Functional characterization of Rasd1 : a novel interaction between Rasd1 and Ear2 (NR2F6) is involved in the regulation of renin transcription

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FUNCTIONAL CHARACTERIZATION OF RASD1:

A NOVEL INTERACTION BETWEEN RASD1 AND EAR2 (NR2F6) IS INVOLVED IN THE REGULATION OF RENIN TRANSCRIPTION

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
AD	Activation domain
AGS1	Activator of G protein signaling 1
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
APP	Amyloid precursor protein
AT1	Angiotensin II type 1
AT2	Angiotensin II type 2
AT4	Angiotensin II type 4
at-RA	All-trans retinoic acid
ANOVA	Analysis of variance
ade	Adenine
bp	Base pairs
β-gal	β-galactosidase
BSA	Bovine serum albumin
CAGE	Chymostatin-sensitive Ang-II-generating enzyme
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CO_2	Carbon dioxide
ChIP	Chromatin immunoprecipitation
coIP	Co-immunoprecipitation
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB	cAMP response element binding protein

cry	cryptochrome
DAPI	4´,6-diamidino-2-phenylindole
Dbh	Domapine-β-hydroxylase
DMT1	Divalent metal transporter 1
DNA-BD	DNA binding domain
Ear2	Erb-A related 2
EDTA	Ethylenediaminetetraacetic acid
EMSA	Eletrophorectic mobility shift assay
EST	Expressed sequence tag
FBS	Fetal bovine serum
GAP	GTPase activating protein
GDP	Guaninosine diphosphate
GPCR	G protein-coupled receptor
GSF	Guanine nucleotide exchange factor
GSH	Glutathione
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HA	Hemagglutinin
His	Histidine
HOX	Homeobox
HRP	Horseradish peroxidase
Ig E	Immunoglobulin E
IgG	Immunoglobulin G
IL-17A	Interleukin-17A
kb	Kilo bases
kDa	Kilo Dalton
LB	Luria-Bertani
LC	Locus coeruleus
Leu	Leucine

LiAC	Lithium acetate
MAPK	mitogen-activated protein kinase
NA	Noradrenaline/ norepinerphrine
NaCl	Sodium chloride
NF1	Nuclear factor 1
NF-Y	Nuclear transcription factor Y
Ni-NTA	Nickel-nitrilotriacetic acid
NMDA	N-Methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
Nr2f6	Nuclear receptor subfamily 2, group F, member 6
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAP7	Peripheral benzodiazepine receptor-associated protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Poly-ethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene difluoride
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
RAS	Renin-angiotensin system
Ras	Rat sarcoma
Rasd1	Ras, dexamethasone-induced 1
RNAi	RNA interference
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Synthetic dropout
SDS	Sodium dodecyl sulfate

Sp 1/3	Specificity protein 1/3
SV40	Simian virus 40
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween-20
TE	Tris-EDTA
Th	Tyrosine hydroxylase
t-PA	tissue plasminogen activator
Trp	Tryptophan
USF-1/2	Upstream stimulatory factor 1/2
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

SUMMARY

Introduction

Rasd1 (Ras dexamethasone-induced 1), also known as Dexras1 and activator of G proteins signaling 1 (AGS1), belongs to the Ras superfamily of GTPase. The characteristic GTP binding and hydrolysis properties of Ras proteins render Rasd1 extremely important in numerous signaling pathways and physiological processes. Rasd1 is known to regulate cell growth, cell differentiation and cell death. Rasd1 is also involved in the regulation of neuronal iron homeostasis and circadian timings. *Rasd1* was first identified in 1998. Although a few of its downstream targets have been identified, its related signaling pathways have not been definitively established.

Aims

This project is focused on the functional characterization of Rasd1. To identify potential proteins that interact with Rasd1, I performed yeast two-hybrid assays using Rasd1 as bait against a mouse brain cDNA library. Any promising proteins that interact with Rasd1 will be studied further to elucidate the functional significance of the interaction and to resolve the molecular mechanisms and signaling pathways involved. Identification of Rasd1 interaction partners will provide important functional link on which future translational research will be based.

Results

Yeast two-hybrid analysis revealed several proteins that specifically interact with Rasd1. I chose to further examine the functional significance of the interaction between Rasd1 and erb-A related 2 (Ear2), also known as nuclear receptor subfamily 2, group F, member 6 (Nr2f6), as Ear2 produced three independent hits in the yeast two-hybrid assay, and both Rasd1 and Ear2 are known to be involved in the regulation of the circadian rhythm and in neurological developments. It was confirmed that Rasd1 and Ear2 interact *in vitro* and form an endogenous complex in living cells. Ear2 is a known repressor of renin transcription. It was demonstrated that Rasd1 is able to alleviate both retinoic dependent and independent Ear2-mediated repression of renin gene transcription. This activity was observed in endogenous renin promoter and transfected renin promoter constructs. The knockdown of Rasd1 by RNAi caused a further suppression in the Ear2-mediated repression of renin promoter activity. Moreover, Ear2 interacted with Rasd1 via its ligand binding domain, and that both the DNA binding and ligand binding domains of Ear2 were crucial in Rasd1- and Ear2-mediated renin gene transcription.

In addition, confocal studies demonstrated that wild type Rasd1 and its constitutively active Rasd1 mutant, Rasd1[A178V], were able to alter the nuclear-cytoplasmic distribution of Ear2. When co-transfected, a significant

amount of Ear2 was translocated from the nucleus to the cytoplasm. On the other hand, the Rasd1 mutants- Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], with defective GTPase activity, GDP-GTP exchange by GEF and isoprenylation and membrane localization motifs respectively, showed a significantly reduced ability to alleviate Ear2-mediated repression of renin transcriptional activity and had reduced physical interactions with Ear2. Confocal studies revealed that these Rasd1 mutants also possessed a markedly reduced ability to translocate Ear2 from the nucleus to the cytoplasm when co-transfected. This study identifies a novel interaction between Rasd1 and Ear2, and a novel regulatory role of Rasd1 in the mediation of renin transcription.

Conclusions

The findings from this study provide us with critical insights on the intrinsic and complex regulation of the renin-angiotensin system. Efforts aimed at generating compounds that target renin expression in an attempt to manage clinical symptoms like hypertension, heart and cardiovascular diseases that arise from the dysregulation of the renin angiotensin system have been intense but futile. This study presents a novel mechanism of renin gene regulation that can serve as potential drug targets.

CHAPTER 1: INTRODUCTION

1.1. Ras superfamily of proteins

Ras superfamily has been identified in several species, and their products exhibit remarkable structural, functional and biochemical homology (1). Based on their sequence and structure identities, the Ras superfamily of guaninosine triphosphate (GTP) hydrolysis-coupled signal transduction proteins can be further subdivided to include the Ras, Rho, Ran, Rab, Rad and Arf subfamilies, as well as a more divergent Gα subfamily (2).

