P < 0.05$. *Number in the column* represents number of embryos measured. **D**, Pie charts demonstrating that postnatal death observed from 140 SvEv-*Ramp2*$^{+/-}$ intercross litters was independent of pup genotype (*left*) and pup gender (*right*). Note that the *white slice of the lower left* pie chart represents a single *Ramp2*$^{-/-}$ mouse that we discovered alive after birth. Consistent with the knockout phenotype, this animal was severely hydropic. **E**, Reciprocal crosses of SvEv-*Ramp2*$^{+/-}$ mice. An average of two to three litters were scored for each time point. Loss of pups throughout pregnancy was observed only when the female was SvEv-*Ramp2*$^{+/-}$.

Figure 3.1

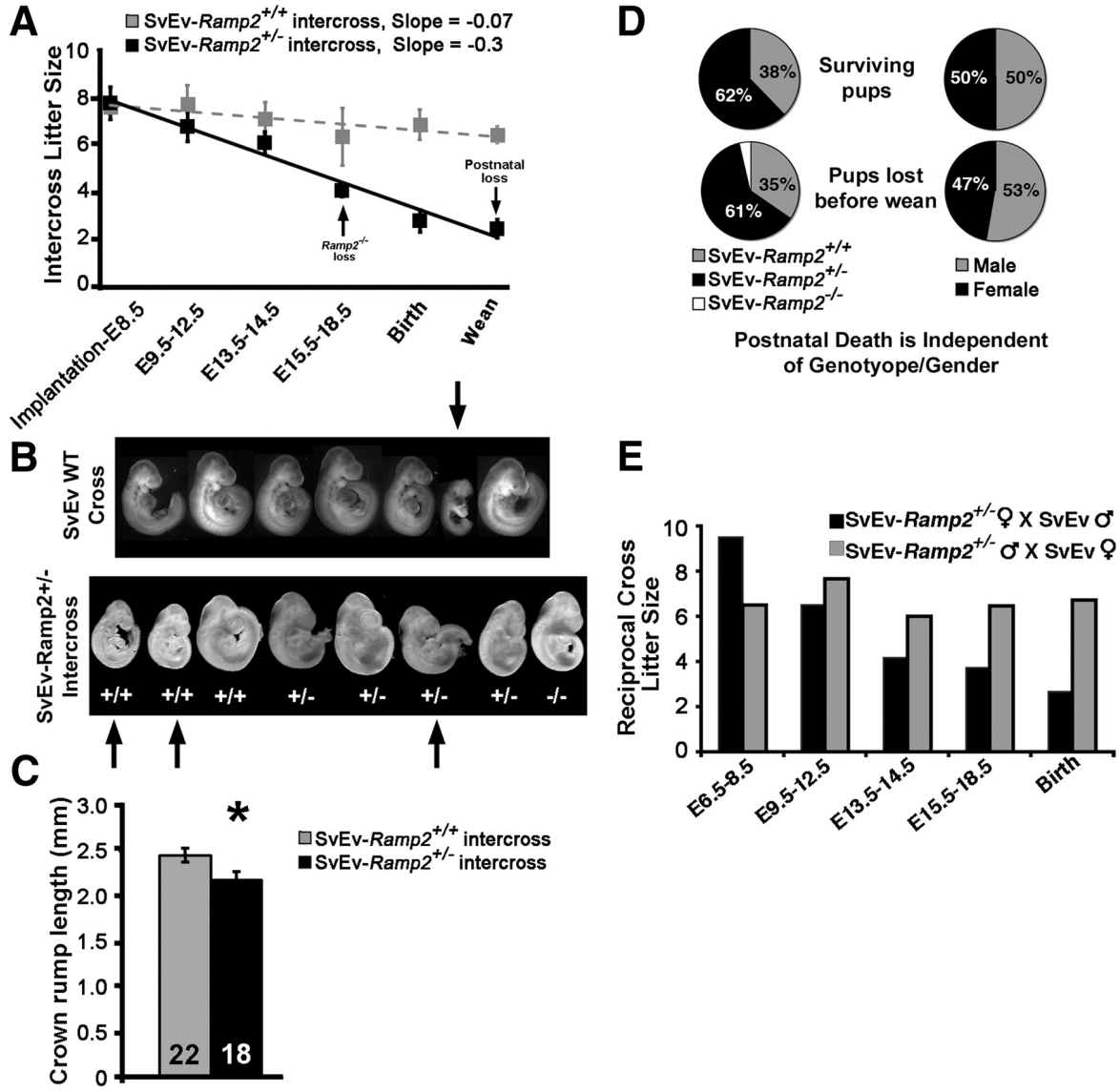

A
SvEv-Ramp2+/+ intercross, Slope = -0.07
SvEv-Ramp2+/- intercross, Slope = -0.3
Intercross Litter Size
Postnatal loss
Ramp2-/- loss
Implantation-E8.5
E9.5-12.5
E13.5-14.5
E15.5-18.5
Birth
Wean
B
SvEv WT Cross
SvEv-Ramp2+/- Intercross
+/+ +/+ +/+ +/- +/- +/- +/- -/-
C
Crown rump length (mm)
SvEv-Ramp2+/+ intercross
SvEv-Ramp2+/- intercross
22
18
D
Surviving pups
38%
62%
50%
50%
Pups lost before wean
35%
61%
47%
53%
SvEv-Ramp2+/+
SvEv-Ramp2+/-
SvEv-Ramp2-/-
Male
Female
Postnatal Death is Independent of Genotype/Gender
E
SvEv-Ramp2+/-♀ X SvEv ♂
SvEv-Ramp2+/- ♂ X SvEv ♀
Reciprocal Cross Litter Size
E6.5-8.5
E9.5-12.5
E13.5-14.5
E15.5-18.5
Birth

Figure 3.2 SvEv-*Ramp2*$^{+/-}$ females exhibit hyperprolactinemia and hyperplastic pituitary glands.

A, Maternal serum prolactin levels in wild-type SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$ intercrosses on E14.5, E16.5, and E18.5 of gestation. *, $P < 0.01$, by one-way ANOVA; n ≥ 5 mice per group and time point. **B**, Basal serum prolactin levels in nonpregnant mice; n = 25 for wild-type SvEv-*Ramp2*$^{+/-}$ females, and n = 14 for SvEv-*Ramp2*$^{+/+}$ females. $P = 0.06$. **C**, Quantitative RT-PCR of *prolactin* gene expression, normalized to *GAPDH* expression, in the anterior pituitary glands of 6-month-old, virgin SvEv-*Ramp2*$^{+/-}$ female mice (*black bars*) compared with the wild-type SvEv-*Ramp2*$^{+/+}$ controls (*gray bars*); n = 8 mice per genotype. *, $P < 0.05$. **D**, Immunohistochemistry on cryosections of pituitary glands from virgin wild-type and SvEv-*Ramp2*$^{+/-}$ female mice using antibodies against six anterior pituitary hormones: ACTH, prolactin (PRL), GH, TSH, FSH, and LH. Images are representative of n = 8 pituitaries analyzed for each genotype. **E**, Images of dissected pituitary glands from wild-type and SvEv-*Ramp2*$^{+/-}$ females, showing enlarged pituitary glands in SvEv-*Ramp2*$^{+/-}$ mice. **F**, Volume of the pituitary gland is significantly larger in SvEv-*Ramp2*$^{+/-}$ female mice (*black bars*) compared with the controls (*gray bars*). *, $P < 0.03$. *Scale bar*, 500 µm. G, Cell density of anterior pituitary gland is significantly increased in SvEv-*Ramp2*$^{+/-}$ female mice (*black bars*) compared with the controls (*gray bars*). *, $P < 0.01$. Mice were 18-wk-old virgin females at time of euthanasia; n ≥ 5 mice per genotype.

Figure 3.2

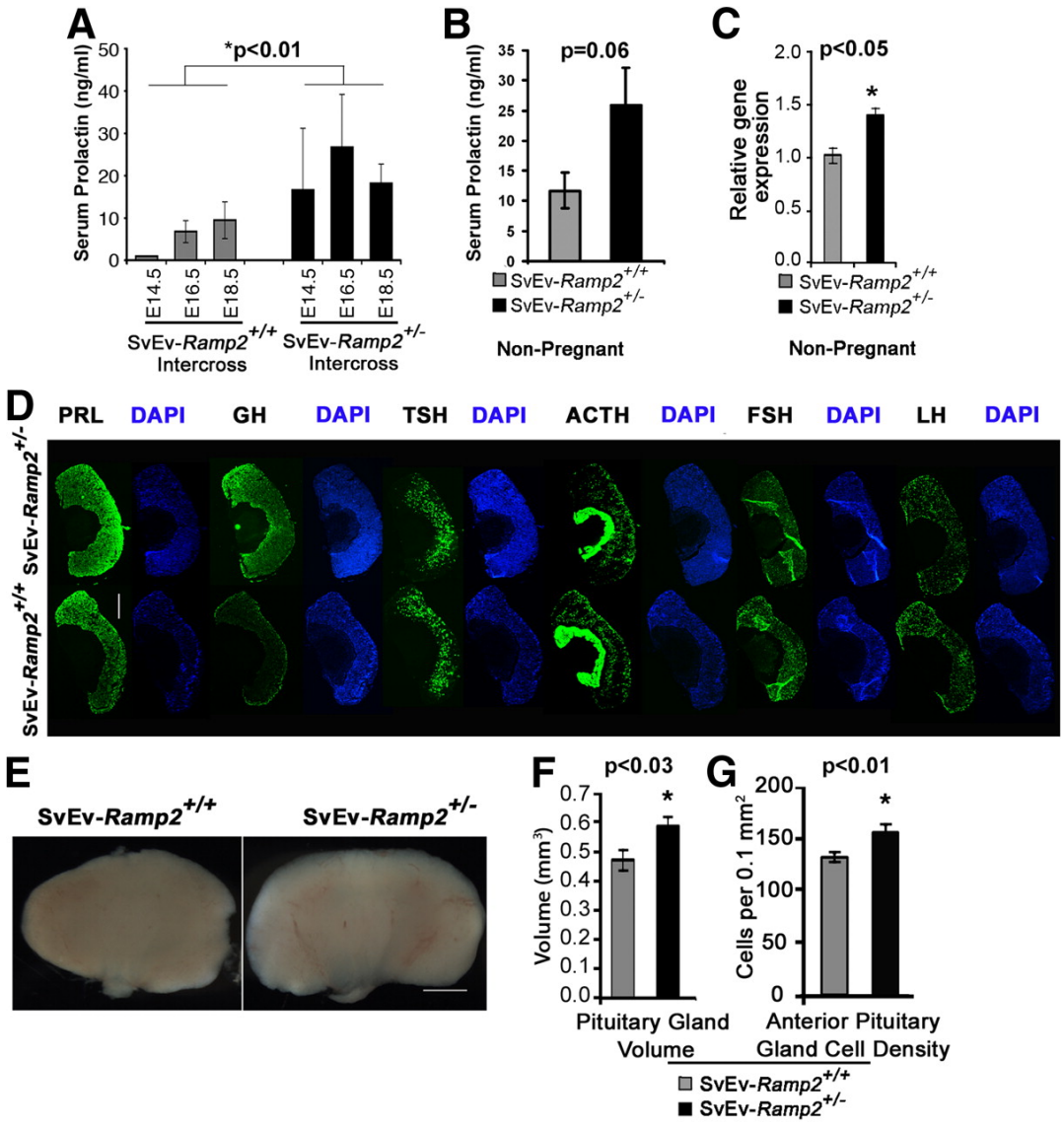

A
*p<0.01
Serum Prolactin (ng/ml)
E14.5
E16.5
E18.5
SvEv-Ramp2+/+ Intercross
SvEv-Ramp2+/- Intercross
B
p=0.06
Serum Prolactin (ng/ml)
SvEv-Ramp2+/+
SvEv-Ramp2+/-
Non-Pregnant
C
p<0.05
Relative gene expression
SvEv-Ramp2+/+
SvEv-Ramp2+/-
Non-Pregnant
D
PRL
DAPI
GH
TSH
ACTH
FSH
LH
SvEv-Ramp2+/-
SvEv-Ramp2+/+
E
SvEv-Ramp2+/+
SvEv-Ramp2+/-
F
p<0.03
Volume (mm3)
Pituitary Gland Volume
G
p<0.01
Cells per 0.1 mm2
Anterior Pituitary Gland Cell Density
SvEv-Ramp2+/+
SvEv-Ramp2+/-

Figure 3.3 Accelerated mammary gland development in SvEv-*Ramp2*$^{+/-}$ female mice.

A, Fourth inguinal mammary glands of 18-wk-old SvEv-*Ramp2*$^{+/-}$ and wild-type virgin female mice were dissected, fixed in Carnoy's fixative, and stained with Carmine dye. Lymph nodes were used as a reference point; n = 15–20 mice per genotype. *Scale bars*, 1 mm (low magnification) and 100 µm (high magnification). **B**, Quantitation of ductal branch points within two independent 30-mm^2 areas per sample/animal. *, $P < 0.01$; n = 9 for SvEv-*Ramp2*$^{+/+}$, and n = 6 for SvEv-*Ramp2*$^{+/-}$ **C**, Quantitation of terminal end buds (TEBs) along a 5-mm peripheral duct. *, $P < 0.05$; n = 9 for SvEv-*Ramp2*$^{+/+}$, and n = 6 for SvEv-*Ramp2*$^{+/-}$.

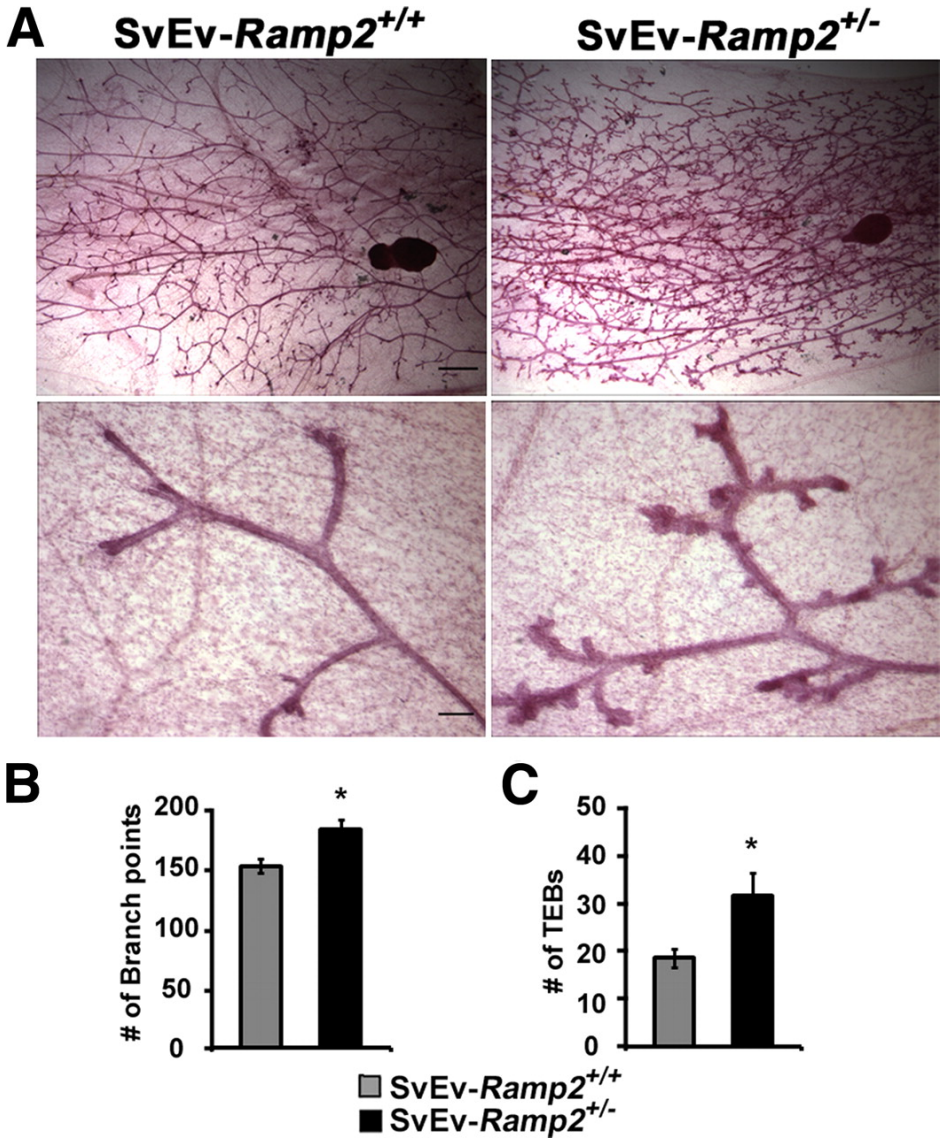


Figure 3.4 *Ramp2*$^{+/-}$ mice have skeletal abnormalities.

A–C, DEXA analyses of femurs showing decreased bone mineral content in milligrams (**A**), decreased bone mineral density in milligrams per square centimeters (**B**) (*, $P < 0.01$) and C) unchanged bone area in square centimeters (**C**); **D**, radiographs of tibiae; **E**, radiographs of femurs; **F**, micro-CT of midregion of femurs; **G**, 3D model of lumbar vertebrae; **H**, tibial and femoral bone volume (*, $P < 0.05$); **I**, skeletal staining by Alcian blue; Alizarin red (unmineralized bone and cartilage in blue, mineralized bone in purple). Animals used for the experiments in A–G are 18-wk-old virgin female SvEv-*Ramp2*$^{+/+}$ and SvEv-*Ramp2*$^{+/-}$ mice; n = 8–10 per genotype for DEXA, and n = 3 for micro-CT analysis and radiographs. Animals used in H and I are 2-d-old pups of SvEv-*Ramp2*$^{+/+}$ (n = 5) and SvEv-*Ramp2*$^{+/-}$ genotypes (n = 3).

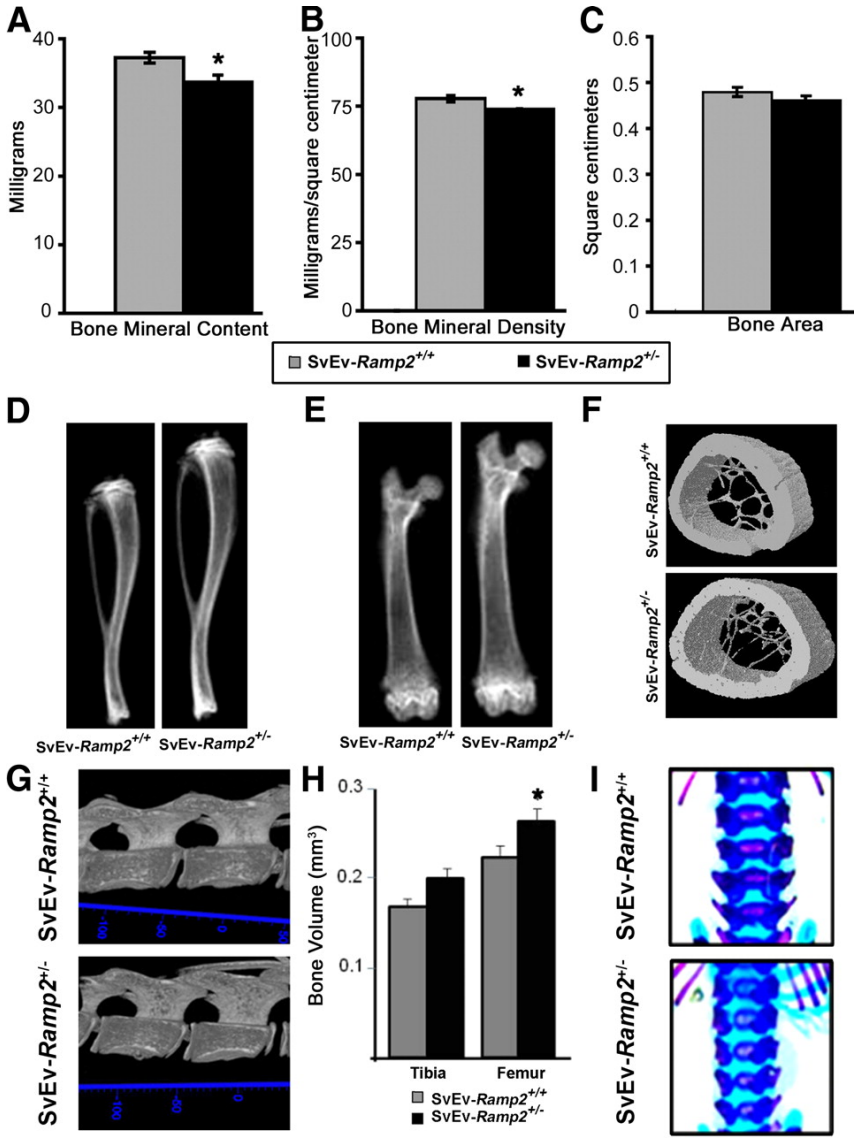
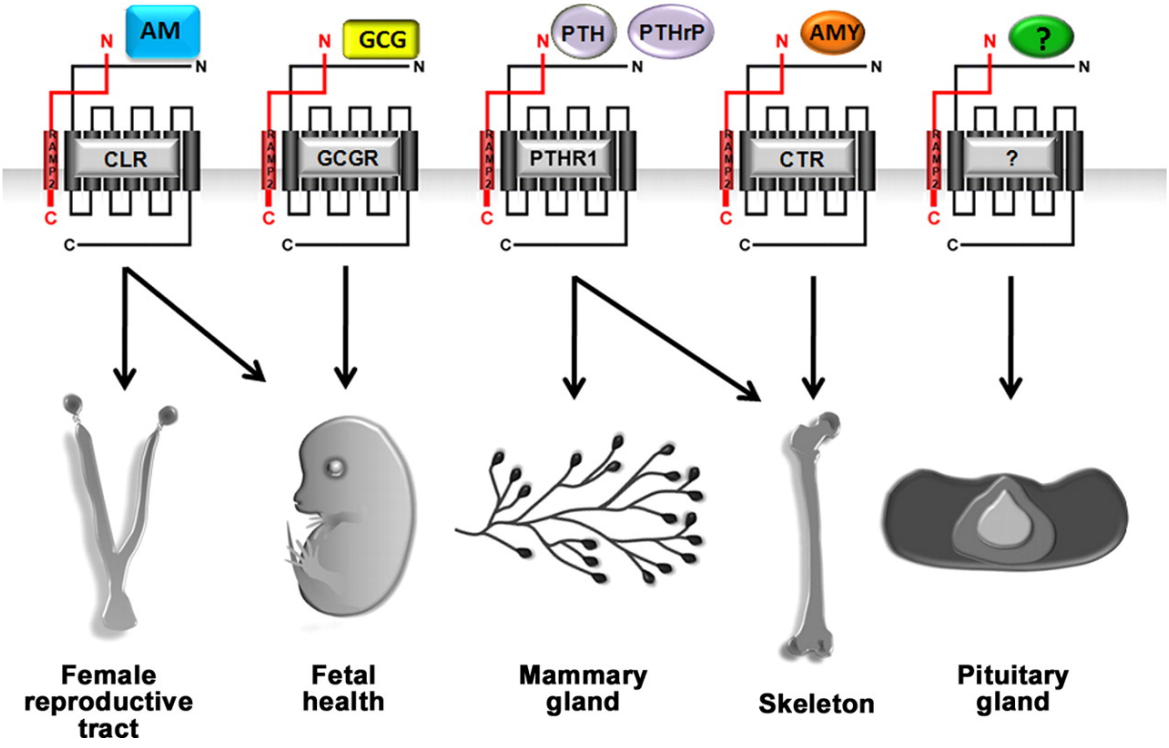


Figure 3.5 Proposed model for phenotypic outcomes in *Ramp2*$^{+/-}$ mice.

The constellation of phenotypes observed in SvEv-*Ramp2*$^{+/-}$ mice is likely due to a combined effect of reduced signaling for many GPCR and ligands and perhaps others that have yet to be identified.



CHAPTER 4

A ROLE FOR RECEPTOR ACTIVITY MODIFYING PROTEIN-2 (RAMP2) AND PARATHYROID HORMONE RECEPTOR 1 (PTHR1) IN PLACENTAL DEVELOPMENT

This research is a manuscript in preparation
Kadmiel M, Espenschied T, Lenhart T, Wilcockson H, Caron KM.
A role for receptor activity modifying protein-2 (RAMP2) and parathyroid hormone receptor 1 (PTHR1) in placental development.

ABSTRACT

Receptor activity-modifying protein-2 (RAMP2) is a single-pass transmembrane protein that regulates the trafficking, ligand binding and signaling of several G protein-coupled receptors (GPCRs). *In vitro*, *RAMP2* has been shown to interact with several GPCRs, including calcitonin receptor-like receptor (CLR), parathyroid hormone receptor (PTHR1), calcitonin receptor (CTR) and glucagon receptor (GCGR). The most well-characterized role of *RAMP2* is in the regulation of adrenomedullin (AM) binding to calcitonin receptor-like receptor (CLR) and our previous *in vivo* studies using knockout mouse models support this canonical signaling paradigm. However, we have also demonstrated previously that *Ramp2*$^{+/-}$ mice exhibit a constellation of endocrine-related phenotypes which are distinct from those of *Adm*$^{+/-}$ and Calcrl$^{+/-}$ mice, demonstrating that *RAMP2* has physiological functions beyond AM/CLR signaling. Here, we characterize *Ramp2*$^{-/-}$ placentas, and show a variety of phenotypes distinct from those of *Adm*$^{-/-}$ *and Calcrl*$^{-/-}$ placentas, further supporting an extended function of RAMP2 with other GPCRs. *In situ* hybridization of *Ramp2* in wild type placentas revealed robust expression in the fetal labyrinth layer. Consistent with this expression pattern, *Ramp2*$^{-/-}$ placentas had a thinner labyrinth layer with significantly fewer trophoblast cells and reduced proliferation compared to littermate *Ramp2*$^{+/-}$ placentas. Interestingly, this phenotype is not observed in *Adm*$^{-/-}$ *and Calcrl*$^{-/-}$ placentas, suggesting that other RAMP2-associated GPCRs may be involved. Because PTHR1 plays a role in proliferation of trophoblast cells, we hypothesized that the cause of placental phenotypes in

Ramp2$^{-/-}$ mice could be partially due to compromised PTHR1 signaling. Using placental extracts from *Ramp2*$^{-/-}$ and control mice, we discovered that the gene and protein expression of PTHR1 was reduced in *Ramp2*$^{-/-}$ *placentas* compared to controls. This work is the first *in vivo* evidence demonstrating an essential role for RAMP2 in placental development related to PTHR1 expression and function.

Introduction

The placenta forms the maternal-fetal interface, a site for nutrient supply, exchange of gases and waste uptake and is a critical organ for the successful outcome of pregnancy. A mature mouse placenta consists of three major layers: a maternal layer which attaches to the uterus, a junctional zone that connects the maternal part of the placenta to the fetus, and finally the fetal compartment, called the labyrinth (72). The labyrinth contains highly branched villi that are composed of trophoblast cells, stromal cells and blood vessels, and it is the site of nutrient exchange. The junctional zone is composed of spongiotrophoblast cells that form a supporting structure lining the labyrinth and the giant trophoblast cells dividing the maternal and the fetal compartments. The maternal side of the placenta, also referred to as the decidua, contains the maternal vasculature through which maternal blood enters the placenta. At mid gestation, spiral arteries of the decidua transform into low-resistance and high capacitance vessels to meet the increasing demands of the growing embryo. A balanced cross-talk between multiple proteins and different cell types is required for a successful pregnancy. Therefore, any perturbation in this balance can result in complications of pregnancy, such as ectopic pregnancy, intrauterine growth

restriction, preterm birth and preeclampsia. Although several studies using genetically engineered mouse models have provided much insight into the intricate communication that occurs at the maternal-fetal interface and have revealed key molecular players involved in placental development (73), there is still plenty to be understood.