1.1.1. Biochemical activities of Ras proteins

Ras proteins are GTP/guaninosine diphosphate (GDP)-binding proteins that share the fundamental biochemical activity of GTP binding and hydrolysis, making them exceptionally suitable to act as binary switches (3). Upon GTP binding, Ras proteins undergo conformational changes that result in the display of a binding surface with a high affinity for downstream effector proteins (4). Binding of an effector protein to an activated GTP-bound Ras protein activates the downstream signal cascade. The structural changes upon GTP binding are mainly confined to two loops of the protein, termed switch 1 and switch 2 (5). The conformational changes are transient, and upon GTP hydrolysis and release of the γ-phosphate, the residues involved in effector binding reorient. Effector

protein is released due to a reduced affinity between the Ras protein and the effector protein and downstream signaling is attenuated (2).

The exchange of Ras-bound GDP for GTP is a slow step, and is often the rate limiting step in the activation of Ras (6). Thus, despite the presence of a high cellular ratio of GTP to GDP (7), Ras proteins favor an inactive steady state. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP and promote the binding of GTP and activation of Ras (3,8,9). Ras proteins have a very low intrinsic GTPase activity. GTP hydrolysis however is greatly enhanced by GTPase activating proteins (GAPs) (3,10). Several GEFs and GAPs may act on a particular Ras protein (3,11), with each responding to a distinct upstream signal; and each regulatory Ras protein itself may in turn affect a variety of downstream effectors (Figure 1). The intracellular location of Ras proteins, which affects the proteins with which Ras proteins can potentially interact, are further regulated by a different class of regulatory proteins. All of these entwine Ras proteins in an intrinsic and complex signaling web, providing multiple signaling avenues for the regulation of Ras proteins, and allowing Ras proteins to integrate an exceptional degree of information and inputs from multiple cellular pathways (3).

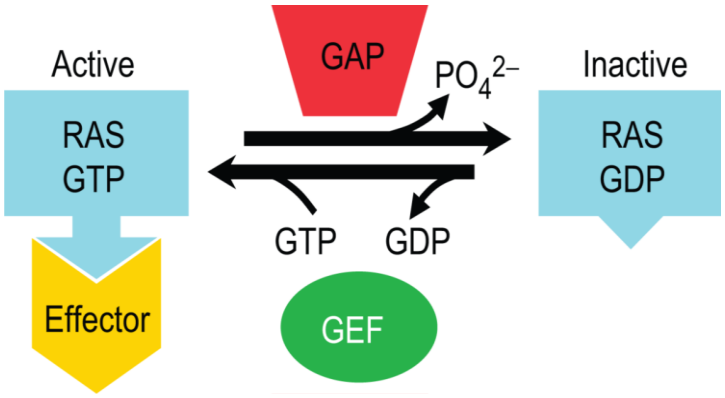

Figure 1. Activation and deactivation of Ras proteins.
Ras proteins exist in equilibrium between active GTP- and inactive GDP-bound forms. GEFs and GAPs regulate the relative amounts of each form. The GTP-bound conformation of RAS shows high affinity interactions with effector proteins that propagate downstream signaling. Figure adapted from (2).

1.1.2. Structural characteristics of Ras proteins

Most proteins in the Ras protein subfamily are small, between 183 to 340 amino acids in length (2). The common function of GTPase activity that is shared by all Ras proteins is reflected in the highly conserved G box sequences, including the G1, G3, G4 and G5 boxes (12,13). The G1 box (aaaGxxxGK(S/T), where a is L, I, V or M and x is any amino acid) contains a purine nucleotide binding motif. The G3 box (blbbDxxGl, where l is hydrophilic and b is hydrophobic) is involved in binding of nucleotide-associated Mg^{2+} ion. Residues that make up the G4 box (bbbb(N/T)(K/D)xD) form hydrogen bonds with the guanine ring of GTP and form stable interactions with residues of the G1 box. The G5 box (bbE(A/C/S/T)SA(K/L)) makes indirect associations with the guanine nucleotide, but is less well conserved among superfamily members.

The G2 box (YPDTIEDSY for Hras) is located in one of the two segments, which changes conformation upon GDP or GTP binding to accommodate for the binding of downstream effectors. The amino acid sequences of the G2 box is the least conserved among the five G boxes- only a threonine residue is highly conserved (2).

Ras proteins can undergo various post-translational modifications which determine their subcelluar localizations. Ras-like GTPases are targeted to the plasma membrane where they undergo post-translational modifications at their C-terminus (14). Prenylation occurs at the CAAX motif, where A is an aliphatic amino acid, and X is the terminal amino acid, followed by proteolytic cleavage of the three C-terminal residues (AAX) and methylation of the lipid modified cysteine (14). Some Ras subfamily proteins like Hras, Nras, Rras, Rap2A, Rap2B and Rap2C contain signals for fatty acid acylation. These proteins have palmitoylated cysteine residues next to their prenylated C-terminus cysteines (2). Other known factors like N-terminal lipidations and the presence of a C-terminal polybasic region also contribute to the localization of Ras proteins (2). There are other post-translational modifications with unknown functions, including phosphorylation (15) and nitrosylation (16,17) of Hras and tyrosine phosphorylation of Rras1 (18).

1.1.3. Biological roles of Ras proteins

The unique biochemical properties of the Ras proteins as binary switches that cycle between active GTP-bound and inactive GDP-bound forms, regulated by a large number of GEFs and GAPs, make them extraordinarily suitable for playing regulatory roles in various physiological processes (1,3,9). Indeed, Ras proteins play critical roles in the control of cell growth (19,20), differentiation (21) and apoptosis (22). They are also involved in the regulation of cytoskeleton (23-25) and are critical players in the integration and transmission of signals among various membrane-bound cellular compartments (26,27). Mutations in the Ras proteins, their effectors, activators and regulators are commonly associated with several pathological conditions, particularly the development of cancer (28,29).

1.2. Rasd1- a member of the Ras superfamily of GTPases

Rasd1 (Ras dexamethasone-induced 1), also known as Dexras1 and activator of G proteins signaling 1 (AGS1) (accession numbers: NM_016084 for *homo sapiens*, and NM_009026 for *mus musculus*) is a 281 amino acid G-protein that belongs to the Ras superfamily of small GTPases. *Rasd1* was originally discovered as a dexamethasone-inducible gene in AtT-20 mouse pituitary cell line (30). *Rasd1* is located on chromosome 17 in humans, and its synergistic region is on the mouse chromosome 7. Its mRNA is detected in several tissues and is up-regulated by glucocorticoids in the brain, heart, kidney, pancreas,

liver and anterior pituitary (30,31). It is most similar to the Ras subfamily that is involved in the regulation of cell growth, proliferation, differentiation and transformation (32). Rasd1 possesses the conserved G boxes necessary for GTP binding and hydrolysis, and a C-terminal CAAX box that is involved in subcellular membrane localization (33). It is however distinguishable from other members of the Ras family by its highly basic net isoelectric point (34). Rasd1 also has a high molecular weight of 34kDa, compared to typical Ras proteins of 22kDa, conferred by its extended C-terminal cationic variable domain (34). Additionally, while most other Ras proteins are membrane associated, Rasd1 can be found in both cytosol and membranes (35).