Receptor activity modifying proteins (RAMP) are type1 membrane proteins that pass the membrane once. There are three mammalian RAMPs encoded by three different genes. RAMPs function to modify G-protein coupled receptor signaling in several ways: i) altering GPCR trafficking to and from the cell surface (1) or from recycling endosomes (6-7), ii) altering GPCR pharmacology by dictating ligand-binding specificity (1), and iii) influencing signaling downstream of GPCRs (3). Calcitonin receptor-like receptor (CLR), the first identified receptor partner for RAMPs, requires RAMPs for it to be trafficked to the plasma membrane. Depending on the RAMP expressed, CLR binds to different ligands. For example, CLR bound to RAMP1 forms a receptor for the neuropeptide, calcitonin gene related peptide (CGRP), while CLR bound to RAMP2 or RAMP3 forms adrenomedullin receptor 1 and adrenomedullin receptor 2 for the peptide hormone Adrenomedullin, respectively. Studies show that RAMPs associate with 7 other GPCRs: Calcitonin receptor (CTR), parathyroid hormone receptor 1 and 2 (PTHR1, PTH2R), type 1 receptor for vasoactive instestinal peptide (VPAC1), glucagon receptor (GCGR), secretin receptor (SCTR) and calcium sensing receptor (CaSR) (2-5); but their expression data (57) suggests a broader role than hitherto known. Since most studies identifying novel RAMP-interacting

GPCR receptor partners have not addressed the physiological significance of the GPCR-RAMP interaction, there is a greater need for investigating the functions of these interactions in normal and pathological conditions. Importantly, based on the wide expression of RAMPs and their drug tractable properties, RAMPs carry therapeutic potential for several disease conditions such as migraine, cardiovascular disease, diabetes, osteoporosis, inflammation, and female fertility (18-19, 22, 25, 42, 74).

In recent years, genetic mouse models of RAMPs have provided great insight on the broad roles of RAMP proteins. A comprehensive review of the various mouse models of RAMPs is described elsewhere (24). Briefly, loss of RAMP2 leads to embryonic lethality (17, 23). More recently, we have shown that haploinsufficiency of *Ramp2* disrupts endocrine homeostasis leading to problems such as subfertility and weakened skeletal system in female mice (25). Although RAMP1 and RAMP3 are not essential for survival, mice lacking *Ramp1* exhibit mild phenotypes involving cardiovascular and inflammatory processes (42), while *Ramp3*$^{-/-}$ mice exhibit a lean phenotype in their old-age (17).

In this study, we utilized *Ramp2*$^{-/-}$ mice to evaluate the role of RAMP2 in placental development. Additionally, we hypothesized that RAMP2 regulates PTHR1 function in the placenta and tested the physiological importance of PTHR1-RAMP2 association. Our findings demonstrate an essential role for RAMP2 in placental development. RAMP2 is required for proliferation of trophoblast cells and for the successful remodeling of spiral arteries. Surprisingly,

loss of RAMP2 compromises PTHR1 expression levels, suggesting an additional, unique layer of regulation of GPCR signaling by a RAMP.

Materials and Methods

Animals: *Ramp2 null* mice were generated and characterized as described previously (17). Genotyping was performed as recently described in the article that characterizes haploinsufficiency of *Ramp2* in the female endocrine system (25). Although the Ramp2 knockout phenotype is conserved in SvEV129/6-TC1 mice and in C57Bl6 mice, the current study involves the characterization of *Ramp2 null* placentas generated on SvEV129/6-TC1 background. To obtain timed-pregnancies, mice were allowed to mate and the morning of vaginal plug detection was considered to be embryonic day 0.5 (E0.5d). Placentas from littermate controls were used in all studies. Amniotic fluid was collected from E13.5 to E14 days embryos by placing the embryo with its yolksac intact in an eppendorf tube and then gently piercing the yolk sac with a fine needle. This allowed the amniotic fluid to drain into the collection tube. Samples were snap frozen on dry ice and stored at -80C until assayed. Calcium measurements were performed as previously described (25). All experiments performed on animals were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Histology, immunohistochemistry and microscopy: The antibodies used in this study are anti-cytokeratin (Dako), anti-BrdU (Zymed), anti-Ki-67 (Dako), anti α-smooth muscle actin (Sigma). All tissues were fixed in 4% paraformaldehyde

overnight, cryoprotected in 30% sucrose overnight, embedded in OCT (Tissue-Tek) and serially sectioned at 8-10µm. Sections were rehydrated in PBS, permeablized in 0.2% Triton-X100, blocked in 4%BSA and incubated in primary antibody overnight. After washing, sections were incubated in appropriate secondary antibody for 1-2hours, washed and mounted for imaging. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER charge-coupled device camera with Metamorph software (Molecular Devices Corp.) and processed with Photoshop.

In situ hybridization: Placentas from embryonic day 14.5 from wildtype 129S6/SvEv intercrosses were collected and processed same as the tissue for immunohistochemistry except that the PFA and sucrose solutions were prepared in diethylypyrocarbonate treated phosphate buffered saline. Placentas were serially sectioned at 16µm thickness. Non-radioactive in situ hybridization was performed as previously described (53) using sense and antisense RNA probes synthesized from plasmids containing cDNA sequences of mouse *Ramp2*.

RNA extraction, cDNA synthesis and Quantitative RT-PCR analyses: RNA was extracted using the Trizol method following the manufacturer's protocol (Ambion, Austin, Texas). Isolated RNA was DNase treated and reverse transcribed using the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, California) and gene expression was measured by MX3000 Q-PCR machine from Stratagene, Santa Clara, California. TaqMan probes purchased from Applied Biosystems (Carlsbad, California) were used to measure the gene expression of *Pth1r,* catalog # Mm00441046_m1. For the rest of the genes

tested, SYBR green master mix purchased from Sigma (St. Louis, Missouri), catalog# 4438, was used. Primer sequences were obtained from previously published references for the following genes: plasma membrane Ca2+ ATPase 1 (PMCA1), Mm_PMCA1_F(CGCCATCTTCTGCACCATT), Mm_PMCA1_R (CAG CCATTGCTCTATTGAAAGTTC) (75); transient receptor potential vanilloid 5 channel (TRPV5), Mm_TRPV5_F(ATTGACGGACCTGCCAATTACAGAG), Mm_TRPV5_R(GTGTTCAACCCGTAAGAACCAACGGTC) (76); transient receptor potential vanilloid 6 channel (TRPV6), TRPV6_F (ATCGATGGCCCTGCGAACT), TRPV6_R(CAGAGTAGAGGCCATCTTG TTGCTG) (76); Calbindin-D_{9k}, Calb-D9K_F(TCCTGCAGAAATGAAGAGC A), Calb-9K_R(GTGCTGTTGAACTCCTTCT) (76); Calbindin D_{28k} Calb-D28K_F(AACTGACAGAGATGGCCAGGTTA), Calb-D28K_R (TGAACTCTTT CCCACACATTTTGAT) (77); vitamin D receptor (VDR), VDR_F(AACGCTATGACCTGTGAAGGC), Mm_VDR_R(CCTGTACTTACG TCTGCACGA) (78); 1α-hydroxylase, 1AH_F(CCAGAGCGCTGTAGTT TCTCATCA), 1AH_R(ATGAAGGTTTCTGTGTCAGGAGGG) (79); and Na-Ca exchanger, NCX1_F(TCCCTACAAAACTATTGAAGGCACA), NCX1_R (TTT CTCATACTCCTCGTCATCGATT) (75). m*GAPDH (25)* was used as a normalizing gene and $\Delta\Delta C_T$ method was used to analyze the relative levels of gene expression (80).

SDS-page and western blotting: Placental extracts were quantified by BCA assay to determine the protein concentration. Samples were lysed in sample buffer, boiled for 5 minutes at 100 degrees centigrade and then loaded onto a 4-

12% polyacrylamide gel and resolved by SDS-PAGE. Protein was then transferred to a nitrocellulose membrane which was subsequently blocked in 5% non-fat dry milk overnight at 4 degrees. The membranes were incubated with the primary antibodies (PTHR1 at 1:100 dilution; from Covance, Princeton, NJ and GAPDH at 1:1000 dilution from Sigma, Saint Louis, MO) overnight 4 degrees centigrade and detected with secondary antibodies as described previously (18). The blot was imaged with the Odyssey infrared scanner and data was obtained using scanner software.

***Statistical Analysis*:** All quantitative analyses were performed using unpaired, two-tailed Student's t-test. Data are represented as standard error of mean (SEM) unless indicated otherwise. Differences were considered significant when the p value was lower than 0.05.

Results

Ramp2 is robustly expressed in the labyrinth layer

To assess the localization and extent of expression of *Ramp2* in the mouse placenta, we performed in situ hybridization on E14.0 *wild type* placentas. As shown in **Fig.**4.1A, there was a robust expression of *Ramp2* in the fetal compartments compared to diffused and weak signal in the maternal decidual regions of the placenta. More specifically, *Ramp2* expression noticed was evident in the chorionic plate, labyrinth, spongiotrophoblast cells and in the giant trophoblast cells, with robust expression in the trophoblast cells of the labyrinth

layer (**Fig.**4.1B). These results suggest a critical role for RAMP2 in the fetal compartment of the placenta and specifically in the labyrinth.

Ramp2$^{-/-}$ placentas exhibit abnormal development of the labyrinth layer

To investigate the consequence of the loss of RAMP2 in placental development, we performed histological analyses of placental sections obtained from timed-pregnancies of *Ramp2*$^{+/-}$ intercrosses. Hemotoxylin and eosin staining of E13.5 placentas revealed the presence of all the layers of the placenta (**Fig.** 4.2A & B). However, the labyrinth layer of the *Ramp2*$^{-/-}$ placenta had distinctly less cellularity, as evidenced by the relative reduction of eosin staining compared to *Ramp2*$^{+/+}$ placentas. Morphometric evaluation of stained sections showed no change in the labyrinth width, but a significant decrease in the thickness of *Ramp2*$^{-/-}$ labyrinth layer compared to that of littermate controls (**Fig.** 4.2.C & D).

Numerous mutant mouse models have been described with a “small labyrinth”, in which the phenotype is manifested by a reduction in labyrinth trophoblast cell number, often leading to fetal growth restriction or death of the fetus due to improper nutrient exchange (reviewed in (81)). We therefore hypothesized that the thin labyrinth layer in *Ramp2*$^{-/-}$ placentas could be caused by fewer trophoblast cells. To test this, we performed immunohistochemistry using an anti-cytokeratin antibody that specifically marks trophoblast cells. We found a markedly reduced density of trophoblast cells in the *Ramp2*$^{-/-}$ placentas compared to their littermate controls (**Fig.**4.2E & F). We also quantified labyrinth

trophoblast cells from E14 day *wild type* and *Ramp2*$^{-/-}$ placentas, based on histological identification. As shown in **Fig** 4.2G, *Ramp2*$^{-/-}$ placentas indeed had fewer trophoblasts in the labyrinth region compared to the controls. These data indicate a role for *RAMP2* in the regulation of trophoblast cell number and also in the proper formation of the labyrinth layer.

Ramp2 plays a role in proliferation of trophoblast cells

To determine if the decreased thickness of the labyrinth and reduced numbers of trophoblast cells were due to a failure of trophoblast cells to proliferate at the normal rate, we performed BrdU incorporation studies in *Ramp2*$^{+/-}$ pregnant mice bred with RAMP2$^{+/-}$ males at day 14 of gestation. Immunohistochemistry with anti-BrdU antibody revealed a striking change in proliferation between wild type and knockout placentas. Particularly, the labyrinth layer of *Ramp2*$^{-/-}$ placentas had a marked reduction in signal compared to their controls (**Fig.**4.3A & B). Consistently, as shown in **Fig.**4.3C & D, Ki-67 staining also showed reduced signal in the labyrinth of *Ramp2*$^{-/-}$ placentas compared to the wild type littermate placentas. Taken together, these results demonstrate that RAMP2 plays an important role in proliferation of trophoblast cells of the labyrinth layer.

***Ramp2* does not influence apoptosis in the placenta**

To eliminate the possibility of increased apoptosis as a factor contributing to the reduction in trophoblast cells, we performed QRT-PCR to determine the expression of pro-apoptotic *Bax* and anti-apoptotic *Bcl2*. The ratio of *Bax* to *Bcl2*

did not differ between the two genotypes (**Fig.**4.3E). Our results demonstrate that loss of RAMP2 in the developing placenta does not alter apoptosis.

Loss of *Ramp2* results in failed spiral artery remodeling

Remodeling of maternal spiral arteries into low resistance, high capacitance vessels is a critical process for maintaining appropriate placental function and promoting fetal health in both rodents and humans. A failure of spiral arteries to remodel is considered a hallmark pathophysiological feature of preeclampsia. Using a smooth muscle actin antibody, we stained decidual sections of *wild type* and *Ramp2*$^{-/-}$ placentas in order to evaluate the extent of vascular smooth muscle coverage surrounding the maternal spiral arteries. As expected, the spiral arteries of *wild type* placentas progressively lost their smooth muscle coverage as they approved the maternal-fetal interface. However, interestingly, there was a prominent amount of smooth muscle coverage left on the spiral arteries of *Ramp2*$^{-/-}$ placentas (**Fig.**4.4A&B).

More interestingly, the spiral artery phenotype of *Ramp2*$^{-/-}$ placentas phenocopies that of *Adm*$^{-/-}$ and *Calcrl*$^{-/-}$ placentas strongly suggests that AM signaling is involved in spiral artery remodeling. Uterine natural killer (uNK) cells have been implicated in spiral artery remodeling. Several studies suggest that factors released by uNK cells trigger spiral arteries to remodel (82-84). Consistent with a failure of spiral arteries to remodel, *Adm*$^{-/-}$ placentas and *Calcrl*$^{-/-}$ placentas show a remarkable decrease in uNK cell number (Li M and Caron K et al, manuscript in preparation). Therefore, to determine if this phenotype was

conserved in *Ramp2*$^{-/-}$ placentas, we performed IHC of DBA lectin on E14.0 *Ramp2*$^{-/-}$ and WT placentas generated from *Ramp2*$^{+/-}$ intercrosses. Contrary to the phenotype observed in *Adm*$^{-/-}$ and *Calcrl*$^{-/-}$ placentas, *Ramp2*$^{-/-}$ placentas had no change in uNK cell number compared to WT littermate placentas (**Fig.**4.4C-E).

Taken together, our results show that RAMP2 is required for maternal spiral artery remodeling in a manner that is independent of uNK cell recruitment and likely distinct from canonical AM/CLR signaling. In addition, the marked labyrinth phenotype of *Ramp2*$^{-/-}$ placentas, which is not observed in *Adm*$^{-/-}$ *and Calcrl*$^{-/-}$ placentas, strongly indicates the involvement of one or more RAMP2-interacting-receptor(s), besides CLR, in the normal development of the labyrinth layer.