1.2.1. Rasd1 and its involvement in signal transduction

Rasd1 is known to modulate several signaling pathways with distinct biological functions in each pathway. Biochemically, Rasd1 functions as a G protein-coupled receptor (GPCR)-independent activator of G proteins and acts as a GEF for both monomeric and heteromeric $G\alpha_{i/o}$ proteins (33,36). Upon activation of G proteins and the release of the $G\beta\gamma$ subunits, Rasd1 selectively transduces a signal to the p42/p44 mitogen-activated protein kinase (MAPK) pathway, and not other protein kinase cascades, leading to transcriptional activation of downstream target genes (33). Alternatively, Rasd1 may work with activated GPCRs to enhance or prolong signaling or may compete for a common pool of heterotrimeric G proteins and disrupt receptor-G protein

signaling by reducing the G proteins available for receptor coupling (37). Rasd1 also inhibits the activity of adenylyl cyclase, a target of Gα signaling, and the subsequent production of cyclic AMP (cAMP) (37).

As a Ras family member, Rasd1 regulates various aspects of cell growth and differentiation, and is actively involved in a signaling pathway that causes growth arrest or apoptosis, and thus plays a role in preventing the development of certain tumors (38).

Rasd1 has also been reported to interact with FE65, an adaptor protein that binds to γ-secretase-derived amyloid precursor protein (APP), to directly suppress FE65-APP-mediated transcription (39), thus affecting FE65-APP-regulated genes like *GSK3β* and *APP*, which are involved in the pathogenesis of Alzhiemer's disease.

Rasd1 is also a physiological nitric oxide (NO) effector (35). It interacts with neuronal nitric oxide synthase (nNOS) via CAPON, and this enhances the ability of nNOS to activate Rasd1 (35). nNOS is activated to form NO by glutamate acting through N-Methyl-D-aspartic acid (NMDA) receptors, and Rasd1 is activated when S-nitrosylated by NO to enhance physiological NO signaling (35). Activated S-nitrosylated Rasd1 interacts with peripheral benzodiazepine receptor-associated protein (PAP7), which in turn binds to

divalent metal transporter 1 (DMT1), an iron import channel to regulate neuronal iron homeostasis (40). This glutamate-NMDA-NO-Rasd1-PAP7-DMT1-iron uptake signaling cascade is important in mediating NMDA neurotoxicity (40).

1.2.2. Rasd1 and the circadian clock

Rasd1 was the first G protein to be isolated as an oscillating gene specific to the suprachiasmatic nucleus (SCN) (41,42). Expression of *Rasd1* is regulated by clock genes, and its rhythmic expression was abolished in the SCN in cryptochrome (cry) 1 and 2 double knockout mice (41). All these point to its potential involvement in the regulation of the circadian timing mechanisms. In addition, Rasd1 regulates the Gi-coupled receptor activation of the ERK-MAPK pathway, through the release of Gβγ subunits (33). Upon photic cues, Rasd1 mediates the activation of the MAPK cascade after glutamate signaling via the retino-hypothalamic tract (43). The involvement of Rasd1 in several signaling cascades, in particular its regulation of the p42/p44 MAPK pathway, provide the possibility of Rasd1 acting as a critical regulator of several input pathways of the circadian regulatory mechanisms.

Rasd1 knockout mice appear healthy and exhibit normal behavior (44). Histological analysis of all tissues also did not show any structural or morphological defects (44). Rasd1 knockout mice however showed a reduced

sensitivity to light and displayed an abnormal phase relationship with external light-dark cycles under dim light conditions, manifested by a reduction in the activation of light-induced p42/p44 MAPK pathway in the SCN (45). This abnormality was abolished in mice that had undergone a period of dark adaptation, and this indicates that Rasd1 plays a role in the adaptation of the circadian timings to the memory of external environmental cues (45). While Rasd1 potentiates response of the circadian clock to photic cues, it suppresses responsiveness of the circadian system to non-photic cues (44). Rasd1 is thus crucial in the operation of the mammalian circadian clock- it plays a crucial role in the entrainment and shaping of the responsiveness of the circadian clock to external stimuli and in the interplay of individual oscillators (45).

1.3. Renin-angiotensin system

The renin-angiotensin system (RAS) plays a crucial role in the complex regulation of blood pressure, fluid volume and electrolyte homeostasis in the body (46). The RAS has also been hailed as a growth factor. In fact, RAS is required for normal mammalian development and remodeling of several organs, and in particular, renal development (47-49). There is also increasing evidence showing that the RAS is involved in the accumulation of extracellular matrix, a precursor to fibrosis and glomerulosclerosis, which can lead to renal diseases (48,50-52).

1.3.1. Renin-angiotensin system: The main players

The classical enzymatic cascade of the RAS starts off with the cleavage of angiotensinogen (AGT) to angiotensin I (Ang I) by renin. Ang I is subsequently cleaved by angiotensin I-converting enzyme (ACE) to form angiotensin II (Ang II), the physiologically active peptide of the system. Ang II acts by binding to specific plasma membrane receptors, Ang II type 1 (AT1) and Ang II type 2 (AT2) (53,54) (Figure 2). Most physiological actions of Ang II are mediated via AT1 receptors (53). AT1 receptors are predominantly involved in the regulation of sodium and fluid retention and vasoconstrictor responses (55,56). AT2 receptors are thought to oppose the actions of AT1 in blood pressure control and in cell proliferation (57). AT2 receptors stimulation also decrease renal sodium reabsorption (58).

Ang II is considered the main effector peptide of the RAS. Ang II causes vasoconstriction directly and indirectly by binding to AT1 receptors present on blood vessels and by increasing the sympathetic tone and vasopressin release. Classically, Ang II regulates blood pressure by directly binding to AT1 receptors in the kidneys (53) or by regulating sodium and water reabsorption in the kidneys (59). Ang II is also able to regulate blood pressure indirectly by stimulating the adrenal glands to produce and release aldosterone (60,61), or by producing the sensation of thirst in the central nervous system (46).

Findings over the years have broadened our understanding of the RAS. For example, further processing of Ang II by aminopeptidase A and aminopeptidase N produces Ang III (Ang 2-8) and Ang IV (Ang 3-8) respectively; and an Ang II type 4 (AT4) receptor that binds Ang 3-8 preferentially to cause vasodilation has also been identified (62). Alternative pathways for the production of Ang II have also been discovered- a renin-independent production of Ang II from AGT has been reported (63,64); the conversion of Ang I to Ang II by other enzymes like chymostatin-sensitive Ang-II-generating enzyme (CAGE), cathepsin G and chymase, and the conversion of AGT to Ang II by cathepsin D, tonin and tissue plasminogen activator (t-PA) has also been reported (65,66) (Figure 2). A renin receptor that binds both renin and its precursor prorenin, and activates ERK1/2 independently of angiotensin production was also recently identified (67).

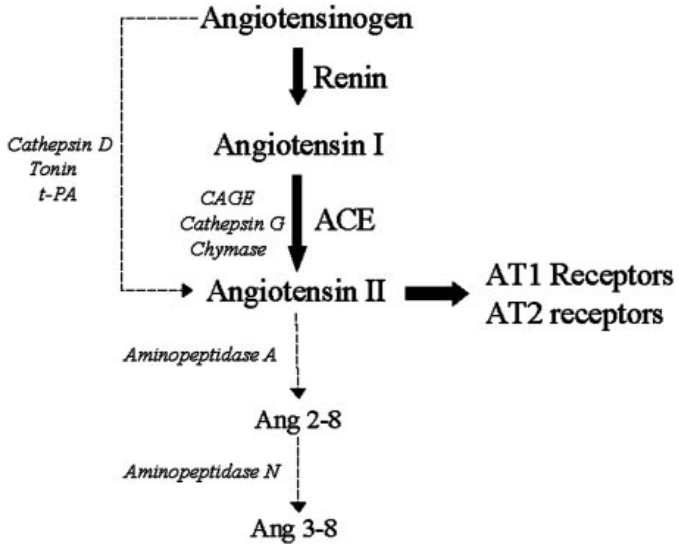

Figure 2. The main player in the RAS pathway.
In the classical RAS (block arrows), AGT is cleaved by renin to form

angiotensin I, which is processed by angiotensin-converting enzyme (ACE) to angiotensin II, which in turn activates the AT1 or AT2 plasma membrane receptor. Findings over the years have broadened our understanding of the RAS. Alternative means for the generation of Ang-II is indicated by dashed arrows. Figure adapted from (68).

1.4. Local Renin-angiotensin systems

For most of its history, the RAS has been considered mainly as a circulating hormone system. However, there has been an increasing amount of evidences in recent years showing that in addition to the circulating systemic RAS, local tissue RASs that regulate tissue specific Ang II production exist. This tissue specific Ang II acts in an autocrine and paracrine manner (68,69). Local tissue RASs have been described in a number of tissues and organs, including the brain (70,71), heart (70,72), vasculature (70), adipose tissue (73), kidney (70), pancreas (74,75), adrenals (76), placenta (77), skin (72,78) and reproductive organs (79) (Figure 3). In some tissues, only some components of RAS are present, which led to the discovery of alternative pathways for the synthesis of Ang II. It is this duality of the RAS, with both tissue specific and circulating hormonal systems working together, that makes the system extremely complex.

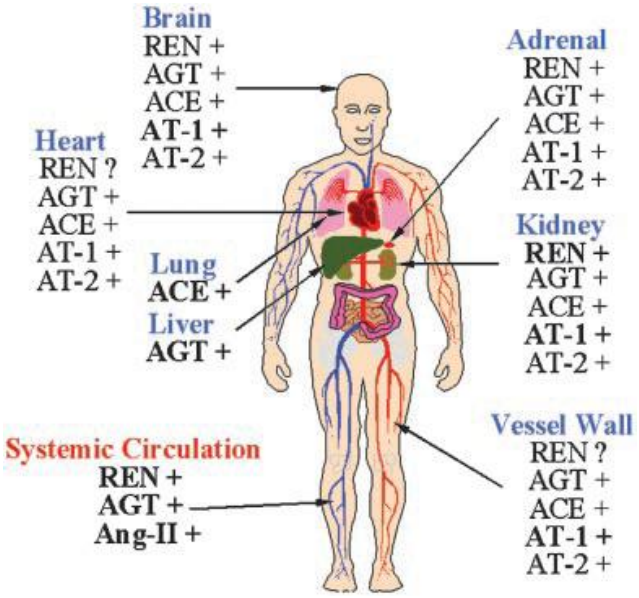

Figure 3. Expression sites of the RAS and its components.
Classical sites of synthesis for the endocrine RAS are in bold. Figure adapted from (68).

1.4.1. Functions of the local RASs

Our understanding of the roles and functions of local tissue RASs is limited, but local RASs have been shown to be involved in normal organ development, cell growth and proliferation, mitogenesis and in diseases such as hypertension and heart diseases, in addition to the traditional functions of RAS in the regulation of fluid, blood and electrolyte balance. More specifically, local RAS in the heart is involved in cardiac remodeling, cell growth and proliferation as well as mediating responses to cardiac stress (80-83); in the vasculature, local RAS affects the growth of vascular cells and binds to AT1 receptors on vascular smooth muscle cells to mediate vasoconstriction (54,84); RAS in the reproductive tract is a mediator involved in ovarian function and follicle

maturation (85), as well as male fertility (86,87) and the ability of the human sperm to penetrate an egg (88); in the skin, RAS has been shown to be involved in wound healing and repair (89-91); in the pancreas, RAS regulates the secretion of pancreatic hormones (92); in adipose tissues, RAS plays a role in sympathetic nervous system-mediated thermogenesis (93).

1.4.2. RAS in the central nervous system

It is well documented and firmly established that the brain has its own complete and intrinsic RAS, which is distinct from peripheral RAS. The central nervous system (CNS) contains all components of the RAS required for the synthesis of the bioactive forms of angiotensin (94). The brain RAS is however, not entirely independent from the peripheral system. In specific areas of the brain not bound by the blood-brain barrier, circulating periphery angiotensins can act and interact with the brain RAS (95).

The brain RAS mediates several classic physiological effects like salt, water, electrolyte and blood pressure homeostasis (95-97). In addition, it is also involved in sexual behaviors (98), regulation of pituitary hormones and maintenance of the blood-brain barrier (99,100). The brain RAS is also involved in neurological functions like stress (101-103), learning and memory (101-103), and behavior and emotions (104,105). There is also increasing

evidence to suggest that the RAS is involved in neurodegenerative disorders like Alzheimer's (106-108) and Parkinson's disease (108,109).

1.5. Renin

Renin, an aspartyl protease, is part of the enzymatic cascade that leads to the formation of the vasoactive peptide, Ang II. It is a commonly accepted fact that the first and rate limiting step in the production of all subsequent angiotensin peptides is the cleavage of AGT by renin. Thus, accordingly, a local RAS can only be present if renin is also present or can somehow gain access to that particular organ (110).