Influence of *Ramp2* on the gene expression of RAMP2-interacting GPCRs

To elucidate which putative GPCRs might be involved in the *Ramp2*$^{-/-}$ placenta phenotype, we performed QRT-PCR on numerous GPCRs that have previously been described to interact with RAMP2 (**Fig.**4.5). We found a robust and statistically significant increase in *Calcrl* gene expression, which is entirely consistent with a potential homeostatic up-regulation of AM signaling components in the absence of RAMP2 and with the presence of excessive vascular smooth muscle cells in *Ramp2*$^{-/-}$ placentas, which express high levels of *Calcrl*. We also observed a modest, yet significant, increase in glucagon receptor; however a function for this receptor in placental development has not

been previously appreciated. Most importantly, we found a dramatic 50% reduction in the expression of Pthr1, the receptor for PTH and PTHrP, which has numerous well-characterized roles in trophoblast proliferation and placental development (85-87).

Loss of *Ramp2* alters PTHR1 protein levels

To determine whether the reduced levels of *Pthr1* mRNA correlated with reduced expression, we determined the levels of PTHR1 in *Ramp2*$^{-/-}$ knockout placentas using both western blotting and immunofluorescence. Consistent with the mRNA data, we found a reduction in protein levels in placental lysates by immunoblotting (**Fig.**4.6A). Immunohistochemistry for PTHR1 further showed that this receptor is highly expressed in the labyrinth layer and that *Ramp2*$^{-/-}$ placentas (**Fig.**4.6C&E) had considerably less staining compared to *wild type* placentas (**Fig.**4.6B&D). Our results provide compelling evidence that loss of RAMP2 alters *Pthr1* gene and protein expression in the placenta. Moreover, this data suggests that the possible explanation for abnormal development of the labyrinth in *Ramp2*$^{-/-}$ mice is the loss of RAMP2 in the placenta compromising the function of PTHR1.

Functional changes downstream of PTHR1 signaling

Since PTHR1 signaling plays a critical role in maternal-fetal calcium transfer (86, 88-89), we measured amniotic fluid calcium content in E14.0 day *Ramp2*$^{-/-}$ yolk sacs and their control littermates; a day before *Ramp2*$^{-/-}$ embryos die due to lymphatic vascular defects. As shown in **Fig.**4.6F, we noticed no

significant change in calcium content between the two genotypes. This finding was not unexpected since the window of active materno-fetal calcium transfer occurs from E16.5 to birth, such that the E14.5 lethality of Ramp2$^{-/-}$ embryos precluded our ability to address this. Nevertheless, we hypothesized that calcium handling genes, which are targets of PTHR1 signaling, would be expressed by E14.5 days of gestation. Therefore, we performed QRT-PCR on WT and *Ramp2*$^{-/-}$ placentas to test the expression of plasma membrane Ca2+ ATPase 1 (*Pmca1*), transient receptor potential vanilloid 5 channel (*Trpv5*), transient receptor potential vanilloid 6 channel (*Trpv6*), Calbindins- D_{9k} and D_{28k}, vitamin D receptor (*Vdr*), 1α-hydroxylase and Na-Ca exchanger. Expression of Calbindin-D9k, VDR and TRPV5 was below detectable levels in placentas of all genotypes. TRPV6 and Calbindin-D_{28k} were expressed but showed no differences between *wild type* and *Ramp2*$^{-/-}$ placentas (data not shown). Interestingly, the plasma membrane Ca2+ ATPase 1, *Pmca1* expression was significantly elevated in *Ramp2*$^{-/-}$ placentas compared to their littermate controls (**Fig.**4.6G). Taken together, our results suggest a compensatory mechanism as a consequence of compromised PTHR1 function, wherein the placenta up-regulates production of plasma membrane Ca2+ ATPase 1 channels in the absence of RAMP2 to maintain homeostasis of placental calcium transfer.

Discussion

The role of RAMP2 in embryogenesis and in the adult female endocrine system has been previously determined (18, 25). However, the role of RAMP2 in placental development has not been understood. Therefore, the goal of this study

was to identify the role of RAMP2 in placentation. In this study, we reported the first evidence that RAMP2, particularly of fetal origin, is essential for normal development of the placenta. Moreover, our study is the first to associate a critical role for a RAMP protein in placental formation. Consistent with the spatial localization of *Ramp2*, loss of *Ramp2* led to a defective labyrinth. *Ramp2*$^{-/-}$ placentas generated from intercrosses of *Ramp2*$^{+/-}$ mice exhibited a 40% reduction in thickness of the labyrinth layer, compared to their WT littermate placentas. This decrease in thickness is likely due to reduced trophoblast cell numbers in the labyrinth. Moreover, as evidenced by BrdU incorporation studies and Ki67 labeling, loss of RAMP2 significantly reduced the proliferation of these trophoblast cells. The absence of labyrinth abnormalities in placentas null for the canonical receptor partner of RAMP2- CLR, or the ligand AM, suggested to us that RAMP2 is associating with another receptor to mediate its role in the labyrinth layer.

Our studies provide evidence for a role for *RAMP2* in preeclampsia. A hallmark sign of PE is a failure of the maternal arteries to shed their smooth muscle coverage to become low resistance vessels with increased capacitance (90). Although *Ramp2*$^{+/-}$ pregnant dams with *Ramp2*$^{-/-}$ embryos do not develop preeclampsia, our studies demonstrate that RAMP2 is involved in spiral artery remodeling, thereby having implications in the pathology of preeclampsia. The most conceivable explanation for the dams not developing PE is because the knockout embryos die embryonically, perhaps before the onset of PE. No change in uNK cell number suggests that there could be alternate mechanism(s) that

govern spiral artery remodeling. It is worth mentioning that this absence of uNK cell phenotype is not conserved in *Adm*$^{-/-}$ and *Calcrl*$^{-/-}$ mice, implying CLR-independent roles for RAMP2 in spiral artery remodeling.

Parathyroid hormone (PTH), its related peptide (PTHrP), and their common receptor PTHR1 are all shown to be expressed in the placenta. Elegant studies performed by Kovacs and colleagues demonstrate the role of PTH and PTHrP in maternal-fetal calcium transfer (86, 88) PTHrP is produced by placental trophoblast cells (91) and has been shown to mediate trophoblast cell proliferation (87). Comparative study of *Ramp2*$^{-/-}$ mice and other mouse models of PTHR1 signaling prompted us to hypothesize that PTHR1 is interacting with RAMP2 to regulate trophoblast cell proliferation and placental calcium transfer. Our data revealed a decline in PTHR1 mRNA and protein levels in the placentas lacking RAMP2. This data is consistent with our hypothesis and further supports the biochemical studies (3) as well as our *in vivo* studies (25) that RAMP2 associates not only with CLR, but with an additional GPCRs *in vivo*. Additionally, our data provides the first evidence that a RAMP protein can regulate GPCR mRNA and protein expression.

Our data showing a change in gene expression of Plasma membrane calcium ATPase 1 suggests compensation to maintain homeostasis of maternal calcium transfer. While we do not notice a change in maternal fetal calcium transfer, as measured by amniotic fluid content, it is likely possible that PTHR1

function in calcium transfer across the placenta would be compromised in *Ramp2*$^{-/-}$ placentas if the embryos had survived beyond mid gestation.

Taken together, this study elucidates the essential role of *RAMP2* in placental development and demonstrates the involvement of RAMP2 in PTHR1 regulation and function. Understanding how RAMP2 regulates PTHR1 gene and protein expression and the physiological outcome of PTHR1-RAMP2 interaction still remains complex. Future studies using *in vitro* approaches in trophoblast cell lines or different mouse models can elucidate the precise significance of this interaction. The previously established therapeutic role of PTH and PTHrP in various diseases like osteoporosis (92-93) and cancer (94-95) further emphasize the significance of understanding the physiological outcome of PTHR1-RAMP2 interaction.

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Figure 4.1 Expression of *Ramp2* in the placenta

In situ hybridization of *Ramp2* in WT embryonic day 14 placentas generated from wild type intercrosses. In situ hybridization reveals that *Ramp2* is robustly expressed in the labyrinth layer of the placenta. **A**) Lower magnification; scale bar = 100 microns. **B**) Higher magnification in the labyrinth; scale bar = 50 microns. Inset images at both magnifications show sense control probes. cp = chorionic plate, lb = labyrinth, sp = spongiotrophoblast cells, dec = decidua; arrows point to giant trophoblast cells.

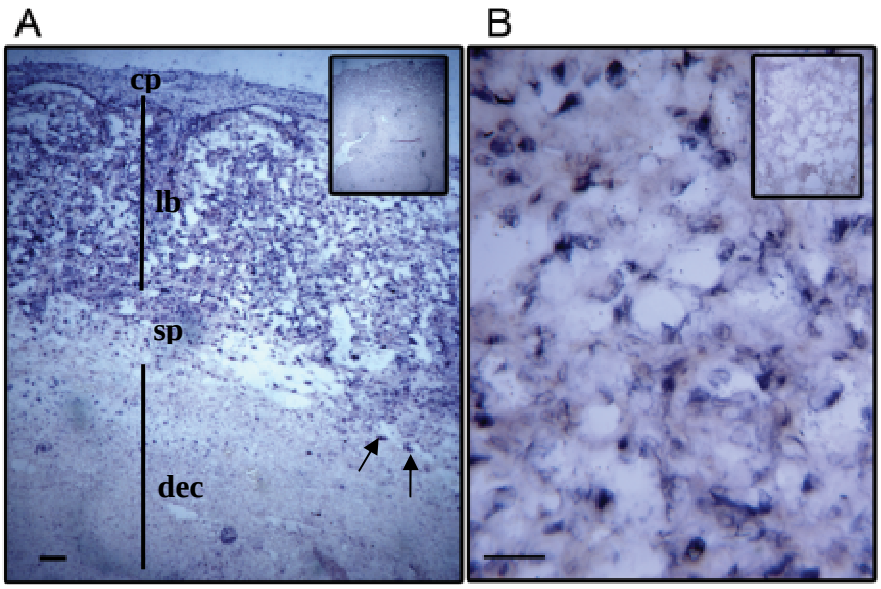


Figure 4.2 RAMP2 is essential for normal development of the labyrinth layer.

Histology and IHC of embryonic day 14 placentas of WT and *Ramp2*$^{-/-}$ littermates generated from *Ramp2*$^{+/-}$ intercrosses. Hematoxylin and eosin stains reveal different layers of the placenta of WT (**A**) and *Ramp2*$^{-/-}$ (**B**) mice; L=labyrinth, J=junctional zone, D=decidua, scale bar = 1mm; Quantification of width of the labyrinth reveals no change between the WT (gray bar) and *Ramp2*$^{-/-}$ (black bar) placentas (**C**); However, labyrinth thickness is significantly decreased in *Ramp2*$^{-/-}$ placentas (black bar) compared to the WT littermate placentas (gray bar) with a p value <0.001 (**D**); Immunohistochemistry of WT (**E**) and *Ramp2*$^{-/-}$ (**F**) placentas using antibodies against pan-cytokeratin (green) marking trophoblast cells, scale bar = 500µm; Quantification of trophoblast cell counts in the labyrinth layer (**G**) shows a significant decrease in trophoblast cell number in *Ramp2*$^{-/-}$ placentas (black bar) compared to WT placentas (gray bar) with a p value <0.01.

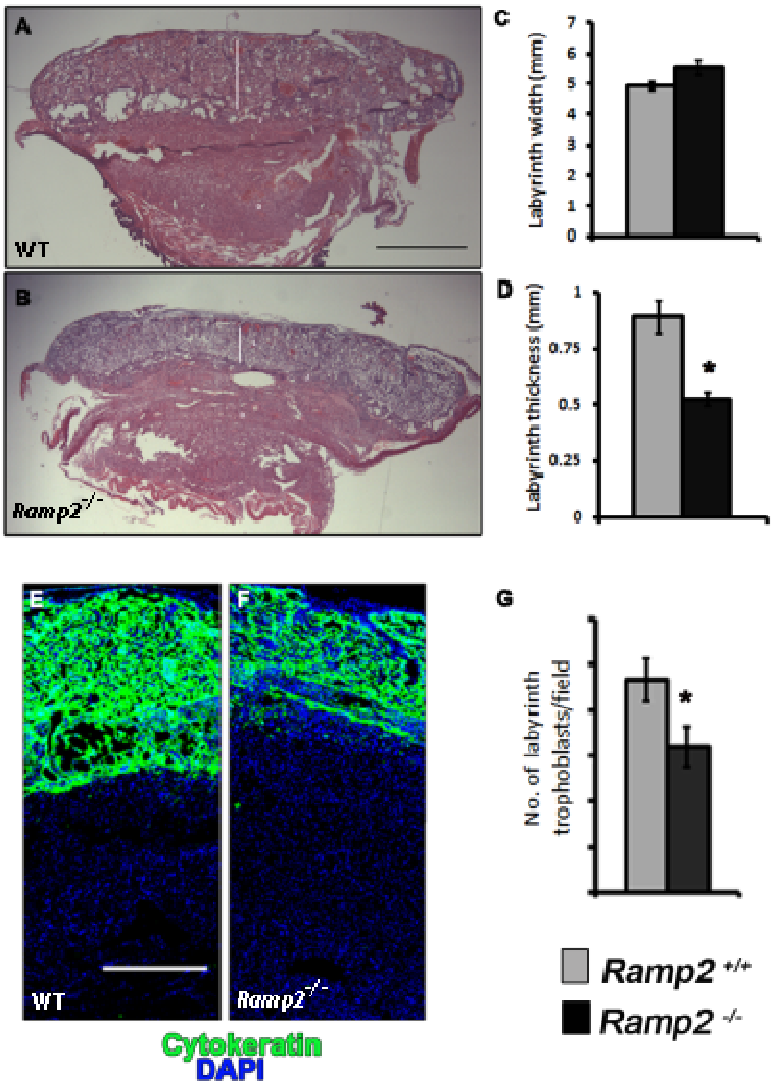


Figure 4.3 Loss of RAMP2 results in a proliferation defect in the labyrinth layer.

Immunohistochemistry of E14day placentas generated from *Ramp2*$^{+/-}$ intercross using antibodies against BrdU (white) and Ki67 (red, merge with DAPI is pink) to evaluate proliferation. *Ramp2*$^{-/-}$ placentas (B&D) show a remarkable decline in proliferation, primarily in the labyrinth region, compared to their controls (A&C), scale bars = 500µm; QRT-PCR revealed no significant changes between the WT (gray bar) and *Ramp2*$^{-/-}$ (black bar) placentas in apoptosis as measured by the ratio of anti-proliferative *Bax* and anti-apoptotic *Bcl2* genes normalized to *Gapdh* (E).