In the systemic circulatory RAS, renin is synthesized and released from the kidney juxtaglomerular cells, located in the afferent arteriole of the glomerulus (59). Factors that influence renin release include sodium chloride balance, arterial blood pressure, extracellular fluid volume, and stress and trauma (111). Renin release is also stimulated via sympathetic nerve stimulation of the adrenergic receptor on the juxtaglomerular apparatus (112). The main tonic regulator of renin secretion is through negative feedback regulation by Ang II, which inhibits the release of renin by acting on AT1 receptors on the juxtaglomerular cells (113).

Renin is produced as pre-prorenin protein, and enters the endoplasmic reticulum where the signal peptide is cleaved off during transfer to yield prorenin. Prorenin is then directed to the Golgi apparatus, and cells export the newly synthesized and post translationally modified renin from the Golgi apparatus to small clear vesicles for immediate secretion by the constitutive pathway. Alternatively, prorenin tagged for the regulated pathway of secretion is contained in protogranules that coagulate to form mature renin granules where the pro-segment is cleaved off to give activated renin. The mature granules are stored and released upon signal induction via regulated exocytosis (114) (Figure 4).

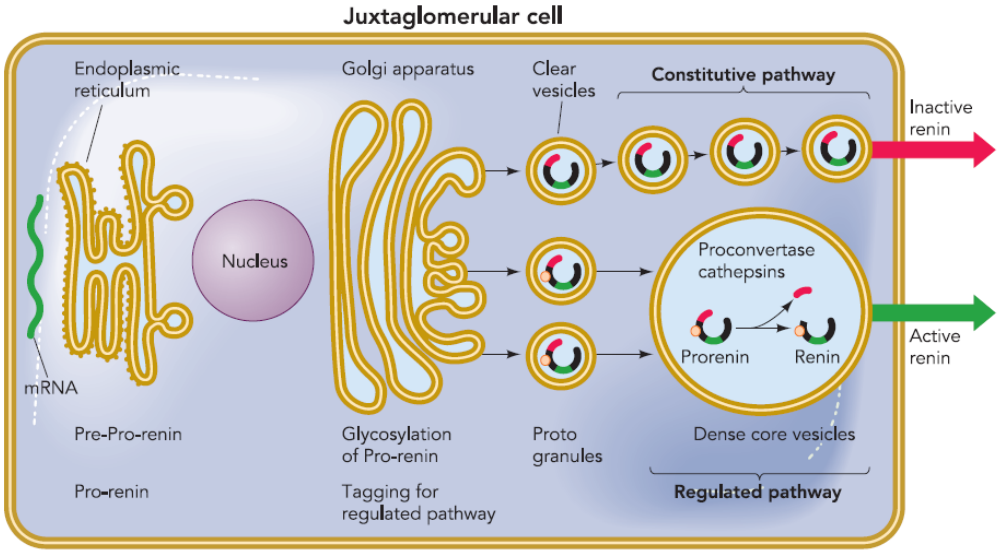

Figure 4. Renin synthesis and renin secretion in juxtaglomerular cell. Renin is synthesized as pre-prorenin which is transferred to the endoplasmic reticulum, then the Golgi apparatus, where it is then either exported to small clear vesicles for immediate secretion via the constituitive pathway or directed to protogranules to be further processed for regulated secretion. Figure adapted from (114).

1.6. Transcriptional regulation of renin

1.6.1. Renin promoter and its regulatory elements

Renin gene expression is largely regulated at the transcriptional level, although post transcriptional regulation has also been reported (115,116). Transgenic studies using GFP or SV40 T antigens as reporters have shown that approximately 4.1 kb of the mouse renin 5' flanking sequence is sufficient for the correct temporal and spatial expression of renin in mouse embryonic, extra-embryonic and adult tissues (117,118). This provides strong evidence that the most important regulatory elements of the renin gene reside within this region.

1.6.2. Renin promoter

The 123 bp mouse renin proximal promoter (-117 to +6) has more than 80% sequence identity with the rat and human renin genes (119,120). The HOX-PBX binding site lies in the region -72 to -50, and is critical for renin expression (121). In addition to the HOX-PBX binding site, there are other important transcription factor binding sites within the renin proximal promoter (122). Especially important is the region -197 to -70, located immediately 5' to the HOX-PBX binding site. In As4.1 cells, deletion of this region in a reporter construct containing 4.1 kb of the renin 5' flanking sequence abolishes renin promoter activity by up to 99%. Six cis-acting elements have been identified to bind to region. Two NF1-binding sites and an Sp1/Sp3 binding site lie in the

distal portion of this region, and three other sites with unknown binding factors are all required for maximum renin gene expression in As4.1 cells (122,123).

In the more distal promoter region, a consensus sequence has been identified as the binding site for CBF1, a nuclear effector of the Notch signaling pathway (123). The Notch signaling pathway may be involved in tissue specificity and developmental regulation of renin gene expression. In addition, a CNRE site (an overlapping cAMP response element and a negative response element) has been identified (124), where LXRα, a member of the nuclear receptor superfamily, binds to and mediates the cAMP response (125).

1.6.3. Renin enhancer

The renin enhancer is a complex element that responds to both stimulatory and inhibitory stimuli (122,123). A 242 bp element (-2866 to -2625) has been identified in the 5' flanking sequence of the renin gene to act as a potent classical transcriptional enhancer of the renin gene (121). This classic enhancer lies about 2.6 kb upstream of the mouse renin gene, and is homologous to a sequence about 11 kb upstream of the human renin gene (121,126,127). The human enhancer shows 71% identity, and the rat enhancer shows 85% identity to the mouse enhancer (121,126,127). The renin gene enhancer works in a position and orientation independent fashion (121). The transcriptional enhancer contains several transcription factor binding sites that have both

excitatory and inhibitory regulatory functions (123,128-130). Within the enhancer, eleven transcription factor binding sites have been identified, among which, a CRE and an adjacent E-box are the most crucial in regulation of basal gene expression of the renin gene (130). CREB/CREM binds at the CRE and USF1/USF2 binds at the E-box. Two TGACCT motifs, separated by a 10 bp, are located downstream of the E-box in the renin enhancer. RAR/RXR bind to these two sites, and mediates the induction of the renin promoter activity by retinoic acid (128). Vitamin D has also been observed to bind to this sequence to downregulate the renin promoter activity (128,131). The orphan nuclear receptor Ear2 has also been shown to bind to these TGACCT motifs (132). Close to the 3' end of the enhancer, and overlapping with the downstream TGACCT motif, is an NF-Y binding site that acts as a transcriptional repressor (131). It has been hypothesized that NF-Y blocks enhancer activity by preventing the binding of transcription factors to the TGACCT motif (129). Within the distal portion of the enhancer, six additional transcription factor binding sites have been identified. These include four NF1-binding, an Sp1/Sp3 binding and an unknown transcription factor-binding site (133). Each transcription factor-binding site is required for enhancer-mediated transcriptional regulation of the renin gene; the regulation of renin transcription is an orchestra of complex interactions between these binding sites, and the factors and cofactors that they recruit.

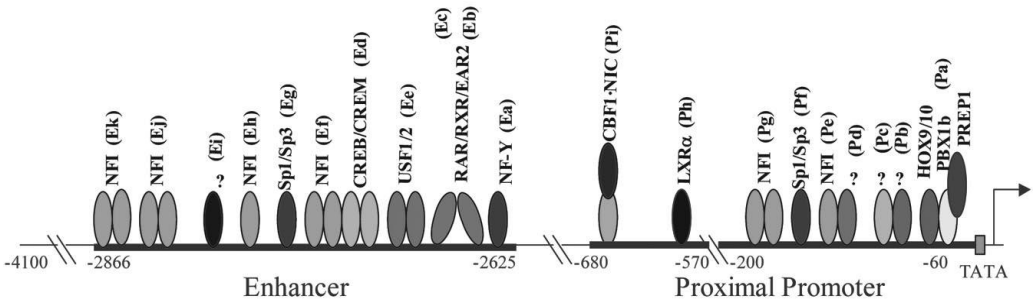

Figure 5. Schematic representation of transcription factor-binding sites within the 5' flanking sequence of the renin gene.
Shown are identified transcription factor-binding sites within the proximal promoter (Pa-Pi) and the enhancer (Ea-Ek). Transcription factors binding to these sites are labeled as described in the text. Unidentified transcription factors are labeled with a question mark (?). Figure adapted from (123).

1.6.4. Regulation of renin transcription by Ear2

The TGACCT motif is recognized by members of the steroid hormone nuclear receptor family. The renin enhancer contains two TGACCT repeats separated by an atypical 10 bp and acts as a retinoic acid response element (RARE) (128). It is required for both baseline activity of the renin enhancer and for retinoic acid-mediated induction of the renin promoter (128). Using EMSA and mutagenesis experiments, orphan nuclear receptor Ear2 has been shown to bind specifically to this RARE site (132). Upon binding, Ear2 attenuated the increase in renin promoter activity, in the presence or absence of retinoic acid induction (132). Ear2 can also regulate the expression of endogenous mouse renin gene in As4.1 cells (132). Ear2 negatively regulates renin expression by directly competing with retinoic acid receptor/ retinoid X receptor (RAR/RXR) for its binding to the RARE of the renin enhancer (132). There are two other

TGACCT motifs and one AGGTCA motif within the 4.1 kb of the mouse renin 5' flanking sequence, and it has been suggested that Ear2 are able to bind to these other RARE half sites as well to further repress renin transcription (132).

1.7. Orphan nuclear receptors

1.7.1. What are orphan nuclear receptors?

Nuclear receptors belong to a large and diverse family of ligand-activated transcription factors that have the ability to bind to hormone response elements on DNA directly to regulate the expression of specific target genes, and hence are extremely crucial to the growth and development, morphogenesis, metabolism, physiology and homeostasis of an organism (134-139). Nuclear hormone receptors are known to mediate response to steroid and thyroid hormones, retinoids and Vitamin D (135,136,140). Nuclear receptors show a high degree of similarity both structurally and functionally, and are defined structurally by a conserved DNA binding domain and a ligand binding domain. These domains are largely responsible for the nuclear receptors function in the regulation of gene expression upon ligand binding (141). There are a vast number of nuclear receptors without known ligands and defined gene families, and these proteins are referred to as orphan nuclear receptors (142).

1.7.2. Biological functions of orphan nuclear receptors

Orphan receptors are thus by definition receptors by which no ligands are known. This in itself is a paradox because the term receptor implies that a physiological ligand should be present. Orphan receptors possibly act in a constitutive manner or are activated by other means such as phosphorylation (137). Following this, the orphan receptors form a highly diverse group- their structures are highly diverse at the ligand binding domain and other domains and they have various modes of interacting with DNA (143-145). However, they are no less important than classic receptors. In fact, they often play essential roles in the development and overall well-being of an organism.

All orphan receptors play a role that is specific to each of them, and they often play an important role in modulating the action of classic liganded receptors (141,146-148). Many orphan receptors play important roles in development (149), cell differentiation (150,151), metabolism (152) and physiology (153). Functional defects in orphan receptors have been associated with different diseases including arthrosclerosis, diabetes and cancer. More recently, orphan receptors are found to be involved in regulation of circadian timing systems (139,154).

1.7.3. COUP-TFs and Ear2

Of the superfamily of nuclear hormone receptors, the orphan receptors COUP-TFI (Ear3), COUP-TFII (Arp-1) and Ear2 (also known as COUP-TFIII or Nr2f6) belong to the Nr2f or COUP-TF subfamily, with Ear2 being more distantly related (155) (Figure 6A). COUP-TFs are arguably one of the best characterized nuclear orphan receptors. COUP-TFs bind to a number of different direct and indirect repeats with variable spacing between the repeats (156) to affect a large repertoire of genes (134,157-159). COUP-TFs are distinguishable from other nuclear receptors on several aspects. These nuclear receptors show a remarkably high degree of evolutionary conservation and homology between species (155). Within the Nr2f subfamily, the homology between the DNA-binding domain and ligand-binding domain is exceptionally high (Figure 6B). All of this strongly suggests that nuclear orphan receptors play important biological functions, even though a ligand has yet to be identified.

COUP-TFs have been shown to inhibit the transcriptional activity of receptors by several mechanisms- passive repression via DNA binding competition (160), hetero-formation with co-activators or co-regulators like RXR thus decreasing their availability (161), interference with formation of preinitiation complex (162) and transrepression of gene activation (162,163). COUP-TFs have also been shown to act as positive regulators of transcription (164,165).