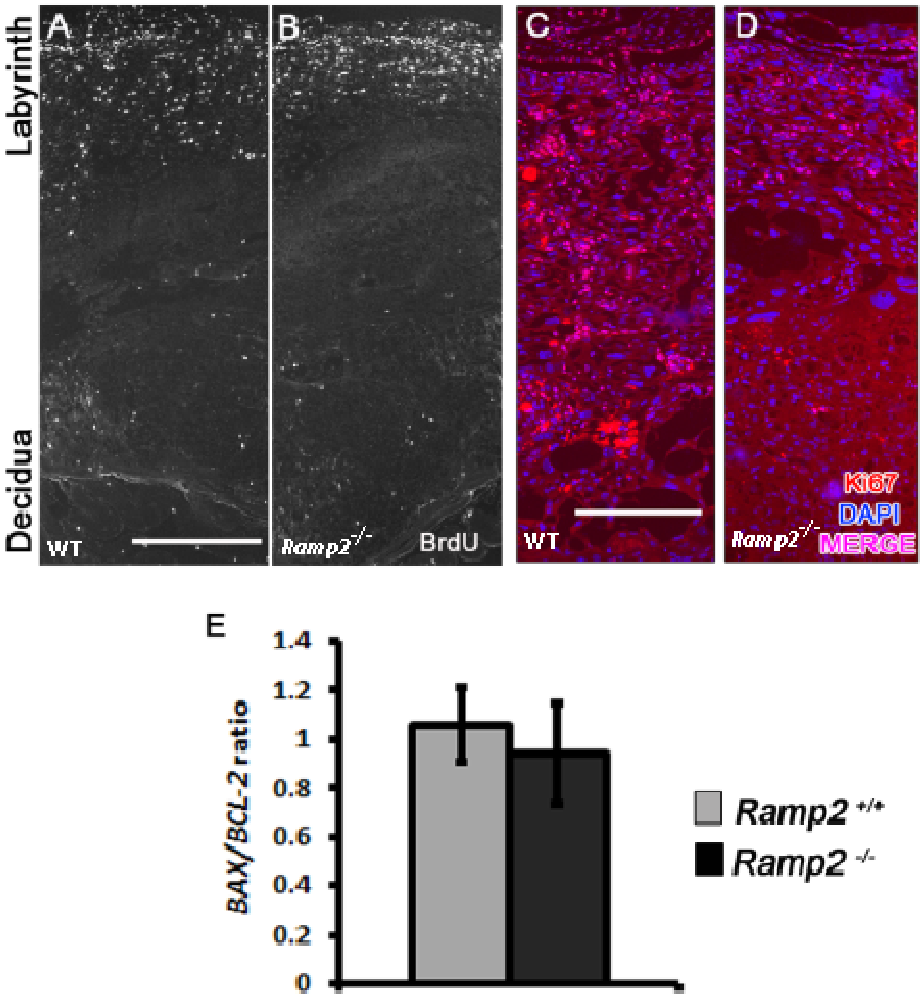


Figure 4.4 Spiral artery defects in *Ramp2*$^{-/-}$ placentas.

Immunohistochemistry of E14days placentas generated from *Ramp2*$^{+/-}$ intercrosses to evaluate spiral artery remodeling and uNK cell number. Staining of WT (**A**) and *Ramp2*$^{-/-}$ (**B**) placentas with antibodies against smooth muscle actin revealed the presence of marked smooth muscle coverage (yellow) still remaining around spiral arteries in *Ramp2*$^{-/-}$ placentas, whereas WT littermate placentas have lost most of their smooth muscle coverage; scale bar = 100µm. DBA lectin staining of uNK cells (green) revealed no change in *Ramp2*$^{-/-}$ placentas (**D**) compared to their littermate controls (**C**), scale bar = 100µm; Quantification of uNK cell number from DBA lectin stained sections (**E**) revealed no change between the number of uNK cells between the WT (gray bar) and *Ramp2*$^{-/-}$ (black bar) placentas;

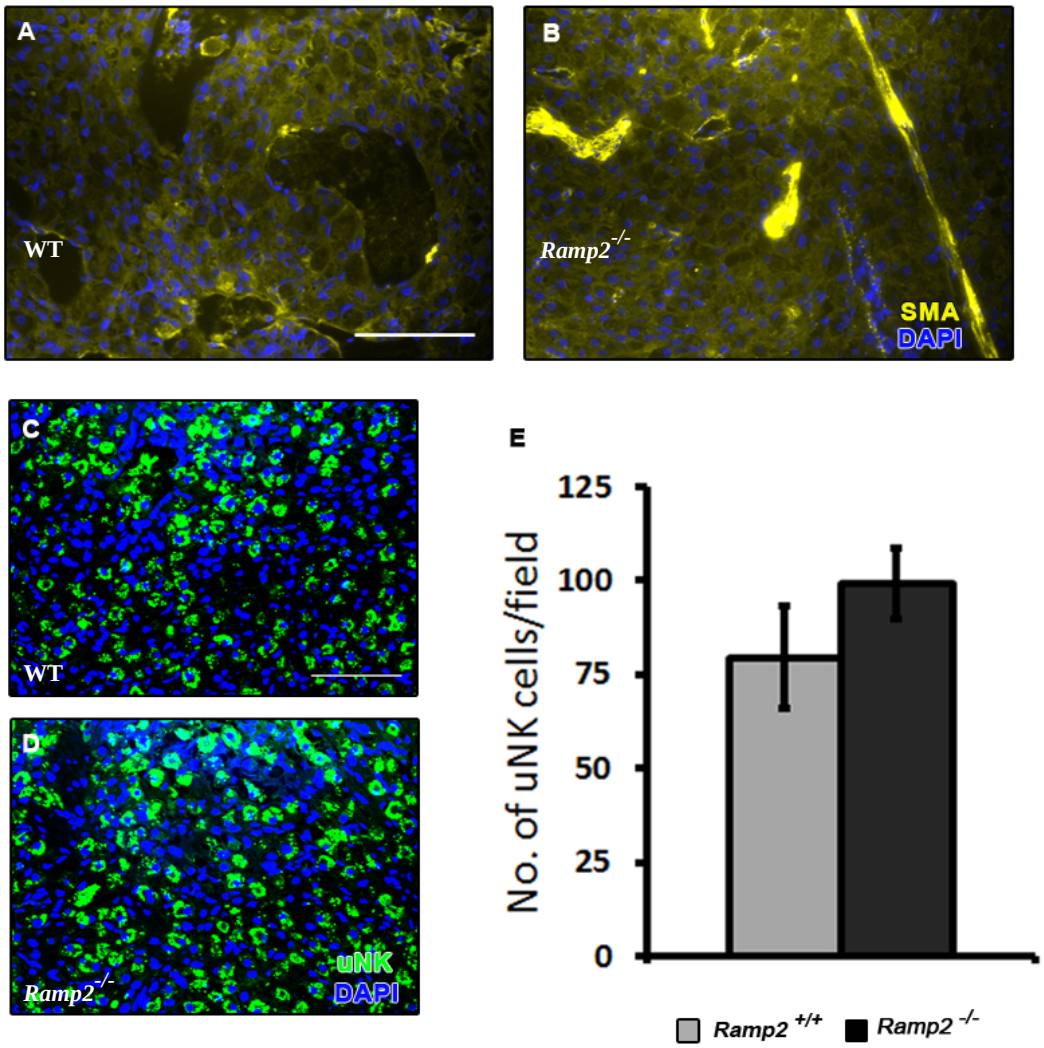


Figure 4.5 Loss of RAMP2 affects mRNA expression of RAMP2-interacting GPCRs.

mRNA expression of RAMP2-interacting GPCRs from WT and *Ramp2*$^{-/-}$ placentas of E13.5 day; *Pthr1* = parathyroid hormone receptor1, *Calcrl* = calcitonin receptor-like receptor, *Ctr* = calcitonin receptor, *Gcgr* = glucagon receptor, *Vpac1r* = vasoactive intestinal peptide receptor 1. QRT-PCR analyses using TaqMan probes revealed a significant reduction in *Pth1r* levels in *Ramp2*$^{-/-}$ placentas (black bar) compared to the WT (gray bar) controls, a significant increase in *Calcrl* was observed in the knockout placentas (black bar) compared to the WT controls, p value < 0.01;

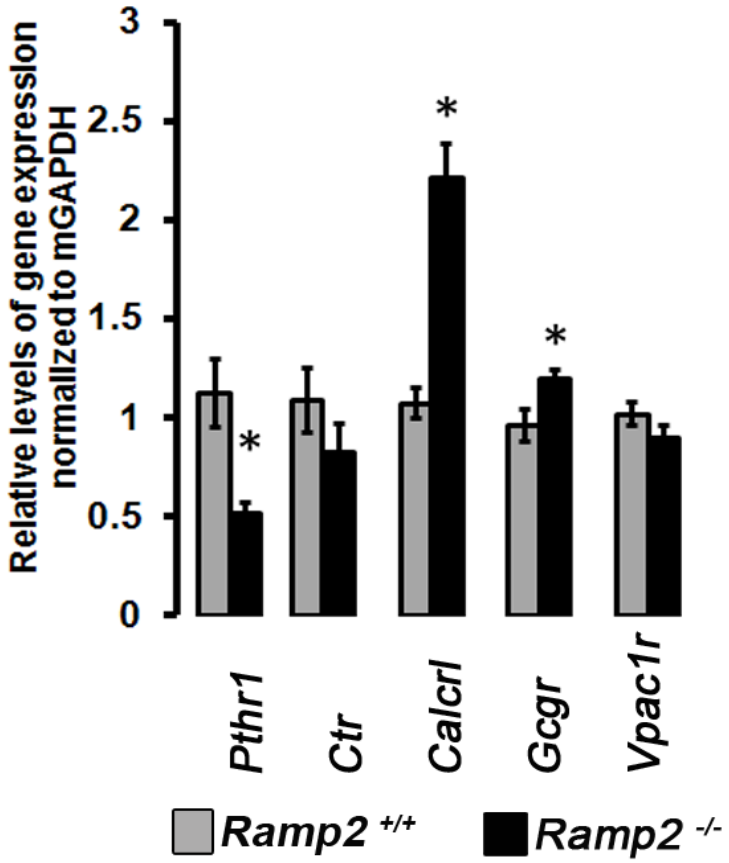
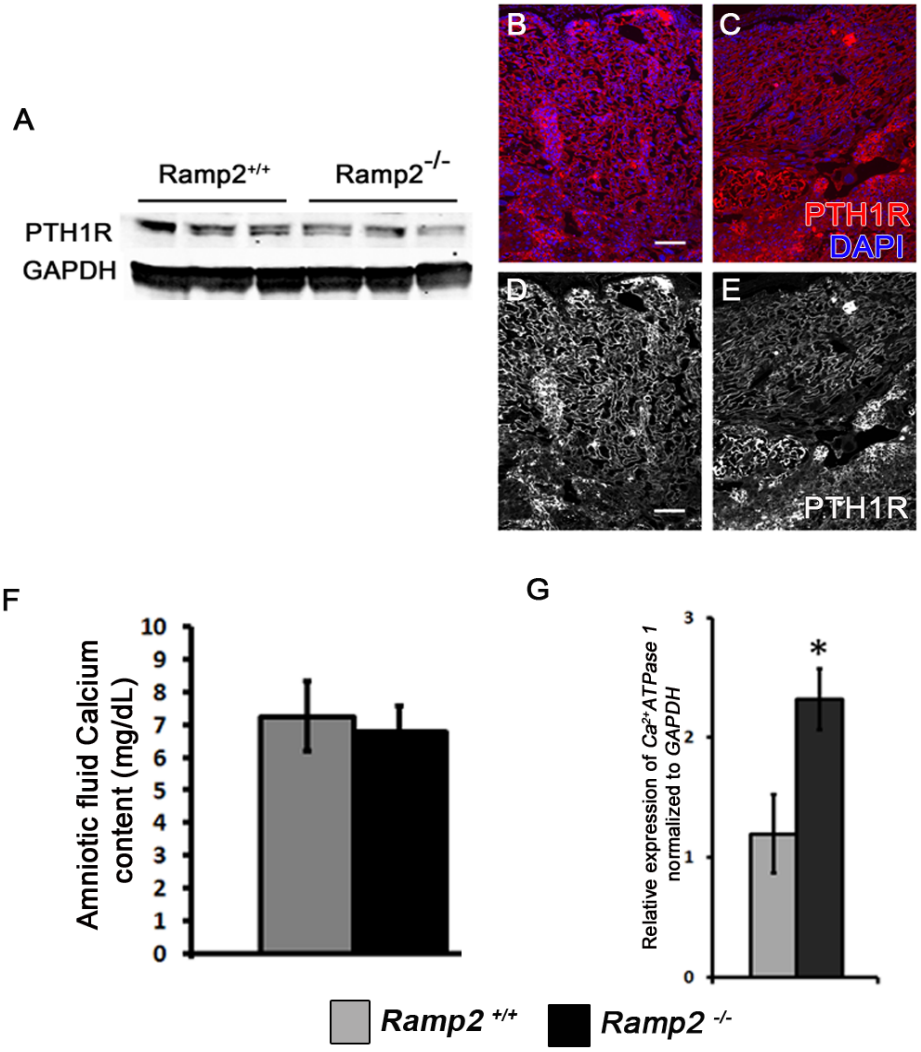


Figure 4.6 Loss of RAMP2 affects PTH1R protein expression and function

A) Evaluation of PTH1R levels by immunoblotting in E 13.5day placentas from WT and *Ramp2*$^{-/-}$ mice reveals a modest reduction in PTH1R expression in tissue lysates from *Ramp2*$^{-/-}$ placentas compared to their controls; Each lane represents an individual placenta; GAPDH was used as the loading control. Immunohistochemistry using antibodies against PTH1R reveals a modest reduction of PTH1R-specific signal (red in B&C and white in D&E) in *Ramp2*$^{-/-}$ (**C&E**) placentas compared to their controls (**B&D**); scale bars = 100µm. Measurement of calcium content in the amniotic fluid of WT (gray bar) and *Ramp2*$^{-/-}$ (black bar) embryos reveals no change (**F**). QRT-PCR analyses revealed a significant increase in gene expression of plasma membrane calcium ATPase 1 (*Pmca1*) in *Ramp2*$^{-/-}$ placentas (black bar) compared to their WT (gray bar) littermate controls (**H**); p value <0.05.