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rCOUP-TFI MAMVVSSWRDPQDDVAGGNPGGPNPAAQAARGGGGGEQQQAGSGAPHTPQTPGQPGAPATPGTAGDKGQG-P--PGSG--   75
rCOUP-TFII MAMVVSTWRDPQDEVPGSQ--GSQ-ASQAPPVPGPPP------GAPHTPQTPGQGGPASTPAQTAAGSQGGPGGPGSDKQ   71
rEAR-2   MAMVTGGWGGP---GGD--------TNGVDKAGGSYPR-----ATEDDSASP--------PGATSDAE------PGDE-E   49

rCOUP-TFI QSQQHIECVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLTYTCRANRNCPIDQHHRNQCQYCRLKKCLKVGMRREAVQR  155
rCOUP-TFII QQQQHIECVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNLSYTCRANRNCPIDQHHRNQCQYCRLKKCLKVGMRREAVQR  151
rEAR-2   RPGLQVDCVVCGDKSSGKHYGVFTCEGCKSFFKRTIRRNLSYTCRSNRDCQIDQHHRNQCQYCRLKKCFRVGMRKEAVQP  129

rCOUP-TFI GRMPPTQPNPGQYALTNGDPLN--GHCYLSGYISLLLRAEPYPTS-RYGSQCMQPNNIMGIENICELAARLLFSAVEWAR  232
rCOUP-TFII GRMPPTQPTHGQFALTNGDPLN--CHSYLSGYISLLLRAEPYPTS-RFGSQCMQPNNIMGIENICELAARMLFSAVEWAR  228
rEAR-2   GPIPHALPGPAACSPPGAAGVEPFAGPPVSELIAQLLRAEPYPAAGRFG----GGGAVLGIDNVCELAARLLFSTVEWAR  205

rCOUP-TFI NIPFFPDLQITDQVSLLRLTWSELFVLNAAQCSMPLHVA-VLAAAGLHASPMSADRVVAFMDHIRIFQEQVEKLKALHVD  311
rCOUP-TFII NIPFFPDLQITDQVALLRLTWSELFVLNAAQCSMPLHVAPLLAAAGLHASPMSADRVVAFMDHIRIFQEQVEKLKALHVD  308
rEAR-2   HAPFFPELPAADQVGLLRLSWSELFVLNAAQAPVPLHTAPLLAAAGLHAGPMAAERAVAFMDQVRAFQEQVDKLGRLQVD  285

rCOUP-TFI SAEYSCLKAIVLFTSDACGLSDAAHIESLQEKSQCALEEYVRSQYPNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVG  391
rCOUP-TFII SAEYSCLKAIVLFTSDACGLSDVAHVESLQEKSQCALEEYVRSQYPNQPTRFGKLLLRLPSLRTVSSSVIEQLFFVRLVG  388
rEAR-2   AAEYGCLKAIALFTPDACGLSDPAHVESLQEKAQVALTEYVRAQYPSQPQRFGRLLLRLPALRAVPASLISQLFFMRLVG  365

rCOUP-TFI KTPIETLIRDMLLSGSSFNWPYMSIQCS  419
rCOUP-TFII KTPIETLIRDMLLSGSSFNWPYMAIQ--  414
rEAR-2   KTPIETLIRDMLLSGSTFNWPYGSG---  390
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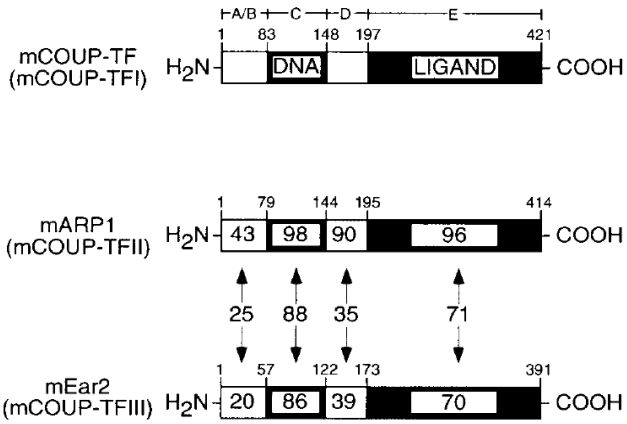


Figure 6. Alignment and relationship between members of the COUP-TF family.

(A) Rat COUP-TFII and EAR2 protein sequences alignment. COUP-TFII and EAR-2 were aligned with COUP-TFI, with the three characters '*', ':' and '.' used to mark strongly conserved positions. The extent of the DNA-binding domain is highlighted with a bar underlining the sequence alignment. Figure adapted from (166).

(B) Relationship between COUP-TF family members. The amino acid identities between domains of mouse COUP-TFI and both mouse ARP1 and mouse Ear2 are indicated as percentages within each schematic domain. The corresponding

identities between ARP1 and Ear2 are indicated within double-headed arrows. Alignments were carried out using Clustal X. Figure adapted from (167).

1.8. Ear2

Although much research has been carried out on other members of the COUP-TF family, little is known regarding the importance of Ear2 and its functions *in vivo*. Ear2 is a 390 amino acid protein that (accession numbers: NM_005234.3 for *homo sapiens*, and NM_010150.2 for *mus musculus*) is expressed in the heart, placenta, liver, skeletal muscle, kidney and pancreas (159), and is especially abundantly expressed in the heart, liver and pancreas, and in the fetal liver and mouse embryo (159,168,169).

Ear2 forms homodimers and heterodimers with COUP-TFI (158,170) and COUP-TFII (167) or with thyroid hormone receptor β (159), and these dimers are able to bind to enhancers present in a wide range of genes (134,157,158). For example, Ear2 binds to COUP-TFII to repress hormonally-induced estrogen-stimulated transcriptional activity of the human oxytocin gene promoter by binding to an estrogen response element (157,171). Ear2 also associates with thyroid hormone receptor β to inhibit binding of thyroid hormone receptor β to the thyroid hormone response elements (159).

Ear2 knockout mice are born alive, viable and fertile, but possess abnormal locus coeruleus (LC) development (139). Ear2 is thus deemed critical for the early development of the LC and acts relatively upstream in a developmental signaling cascade that specifies the noradrenergic cell fate of LC neurons (139). The LC is the source of many neurotransmitters, and expresses domapine-β-hydroxylase (Dbh) and tyrosine hydroxylase (Th), which are required for the synthesis of noradrenaline/ norepinerphrine (NA), the main neurotransmitter of the LC (172). The LC is the main source of NA in the mammalian brain (173). The LC has an extensive web of neuronal connections that allows NA and other LC neurotransmitters to reach nearly all regions of the central nervous system, including the cortex and spinal cord (174-176). Thus, the LC influences a broad spectrum of behavioral and physiological processes including nociception, sleep/wake cycle, alertness, arousal, stress, cognition, memory and attention (176-179). It is not surprising that deficits in LC function are associated with severe neurological conditions such as Parkinson syndrome, depression, epilepsy and attention deficit hyperactivity disorder (173,177).

The LC possesses neuronal connections to the central circadian clockwork of the suprachiasmatic nucleus (SCN) of the hypothalamus, and to various areas of the brain (174,175). Interestingly, the firing rate of the LC neurons exhibits a circadian rhythm (174). In addition to lacking about 70% of the LC, Ear2 knockout mice also possess a spectrum of circadian defects (139). Ear2

knockout mice show a reduction in anticipatory locomotor activity, delayed re-entrainment to a shifted LD cycle, abolished circadian expression pattern of clock gene Per1 in the cortex, dampened Per2 expression, reduced rhythm accuracy, and a significant reduction of cortical NA concentration (139). Ear2 knockout mice also exhibit increased thermal nociception (139).