CHAPTER 5

GENERAL DISCUSSION

Overview of results

The goal of the research presented in this dissertation is to understand the physiological role of RAMP2 in the female endocrine system. To this end, we have employed a gene targeted mouse model of *Ramp2.* Previous studies from our laboratory have demonstrated that loss of *Ramp2* results in midgestational embryonic lethality associated with abnormalities in the lymphatic vascular system (18). These studies have demonstrated an essential role for RAMP2 in embryonic vascular development. However, we were also interested in determining whether RAMP2 may play important physiological functions during adulthood. The research presented in **Chapter 3** of this dissertation addresses the role of RAMP2 in adult mice, utilizing mice heterozygous for *Ramp2*. We have identified previously unknown functions of RAMP2 in the female endocrine system. In particular, we found that haploinsufficiency of *Ramp2* in female mice leads to intrauterine growth restriction, fetal demise and severe postnatal lethality. Furthermore, we have shown that female *Ramp2*$^{+/-}$ mice develop mild hyperprolactinemia, hyperplastic anterior pituitary glands, precociously developed mammary glands and a poorly mineralized skeletal system. It is important to recognize that these endocrine phenotypes are not observed in mice that are haploinsufficient for *Adm* or *Calcrl*; thus supporting the notion that RAMP2 has important physiological functions that extend beyond the canonical AM/CLR signaling paradigm. Although the precise mechanisms underlying these endocrine phenotypes requires further investigation, our studies have

substantially driven the field forward by elucidating novel functions of RAMP2 in numerous endocrine organs.

Additional studies, presented in **Chapter 4**, made use of tissues that are null for *Ramp2,* with the expectation that a complete absence of RAMP2 protein might exacerbate phenotypes and help lead to the discovery of novel RAMP2-interacting GPCRs. Indeed, we found that RAMP2 can alter the expression of the GPCR, PTHR1, in the *Ramp2*$^{-/-}$ placenta. Specifically, loss of RAMP2 reduced *Pthr1* gene expression and PTHR1 protein expression in *Ramp2*$^{-/-}$ placental lysates, compared to littermate control placentas. We also discovered marked defects in the development of the placenta including reduced labyrinth size, reduced cellularity and reduced proliferation of labyrinthine trophoblast cells. Again, these phenotypes were not observed in *Adm*$^{-/-}$ and *Calcrl*$^{-/-}$ placentas. More importantly, these phenotypes are consistent with the previously described function of PTHR1 signaling in regulating trophoblast cell proliferation. Therefore, our studies using placental tissue that is null for RAMP2 provides the first *in vivo* evidence to support the biochemical studies that identified PTHR1 as a novel receptor partner for RAMP2. Our results also suggest that RAMPs may regulate GPCR signaling, not only by regulating receptor trafficking and ligand binding specificity, but also by regulating GPCR transcription and protein levels.

Collectively, the work presented in this dissertation has uncovered numerous physiological roles for RAMP2 in embryonic and placental

development, infertility and prolactin regulation, and in the development of the pituitary gland, mammary gland and the skeletal system.

Future Directions

The research presented in this dissertation has shed light on the numerous roles of RAMP2 in maintaining endocrine homeostasis in female mice. While these studies provide phenotypic characterization of *Ramp2*$^{+/-}$ female mice and *Ramp2*$^{-/-}$ placentas compared to their littermate controls, the specific receptor signaling mechanisms that were disrupted still remain to be elucidated. To address the potential mechanisms responsible for the observed phenotypes in *Ramp2*$^{+/-}$ mice, the following future directions can be taken:

i) Generation and characterization of compound heterozygous mice of *Ramp2* and the potential GPCR partners (*Calcrl*, *Pthr1*, *Gcgr* and *Ctr*) to evaluate if the phenotypes are conserved or exacerbated. The rationale for this approach is that any moderate phenotypes that are elicited by haploinsufficiency of RAMP2 may be exacerbated if and when the associating GPCR is also reduced by half. Additionally, because the RAMP2 phenotype is likely caused by altered signaling of numerous GPCRs in different tissues, a combinatorial approach may help distinguish phenotypes in an organ-specific way that can be related to the expression patterns of RAMP2-interacting GPCRs.

In fact, phenotypic evaluation of gene targeted mouse lines for several of the RAMP2-interacting receptors has revealed numerous phenotypes (**Table** 5.1), suggesting that a combinatorial breeding approach for generating compound gene targeted lines would likely result in alterations of phenotypes.

Of course, there are also several limitations to this approach, not the least of which are obtaining a large cohort of gene targeted mice from multiple investigators and establishing large breeding and phenotyping colonies. However, perhaps the more challenging aspect of this approach stems from what I have discovered as part of my thesis research regarding the strain-specific phenotypes of *Ramp2* mice. The studies presented in this dissertation made exclusive use of *Ramp2* mice that were bred and maintained on an isogenic 129/S6-SvEv genetic background. The genetic robustness of using animals on an isogenic background is well-evidenced by the marked phenotypes which showed very little variability between individual animals. However, during the course of my studies, I discovered that many of the endocrine phenotypes of *Ramp2*$^{+/-}$ mice are completely rescued when 129/S6-SvEv *Ramp2*$^{+/-}$ mice are bred onto a C57Bl/6 genetic background for one generation. Clearly, the fact that a single back-cross onto a mixed genetic background can ameliorate many of the *Ramp2*$^{+/-}$ endocrine phenotypes observed on the 129/S6-SvEv genetic background demonstrates that there must exist potent genetic modifier alleles on the C57Bl/6 genetic background that modulate RAMP2 function. (Of course, this in itself is an exciting discovery that warrants further exploration.) However, within the context of the proposed approach of breeding *Ramp2*$^{+/-}$ mice onto mice with haploinsufficiency or deletion of several GPCRs, this strain-dependent phenotype of *Ramp2*$^{+/-}$ mice will likely introduce the caveat of genetic strain variation, making the correct interpretation of the findings very difficult.

ii) Generate tissue-specific *Ramp2* knockout mice that may survive beyond E14.5, to investigate a specific role for RAMP2 in organ development. During my thesis work, I have already made substantial progress toward this goal. Specifically, I have designed and cloned a targeting vector for the conditional targeting of the Ramp2 locus, integrating *loxP* recombination sites within introns. Briefly, the mouse *Ramp2* knockout construct was generated by screening the 129S6/SvEv genomic library for phage clones containing the 5' portions of the gene. By using suitable restriction sites within the genomic clone, 5' and 3' regions of homology were subcloned into the multiple cloning site of the gene-targeting vector. To achieve conditional deletion of *Ramp2*, *loxP* sites flanking exons 1 and 2 were inserted in the targeted vector. The same exons were deleted in the gene-targeted mouse model of *Ramp2* that has been characterized in Chapters III and IV of this dissertation. Neomycin for positive selection and PGK-DTA (diphtheria toxin A) for negative selection were included in the vector as selection markers. The final targeted vector was sequenced and verified to be accurate.

Given the complex and robust phenotypes of *Ramp2*$^{+/-}$ mice in a variety of endocrine organs, a challenging next step in generating conditional *Ramp2* mouse lines is determining which tissue specific *Cre* line to use. For example, while an osteoblast specific approach may be useful for better understanding the functions of RAMP2 in bone, a mammary gland specific deletion might be more valuable to understanding the role of RAMP2 in branching morphogenesis of the mammary epithelium. However, an even more complex caveat to this approach

is the likelihood that the endocrine phenotypes of *Ramp2*$^{+/-}$ mice are inter-connected. For example, at this time, our studies do not rule out the possibility that the mammary gland and bone phenotypes of *Ramp2*$^{+/-}$ mice are coupled to the modest hyperprolactinemia that is elicited in the pituitary. Therefore, choosing the appropriate tissue-specific approach to conditionally delete *Ramp2* in adult animals presents a challenging puzzle, for which a tremendous amount of resources and future studies will need to be dedicated before embarking on a particular cell or organ type.

iii) Generate a transgenic mouse line overexpressing *Ramp2* exclusively in endothelial cells and then cross this line onto *Ramp2*$^{+/-}$ mice to eventually yield mice *null* for *Ramp2* in all tissues except endothelial cells. This approach builds upon our current understanding that the expression of *Calcrl* (and by extension, the expression of *Ramp2*) in developing endothelium is absolutely required for normal embryonic development, as observed by the phenotypes of mice with conditional Cre-loxP-mediated deletion of *Calcrl* in endothelium (18). If we are able to replace the expression of *Ramp2* specifically in endothelium of a *Ramp2*$^{-/-}$ mouse, then we may be able to generate a global *Ramp2* null mouse that survives to adulthood by virtue of the expression of RAMP2 in the endothelium. Our prediction is that the endocrine phenotypes of the *Ramp2*$^{+/-}$ mice may be substantially exacerbated when the animal is bred to homozygosity.

In fact, other members of the Caron laboratory are currently working towards the generation of an endothelial-specific *Ramp2* transgenic mouse, with promising results. Using the endothelial-specific VE-cadherin promoter, a

RAMP2 cDNA including an HA-tag was cloned downstream of the promoter, using conventional molecular biology approaches. To date, several transgenic founder animals have been generated and we are currently evaluating these animals for expression and passage of the *Ramp2* transgene onto offspring. An additional advantage to this approach is that several primary cell types null for *Ramp2* could be isolated from the surviving adult mice. For example, *Ramp2*$^{-/-}$ osteoblasts would be very beneficial for studying the role of *Ramp2* on CTR and PTHR1 signaling while primary mammary epithelial cells could be used to elucidate the function of RAMP2 in PTHR1 signaling.

Our studies suggest that PTHR1 function is compromised in *Ramp2*$^{-/-}$ placentas. In addition to lymphatic vascular abnormalities, perturbed PTHR1 function may also contribute, at least in part, to the embryonic lethality in *Ramp2*$^{-/-}$ mice. However, further studies employing both *in vivo* and *in vitro* approaches are needed to confirm that loss of RAMP2 and PTHR1 function results in placental dysfunction leading to fetal demise. To address this, the following future directions can be taken:

i) Generation and characterization of compound heterozygous mice by crossing *Ramp2*$^{+/-}$ and *Pthr1*$^{+/-}$ mice (as suggested above). A phenocopy or exacerbation of placental abnormalities in the offspring *null* for *Ramp2* and *Pthr1* would confirm our current observations and establish that PTHR1 and RAMP2 form a functional receptor in a mouse placenta. Additional further studies would

then attempt to determine if PTH, PTHrP or both ligands signal through PTHR1-RAMP receptor complex.

ii) Experiments involving the culturing of primary trophoblast cells null for *Ramp2* are challenging due to the dramatically small litter sizes of *Ramp2*$^{+/-}$ intercrosses. However, the isolation of *Ramp2*$^{-/-}$ trophoblast stem cell lines from *Ramp2*$^{-/-}$ blastocysts may be possible. The *Ramp2*$^{-/-}$ trophoblast stem cells could then be differentiated along several trophoblast lineage pathways in culture and the extent of PTHR1 signaling could be evaluated in the absence of RAMP2. Moreover, microarray or proteomic approaches could be used to investigate pathways affected by loss of RAMP2. This approach would also shed light on the potential molecular players downstream of PTHR1-RAMP2 in trophoblast cells.

iii) *In vitro* studies in trophoblast cell lines such as the human trophoblast cell line, HTR-8/Sv-Neo, to determine the influence of RAMP2 on downstream signaling of PTHR1 in response to PTH or PTHrP. Indeed, during my thesis work, I have already made extensive use of the HTR-8/Sv-Neo trophoblast cell line to explore the potential functions of RAMP2 in PTHR1 signaling. My current studies are focused on establishing the downstream signaling cascades that are activated by either PTH or PTHrP ligands in HTR-8/Sv-Neo cells. Once these pathways are established for the HTR-8/Sv-Neo cell line, my hope is to perform siRNA-mediated knockdown of *Ramp2* in order to determine whether the loss of RAMP2 influences PTHR1 signaling events in trophoblast cells. These studies would help provide additional molecular insights into the potential cellular

mechanisms that underlie the trophoblast cell proliferation defects observed in *Ramp2*$^{-/-}$ placentas.

A potential caveat to this approach is that the phenotypes of the *Ramp2*$^{-/-}$ placentas predominantly affect the trophoblast cells of the labyrinth lineage. However the HTR-8/Sv-Neo cell line represents a distinct and further differentiated trophoblast lineage that is more similar to the mouse trophoblast giant cell (invasive trophoblast cells that form the inner-most border of the maternal-fetal interface). Since we do not yet have a full appreciation for the expression pattern or function of PTHR1 signaling in different trophoblast lineages, we cannot exclude the possibility that results obtained from HTR-8/Sv-Neo cell lines may not be directly applicable to the observed *in vivo* phenotype involving labyrinthine trophoblast cells. Unfortunately, robust labyrinthine trophoblast cells that can be easily transfected do not, to my knowledge, currently exist. Nevertheless, a focused evaluation of the function of RAMP2 in PTHR1 signaling of trophoblast cells is highly warranted.

iv) Determine whether *Pthr1* gene expression and protein levels are regulated directly by RAMP2. An interesting, and somewhat unexpected finding of my thesis work is that the gene expression and protein levels of *Pthr1* are markedly reduced in *Ramp2*$^{-/-}$ placentas. A potential explanation for this finding is that the reduction in labyrinthine trophoblast cells results in reduction of PTRH1 expression, by virtue of the fact that PTHR1 is expressed in this trophoblast lineage. However, an alternative explanation is that RAMP2 has important functions in regulating the gene expression and protein levels of its

associating GPCRs. Our laboratory has already uncovered homeostatic compensations of GPCRs in response to changes in *Ramp* gene expression. For example, the expression of *Calcrl* is modestly, yet significantly, increased in the hearts of mice that are null for *Ramp3*. While this data suggests a compensatory or homeostatic mechanism for regulating *Calcrl* signaling, the possibility that the mRNA and protein expression of other GPCRs is directly modulated by RAMP proteins is novel, exciting and worth pursuing.

Clinical implications of RAMP2

RAMP2 could be a viable target for diseases such as hyperprolactinemia, osteoporosis and infertility. Although dopamine agonists are the first line of treatment of hyperprolactinemia, the numerous side effects of dopamine based treatment (96) such as nausea, vomiting, headache, dizziness, drowsiness and other rare complications affecting the lung and heart function (97), keep the search ongoing for developing a more suitable drug.

Interestingly, treatment of osteoporosis also remains challenging. The presence of RAMPs in osteoblasts (98)) and osteoclasts (99), as well as osteoporosis-like phenotypes in *Ramp2*$^{+/-}$ (25) and *Amy*$^{+/-}$ (100) mice suggest that targeting RAMP2, perhaps at the CTR-RAMP2 interface, might be beneficial in treating conditions such as osteoporosis. Lastly, our work has demonstrated that RAMP2 has implications in pregnancy in mice; playing crucial roles in fetal growth, placental development and postnatal growth of pups. Therefore, RAMP2 needs to be exploited for its role in the successful outcome of a pregnancy. Since

we lack knowledge about the precise GPCRs, besides CLR, that might be involved in the RAMP2-mediated functions in the female reproductive system, future studies addressing these gaps in our understanding would become extremely important. Additionally, the fact that multiple organ systems can be affected by the deficiency or the complete absence of RAMP2 has to be taken into consideration when creating a therapeutic against RAMP2, in order to develop a drug with greater specificity.

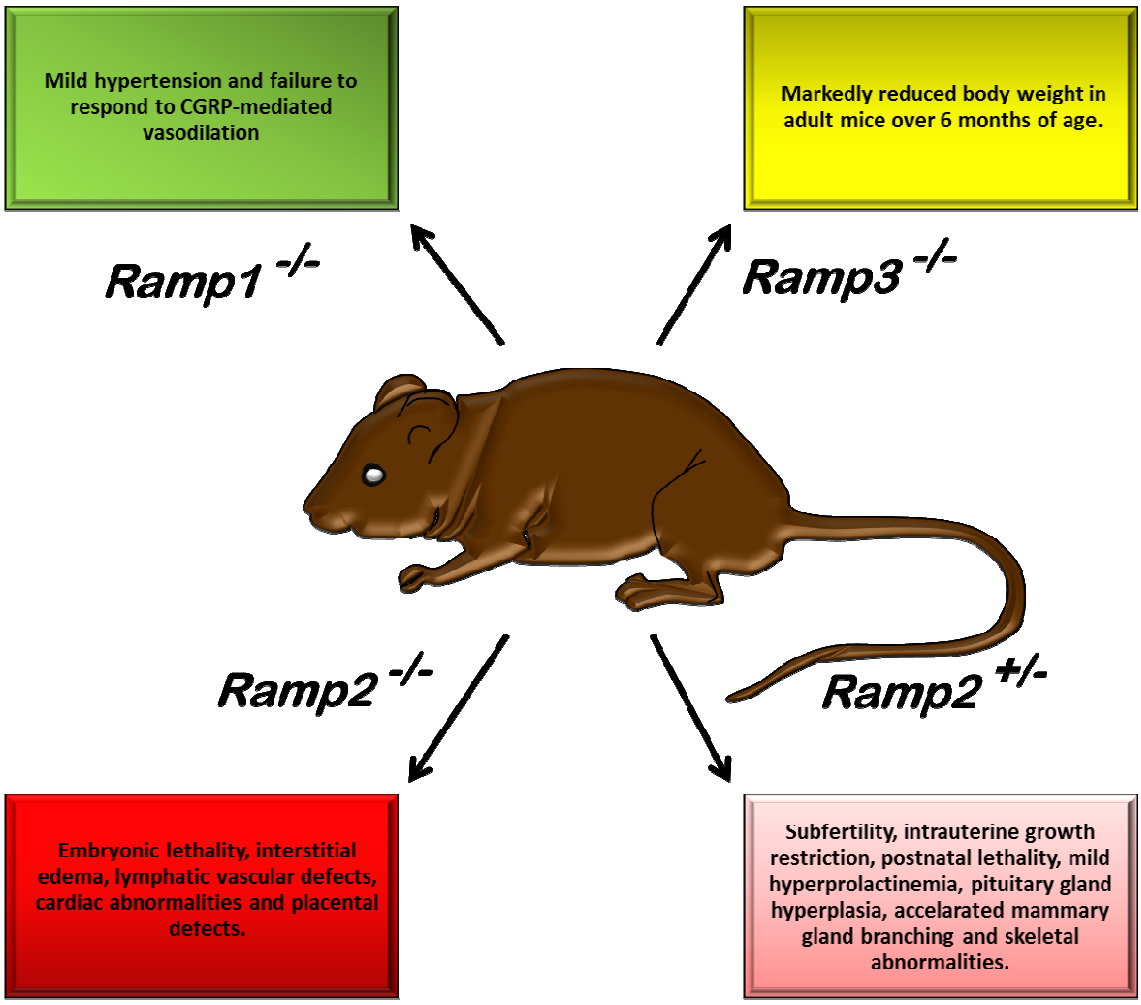
Conclusions

In summary, the findings presented in this dissertation have significantly advanced the field of RAMP research by providing an *in vivo* model that supports previously reported biochemical investigations. In addition, as reviewed in **Chapter 2** and as shown in **Fig 5.1**, this dissertation provides a summary of the phenotypes of gene targeted mouse models of all the RAMPs. More importantly, the phenotypic characterization of a mouse model of *Ramp2* presented in **Chapters 3** and **4** have provided novel insights on the role of RAMP2 in postnatal development as well as a role in placentation. Furthermore, these studies enhance our understanding of the *in vivo* role of *Ramp2* and should bear significance in the development of RAMP2-based therapeutics.

Table 5.1 Phenotypes of Mouse Models of RAMP2-interacting GPCRs and their ligands

Knockout Phenotype	Adrenomedullin	Calcitonin receptor-like receptor	Parathyroid hormone	Parathyroid hormone related peptide	Parathyroid hormone receptor 1	Glucagon	Glucagon Receptor	Amylin	Calcitonin Receptor	RAMP2
Embryonic lethality	Midgestational lethality	Midgestational lethality	Viable	Perinatal lethality	Midgestational lethality	Postnatal lethality	Late-gestational to perinatal lethality	Viable	Midgestational lethality	Midgestational lethality
Female Fertility	Heterozygotes are subfertile	Heterozygotes are subfertile	Normal	Heterozygotes are fertile	Heterozygotes are fertile	Normal	Subfertility; perinatal and postnatal lethality in pups born to Kos.	Normal	Heterozygotes are subfertile (unpublished)	Severe subfertility in heterozygotes
Placenta	Defective with failure of spiral artery remodelling	Phenocopy $AM^{-/-}$ placentas	Placental calcium transfer normal; reduced expression of TRPV6, calbindin D-9K, and VDR.	Impaired intraplacental yolk sac; increased placental calcium transfer	Decreased placental calcium transfer	Not described	Impaired glucose transport; fetal growth restriction	Not described	Not described	Proliferative defect in labyrinthine trophoblasts and failure of spiral artery remodeling
Prolactin	No change in basal serum (unpublished); not tested during pregnancy	No change in basal serum (unpublished); not tested during pregnancy	Not described	Not described	Not described	Not described	Not described	Not described	Normal (unpublished)	Mild hyperprolactenemia (basal & during pregnancy) in heterozygotes
Pituitary gland	Not described	Normal (Unpublished)	Not described	Not described	Not described	Not described	Not described	Not described	Not described	Heterozygotes exhibit anterior pituitary gland hyperplasia
Mammary gland	Mild hypoplasia (unpublished)	Mild hypoplasia (unpublished)	Not described	Failure to form mammary epithelial duct	Phenocopy *Pthrp* $^{-/-}$ mice	Not described	Not described	Not described	Not described	Heterozygotes have precocious branching
Serum Calcium	Not described	Not described	Hypocalcemia	Hypocalcemia	Hypocalcemia	Not described	Not described	Not described	Normal	Normal
Bone	Not described	Not described	Reduced cartilage mineralization and enhanced bone mineralization	Accelarated differentiation of chondrocytes of the growth plate	Phenocopy *Pthrp* $^{-/-}$ mice	Not described	Not described	Low bone mass due to icreased resorption in heterozygotes	Increased bone mass due to icreased bone formation	Reduced minrealization in the heterozygotes

Figure 5.1 Summary of phenotypes of gene targeted mouse models of RAMPs



REFERENCES

1. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM 1998 RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393:333-339

2. Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, Fraser NJ, Main MJ, Foord SM, Sexton PM 1999 Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol 56:235-242

3. Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A, Kuwasako K, Tilakaratne N, Sexton PM 2003 Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 278:3293-3297

4. Harikumar KG, Simms J, Christopoulos G, Sexton PM, Miller LJ 2009 Molecular Basis of Association of Receptor Activity-Modifying Protein 3 with the Family B G Protein-Coupled Secretin Receptor. Biochemistry

5. Bouschet T, Martin S, Henley JM 2005 Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane. J Cell Sci 118:4709-4720

6. Bomberger JM, Spielman WS, Hall CS, Weinman EJ, Parameswaran N 2005 Receptor activity-modifying protein (RAMP) isoform-specific regulation of adrenomedullin receptor trafficking by NHERF-1. J Biol Chem 280:23926-23935

7. Bomberger JM, Parameswaran N, Hall CS, Aiyar N, Spielman WS 2005 Novel function for receptor activity-modifying proteins (RAMPs) in post-endocytic receptor trafficking. J Biol Chem 280:9297-9307

8. Morfis M, Tilakaratne N, Furness SG, Christopoulos G, Werry TD, Christopoulos A, Sexton PM 2008 Receptor activity-modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. Endocrinology 149:5423-5431

9. Fraser NJ, Wise A, Brown J, McLatchie LM, Main MJ, Foord SM 1999 The amino terminus of receptor activity modifying proteins is a critical determinant of glycosylation state and ligand binding of calcitonin receptor-like receptor. Mol Pharmacol 55:1054-1059

10. Qi T, Simms J, Bailey RJ, Wheatley M, Rathbone DL, Hay DL, Poyner DR 2010 Structure-function analysis of RAMP1-RAMP3 chimeras. Biochemistry 49:522-531
11. Heroux M, Hogue M, Lemieux S, Bouvier M 2007 Functional calcitonin gene-related peptide receptors are formed by the asymmetric assembly of a calcitonin receptor-like receptor homo-oligomer and a monomer of receptor activity-modifying protein-1. J Biol Chem 282:31610-31620

12. Hoare SR 2005 Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. Drug Discov Today 10:417-427

13. Kusano S, Kukimoto-Niino M, Akasaka R, Toyama M, Terada T, Shirouzu M, Shindo T, Yokoyama S 2008 Crystal structure of the human receptor activity-modifying protein 1 extracellular domain. Protein Sci 17:1907-1914

14. ter Haar E, Koth CM, Abdul-Manan N, Swenson L, Coll JT, Lippke JA, Lepre CA, Garcia-Guzman M, Moore JM 2010 Crystal structure of the ectodomain complex of the CGRP receptor, a class-B GPCR, reveals the site of drug antagonism. Structure 18:1083-1093

15. Archbold JK, Flanagan JU, Watkins HA, Gingell JJ, Hay DL 2011 Structural insights into RAMP modification of secretin family G protein-coupled receptors: implications for drug development. Trends Pharmacol Sci 32:591-600

16. Simms J, Hay DL, Bailey RJ, Konycheva G, Bailey G, Wheatley M, Poyner DR 2009 Structure-function analysis of RAMP1 by alanine mutagenesis. Biochemistry 48:198-205

17. Dackor R, Fritz-Six K, Smithies O, Caron K 2007 Receptor activity-modifying proteins 2 and 3 have distinct physiological functions from embryogenesis to old age. J Biol Chem 282:18094-18099

18. Fritz-Six KL, Dunworth WP, Li M, Caron KM 2008 Adrenomedullin signaling is necessary for murine lymphatic vascular development. J Clin Invest 118:40-50

19. Zhang Z, Liu X, Morgan DA, Kuburas A, Thedens DR, Russo AF, Rahmouni K 2011 Neuronal receptor activity-modifying protein 1 promotes energy expenditure in mice. Diabetes 60:1063-1071

20. Chrissobolis S, Zhang Z, Kinzenbaw DA, Lynch CM, Russo AF, Faraci FM 2010 Receptor activity-modifying protein-1 augments cerebrovascular responses to calcitonin gene-related peptide and inhibits angiotensin II-induced vascular dysfunction. Stroke 41:2329-2334

21. Sabharwal R, Zhang Z, Lu Y, Abboud FM, Russo AF, Chapleau MW 2010 Receptor activity-modifying protein 1 increases baroreflex sensitivity and attenuates Angiotensin-induced hypertension. Hypertension 55:627-635

22. Zhang Z, Winborn CS, Marquez de Prado B, Russo AF 2007 Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. J Neurosci 27:2693-2703

23. Ichikawa-Shindo Y, Sakurai T, Kamiyoshi A, Kawate H, Iinuma N, Yoshizawa T, Koyama T, Fukuchi J, Iimuro S, Moriyama N, Kawakami H, Murata T, Kangawa K, Nagai R, Shindo T 2008 The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. J Clin Invest 118:29-39

24. Kadmiel M, Fritz–Six K, Caron K eds. 2011 Understanding RAMPs Through Genetically Engineered Mouse Models. Austin: Landes Bioscience

25. Kadmiel M, Fritz-Six K, Pacharne S, Richards GO, Li M, Skerry TM, Caron KM 2011 Research resource: Haploinsufficiency of receptor activity-modifying protein-2 (RAMP2) causes reduced fertility, hyperprolactinemia, skeletal abnormalities, and endocrine dysfunction in mice. Mol Endocrinol 25:1244-1253

26. Cueille C, Pidoux E, de Vernejoul MC, Ventura-Clapier R, Garel JM 2002 Increased myocardial expression of RAMP1 and RAMP3 in rats with chronic heart failure. Biochem Biophys Res Commun 294:340-346

27. Totsune K, Takahashi K, Mackenzie HS, Murakami O, Arihara Z, Sone M, Mouri T, Brenner BM, Ito S 2000 Increased gene expression of adrenomedullin and adrenomedullin-receptor complexes, receptor-activity modifying protein (RAMP)2 and calcitonin-receptor-like receptor (CRLR) in the hearts of rats with congestive heart failure. Clin Sci (Lond) 99:541-546

28. Oie E, Vinge LE, Yndestad A, Sandberg C, Grogaard HK, Attramadal H 2000 Induction of a myocardial adrenomedullin signaling system during ischemic heart failure in rats. Circulation 101:415-422

29. Nishikimi T, Wang X, Akimoto K, Tadokoro K, Mori Y, Ishikawa Y, Ishimura K, Yoshihara F, Minamino N, Kangawa K, Matsuoka H 2005 Alteration of renal adrenomedullin and its receptor system in the severely hypertensive rat: effect of diuretic. Regul Pept 124:89-98

30. Nishikimi T, Yoshihara F, Kanazawa A, Okano I, Horio T, Nagaya N, Yutani C, Matsuo H, Matsuoka H, Kangawa K 2001 Role of increased circulating and renal adrenomedullin in rats with malignant hypertension. Am J Physiol Regul Integr Comp Physiol 281:R2079-2087

31. Nagae T, Mukoyama M, Sugawara A, Mori K, Yahata K, Kasahara M, Suganami T, Makino H, Fujinaga Y, Yoshioka T, Tanaka I, Nakao K 2000 Rat receptor-activity-modifying proteins (RAMPs) for adrenomedullin/CGRP receptor: cloning and upregulation in obstructive nephropathy. Biochem Biophys Res Commun 270:89-93

32. Hiragushi K, Wada J, Eguchi J, Matsuoka T, Yasuhara A, Hashimoto I, Yamashita T, Hida K, Nakamura Y, Shikata K, Minamino N, Kangawa K, Makino H 2004 The role of adrenomedullin and receptors in glomerular hyperfiltration in streptozotocin-induced diabetic rats. Kidney Int 65:540-550

33. Thota C, Gangula PR, Dong YL, Yallampalli C 2003 Changes in the expression of calcitonin receptor-like receptor, receptor activity-modifying protein (RAMP) 1, RAMP2, and RAMP3 in rat uterus during pregnancy, labor, and by steroid hormone treatments. Biol Reprod 69:1432-1437

34. Kitamuro T, Takahashi K, Totsune K, Nakayama M, Murakami O, Hida W, Shirato K, Shibahara S 2001 Differential expression of adrenomedullin and its receptor component, receptor activity modifying protein (RAMP) 2 during hypoxia in cultured human neuroblastoma cells. Peptides 22:1795-1801

35. Qing X, Svaren J, Keith IM 2001 mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. Am J Physiol Lung Cell Mol Physiol 280:L547-554

36. Doods H, Arndt K, Rudolf K, Just S 2007 CGRP antagonists: unravelling the role of CGRP in migraine. Trends Pharmacol Sci 28:580-587

37. Benemei S, Nicoletti P, Capone JA, Geppetti P 2007 Pain pharmacology in migraine: focus on CGRP and CGRP receptors. Neurol Sci 28 Suppl 2:S89-93

38. Sexton PM, Morfis M, Tilakaratne N, Hay DL, Udawela M, Christopoulos G, Christopoulos A 2006 Complexing receptor pharmacology: modulation of family B G protein-coupled receptor function by RAMPs. Ann N Y Acad Sci 1070:90-104

39. Kunz TH, Mueller-Steiner S, Schwerdtfeger K, Kleinert P, Troxler H, Kelm JM, Ittner LM, Fischer JA, Born W 2007 Interaction of receptor-activity-modifying protein1 with tubulin. Biochim Biophys Acta 1770:1145-1150

40. Sexton PM, Poyner DR, Simms J, Christopoulos A, Hay DL 2009 Modulating receptor function through RAMPs: can they represent drug targets in themselves? Drug Discov Today 14:413-419

41. Hay DL, Poyner DR, Sexton PM 2006 GPCR modulation by RAMPs. Pharmacol Ther 109:173-197

42. Tsujikawa K, Yayama K, Hayashi T, Matsushita H, Yamaguchi T, Shigeno T, Ogitani Y, Hirayama M, Kato T, Fukada S, Takatori S, Kawasaki H, Okamoto H, Ikawa M, Okabe M, Yamamoto H 2007 Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. Proc Natl Acad Sci U S A 104:16702-16707

43. Caron KM, Smithies O 2001 Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. Proc Natl Acad Sci U S A 98:615-619

44. Dackor RT, Fritz-Six K, Dunworth WP, Gibbons CL, Smithies O, Caron KM 2006 Hydrops fetalis, cardiovascular defects, and embryonic lethality in mice lacking the calcitonin receptor-like receptor gene. Mol Cell Biol 26:2511-2518

45. Dunworth WP, Fritz-Six KL, Caron KM 2008 Adrenomedullin stabilizes the lymphatic endothelial barrier in vitro and in vivo. Peptides 29:2243-2249

46. Oliver G 2004 Lymphatic vasculature development. Nat Rev Immunol 4:35-45

47. Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K 2002 Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. Embo J 21:4593-4599

48. Hirakawa S, Hong YK, Harvey N, Schacht V, Matsuda K, Libermann T, Detmar M 2003 Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. Am J Pathol 162:575-586

49. Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Skobe M 2002 Molecular characterization of lymphatic endothelial cells. Proc Natl Acad Sci U S A 99:16069-16074

50. Tammela T, Petrova TV, Alitalo K 2005 Molecular lymphangiogenesis: new players. Trends Cell Biol 15:434-441

51. Kahn ML 2008 Blood is thicker than lymph. J Clin Invest 118:23-26

52. Li M, Yee D, Magnuson TR, Smithies O, Caron KM 2006 Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. J Clin Invest 116:2653-2662

53. Li M, Wu Y, Caron KM 2008 Haploinsufficiency for Adrenomedullin Reduces Pinopodes and Diminishes Uterine Receptivity in Mice. Biol Reprod

54. Tam CW, Husmann K, Clark NC, Clark JE, Lazar Z, Ittner LM, Gotz J, Douglas G, Grant AD, Sugden D, Poston L, Poston R, McFadzean I, Marber MS, Fischer JA, Born W, Brain SD 2006 Enhanced vascular responses to adrenomedullin in mice overexpressing receptor-activity-modifying protein 2. Circ Res 98:262-270

55. Olesen J, Diener HC, Husstedt IW, Goadsby PJ, Hall D, Meier U, Pollentier S, Lesko LM 2004 Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. N Engl J Med 350:1104-1110

56. Ho TW, Ferrari MD, Dodick DW, Galet V, Kost J, Fan X, Leibensperger H, Froman S, Assaid C, Lines C, Koppen H, Winner PK 2008 Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. Lancet 372:2115-2123

57. Ho TW, Mannix LK, Fan X, Assaid C, Furtek C, Jones CJ, Lines CR, Rapoport AM 2008 Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. Neurology 70:1304-1312

58. Paone DV, Shaw AW, Nguyen DN, Burgey CS, Deng JZ, Kane SA, Koblan KS, Salvatore CA, Mosser SD, Johnston VK, Wong BK, Miller-Stein CM, Hershey JC, Graham SL, Vacca JP, Williams TM 2007 Potent, Orally Bioavailable Calcitonin Gene-Related Peptide Receptor Antagonists for the Treatment of Migraine: Discovery of N-[(3R,6S)-6-(2,3-Difluorophenyl)-2-oxo-1- (2,2,2-trifluoroethyl)azepan-3-yl]-4- (2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin- 1-yl)piperidine-1-carboxamide (MK-0974). J Med Chem 50:5564-5567

59. Gomez C, David V, Peet NM, Vico L, Chenu C, Malaval L, Skerry TM 2007 Absence of mechanical loading in utero influences bone mass and architecture but not innervation in Myod-Myf5-deficient mice. J Anat 210:259-271

60. Grattan DR, Steyn FJ, Kokay IC, Anderson GM, Bunn SJ 2008 Pregnancy-induced adaptation in the neuroendocrine control of prolactin secretion. J Neuroendocrinol 20:497-507

61. Fisher RA 1972 Statistical Methods, Experimental Design, and Scientific Inference. 14th ed. New york: Hafner Publishing Compnay

62. El-Hashash AH, Esbrit P, Kimber SJ 2005 PTHrP promotes murine secondary trophoblast giant cell differentiation through induction of endocycle, upregulation of giant-cell-promoting transcription factors and suppression of other trophoblast cell types. Differentiation 73:154-174

63. El-Hashash AH, Kimber SJ 2006 PTHrP induces changes in cell cytoskeleton and E-cadherin and regulates Eph/Ephrin kinases and RhoGTPases in murine secondary trophoblast cells. Dev Biol 290:13-31

64. Wysolmerski JJ, McCaughern-Carucci JF, Daifotis AG, Broadus AE, Philbrick WM 1995 Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. Development 121:3539-3547

65. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Broadus AE 1998 Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. Development 125:1285-1294

66. Amizuka N, Karaplis AC, Henderson JE, Warshawsky H, Lipman ML, Matsuki Y, Ejiri S, Tanaka M, Izumi N, Ozawa H, Goltzman D 1996 Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. Dev Biol 175:166-176

67. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC 1994 Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol 126:1611-1623

68. Karaplis AC 2001 PTHrP: novel roles in skeletal biology. Curr Pharm Des 7:655-670

69. Lanske B, Amling M, Neff L, Guiducci J, Baron R, Kronenberg HM 1999 Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. J Clin Invest 104:399-407

70. Vuguin PM, Kedees MH, Cui L, Guz Y, Gelling RW, Nejathaim M, Charron MJ, Teitelman G 2006 Ablation of the glucagon receptor gene increases fetal lethality and produces alterations in islet development and maturation. Endocrinology 147:3995-4006

71. Cornish J, Callon KE, Coy DH, Jiang NY, Xiao L, Cooper GJ, Reid IR 1997 Adrenomedullin is a potent stimulator of osteoblastic activity in vitro and in vivo. Am J Physiol 273:E1113-1120

72. Rossant J, Cross JC 2001 Placental development: lessons from mouse mutants. Nat Rev Genet 2:538-548

73. Hu D, Cross JC 2010 Development and function of trophoblast giant cells in the rodent placenta. Int J Dev Biol 54:341-354

74. Udawela M, Hay DL, Sexton PM 2004 The receptor activity modifying protein family of G protein coupled receptor accessory proteins. Semin Cell Dev Biol 15:299-308

75. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G 2001 Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci U S A 98:13324-13329

76. Suzuki Y, Kovacs CS, Takanaga H, Peng JB, Landowski CP, Hediger MA 2008 Calcium channel TRPV6 is involved in murine maternal-fetal calcium transport. J Bone Miner Res 23:1249-1256

77. van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, Pols HA, Bindels RJ, van Leeuwen JP 2005 The epithelial Ca2+ channel TRPV5 is essential for proper osteoclastic bone resorption. Proc Natl Acad Sci U S A 102:17507-17512

78. Hu XD, Jiang SL, Liu CH, Hu YY, Liu C, Sun MY, Chen GF, Liu P 2010 Preventive effects of 1,25-(OH)2VD3 against ConA-induced mouse hepatitis through promoting vitamin D receptor gene expression. Acta Pharmacol Sin 31:703-708

79. Woodrow JP, Sharpe CJ, Fudge NJ, Hoff AO, Gagel RF, Kovacs CS 2006 Calcitonin plays a critical role in regulating skeletal mineral metabolism during lactation. Endocrinology 147:4010-4021

80. Livak KJ, Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

81. Watson ED, Cross JC 2005 Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) 20:180-193

82. Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C, Cross JC 2002 Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. Dev Biol 250:358-373

83. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I, Gazit R, Yutkin V, Benharroch D, Porgador A, Keshet E, Yagel S, Mandelboim O 2006 Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med 12:1065-1074

84. Eastabrook G, Hu Y, von Dadelszen P 2008 The role of decidual natural killer cells in normal placentation and in the pathogenesis of preeclampsia. J Obstet Gynaecol Can 30:467-476

85. Lafond J, Goyer-O'Reilly I, Laramee M, Simoneau L 2001 Hormonal regulation and implication of cell signaling in calcium transfer by placenta. Endocrine 14:285-294

86. Simmonds CS, Karsenty G, Karaplis AC, Kovacs CS 2010 Parathyroid hormone regulates fetal-placental mineral homeostasis. J Bone Miner Res 25:594-605

87. Clemens TL, Cormier S, Eichinger A, Endlich K, Fiaschi-Taesch N, Fischer E, Friedman PA, Karaplis AC, Massfelder T, Rossert J, Schluter KD, Silve C, Stewart AF, Takane K, Helwig JJ 2001 Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. Br J Pharmacol 134:1113-1136

88. Kovacs CS, Lanske B, Hunzelman JL, Guo J, Karaplis AC, Kronenberg HM 1996 Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. Proc Natl Acad Sci U S A 93:15233-15238

89. Kovacs CS, Chafe LL, Fudge NJ, Friel JK, Manley NR 2001 PTH regulates fetal blood calcium and skeletal mineralization independently of PTHrP. Endocrinology 142:4983-4993

90. Redman CW 2011 Preeclampsia: a multi-stress disorder. Rev Med Interne 32 Suppl 1:S41-44

91. Tucci J, Hammond V, Senior PV, Gibson A, Beck F 1996 The role of fetal parathyroid hormone-related protein in transplacental calcium transport. J Mol Endocrinol 17:159-164

92. Mosekilde L, Torring O, Rejnmark L 2011 Emerging anabolic treatments in osteoporosis. Curr Drug Saf 6:62-74

93. Toulis KA, Anastasilakis AD, Polyzos SA, Makras P 2011 Targeting the osteoblast: approved and experimental anabolic agents for the treatment of osteoporosis. Hormones (Athens) 10:174-195

94. Abdelgadir Adam M, Untch BR, Olson JA, Jr. 2010 Parathyroid carcinoma: current understanding and new insights into gene expression and intraoperative parathyroid hormone kinetics. Oncologist 15:61-72

95. Kremer R, Li J, Camirand A, Karaplis AC 2011 Parathyroid Hormone Related Protein (PTHrP) in Tumor Progression. Adv Exp Med Biol 720:145-160

96. Gillam MP, Molitch ME, Lombardi G, Colao A 2006 Advances in the treatment of prolactinomas. Endocr Rev 27:485-534

97. Zanettini R, Antonini A, Gatto G, Gentile R, Tesei S, Pezzoli G 2007 Valvular heart disease and the use of dopamine agonists for Parkinson's disease. N Engl J Med 356:39-46

98. Naot D, Callon KE, Grey A, Cooper GJ, Reid IR, Cornish J 2001 A potential role for adrenomedullin as a local regulator of bone growth. Endocrinology 142:1849-1857

99. Uzan B, de Vernejoul MC, Cressent M 2004 RAMPs and CRLR expressions in osteoblastic cells after dexamethasone treatment. Biochem Biophys Res Commun 321:802-808

100. Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson DL, Zajac JD, Karsenty G 2004 Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo. J Cell Biol 164:509-514