On top of the roles it plays in the central nervous system, Ear2 is also involved in the regulation of autoimmunity and peripheral immunological tolerance (180). Ear2 was found to suppress lymphocyte activation and T Helper 17-dependent autoimmunity by interfering with the nuclear factor of activated T-cells-dependent transcription of cytokine interleukin-17A (IL-17A) (180). Ear2 knockout mice displayed hyperactive immune phenotypes- they possessed splenomegaly, had high levels of serum immunoglobulin G (IgG) and immunoglobulin E (IgE), and developed a late-onset of lupus-like autoimmune disease (180). Thus, Ear2 is also involved in the modulation of unwarranted inflammation.

CHAPTER 2: AIMS AND SIGNIFICANCE

Rasd1 was first discovered in 1998 as a dexamethasone-inducible gene in AtT-20 cells (30). But till now, its downstream targets remain elusive and its related signaling pathways have not been definitively established. Rasd1 is extremely important because as a binary switch that is entwined in a complex web of signaling pathways, it is involved in the transcriptional regulation of several target genes (33). Rasd1 regulates important physiological processes, including cell growth, proliferation differentiation and transformation (32), and is also involved in the control of the circadian clock (41,42).

The aim of my project is to characterize the functions and molecular mechanisms of Rasd1. The first step lies in the identification of proteins that interact with Rasd1. Using yeast two-hybrid analysis, several proteins that specifically interact with Rasd1 have been identified. The functional significance of the interaction between Rasd1 and Ear2 was chosen for further study as we believe that it was of no coincidence that both Rasd1 and Ear2 are involved in neurological developments and in the regulation of the circadian rhythm.

Firstly, the physical interaction between Rasd1 and Ear2, both *in vitro* and *in vivo*, was confirmed. As Ear2 is a negative regulator of renin gene transcription (132), I proceeded to determine the potential role that Rasd1 might play in the Ear2-mediated regulation of renin gene transcription. The aim is to elucidate their interacting domains and the critical domains required for functionality, determine the biochemical activities of Rasd1 involved and attempt to explain the implications of the interaction of Rasd1 and Ear2 in the regulation of renin gene transcription and propose a model for the molecular mechanisms involved.

Understanding the roles and mechanisms that Rasd1 plays in the regulation of Ear2-mediated renin transcription will provide us with critical insight on the intrinsic and complex regulation of the RAS. Only with concrete genetic and molecular knowledge of the signaling and regulatory pathways can we hope to understand the RAS better and be in a better position to propose new drug targets that aim to alleviate, or more ideally to eradicate, the clinical manifestations of some of the phenotypes, including hypertension, heart and cardiovascular diseases that arise from the dysregulation of the RAS.

CHAPTER 3: MATERIALS AND METHODS

3.1. Yeast two-hybrid assay

3.1.1. Yeast strains and vectors used in yeast two-hybrid assay

Two yeast strains, Y187 and AH109, and two commercial vectors, pACT2 and pGBKT7, were used in the yeast two-hybrid assay.

The *Saccharomyces cerevisiae* strains used are listed below.

Strain	Genotype	Reporter(s)	Transformation markers	References
Y187	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met$^-$, gal80Δ, URA3::GAL1$_{UAS}$-GAL$_{TATA}$-lacZ, MEL1	lacZ, MEL1	trp1, leu2	(181)
AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, MEL1, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ	HIS3, ADE2, lacZ, MEL1	trp1, leu2	(182)

MATCHMAKER two hybrid system cloning vectors used are listed below.

Vector	Description	Selection on SD medium	Size (kb)	References
pACT2	$GAL4_{(768-881)}$AD, LEU2, amp^r, HA epitope tag	-Leu	8.1	(183)
pGBKT7	$GAL4_{(1-147)}$ DNA-BD, TRP1, amp^r	-Trp	7.3	Clonetech; (184)

Table 1. Yeast strains and expression vectors used in yeast two-hybrid screening.

Bait plasmid pGBKT7-Rasd1 was generated by inserting PCR generated 843 bp full length mouse *Rasd1* cDNA into *Nde*I and *Eco*RI digested pGBKT7 as an in- frame fusion with the DNA binding domain (DNA-BD) of GAL4 at its 3' end. Mouse brain MATCHMAKER cDNA library culture (Clonetech) was cloned into pACT2 vector.

3.1.2. Yeast two-hybrid screening

Yeast strain AH109 was transformed with pGBKT7-Rasd1 and nutritionally selected on synthetic dropout (SD) –trp plates. Mouse brain cDNA library cloned into pACT2 was then co-transformed into the AH109 cells that contained pGBKT7-Rasd1 and plated on SD-trp-leu medium to nutritionally select for the yeast cells successfully transformed with both plasmids. Putative positive clones were subjected to a second round of more stringent nutritional selection on SD-ade-his-trp-leu plates. After two rounds of nutritional selection,

colonies were assayed for β-galactosidase (β-gal) activity by use of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate.

Positive colonies from β-gal assay were sub-cultured and grown to saturation in SD-leu medium to expel bait plasmid pGBKT7-Rasd1. Library plasmids from positive interacting colonies were recovered and amplified in *E.coli*. They were then analyzed by PCR with primers MATCHMAKER 5' AD LD-Insert screening amplimer and MATCHMAKER 3' AD LD-Insert screening amplimer, and restriction digests. The clones were sequenced using GAL4 activation domain sequencing primer. BLAST searches (National Centre for Biotechnology Information, NCBI) were performed to identify these clones.

3.1.3. Confirming the specificity of interaction

The specificity of protein-protein interaction was further tested by yeast mating. Y187 was transformed with either pGBKT7-Rasd1 or pGBKT7 vector alone and nutritionally selected in medium lacking tryptophan. AH109 was transformed with either pACT2 vector containing putative positively interacting clones or pACT2 vector alone and grown in medium lacking leucine. To verify the putative positively interacting clones, the transformed yeast strain AH109(MATa) were then mated with the transformed Y187(MATα) and grown in medium lacking tryptophan and leucine. The cells were then replica plated

on SD-ade-his-trp-leu for a second round of nutritional selection and re-assayed for β-galactosidase activity.

3.1.4. Yeast transformation using LiAc/PEG method

Yeast transformations were carried out using high efficiency lithium acetate (LiAc)/ poly-ethylene glycol (PEG) method (185). Yeast cells were grown in 50 ml of appropriate SD medium for 16-18 hours with shaking at 250 rpm to stationary phase, OD_{600}>1.5. The culture was sub-cultured to an OD_{600} of between 0.2 and 0.3. After incubation at 30^{o}C for 3 hours with shaking at 230 rpm, the OD_{600} should be between 0.4 and 0.6. The yeast competent cells were then harvested by centrifuging and re-suspending in distilled water twice at 1,000 x g for 5 minutes at room temperature. Yeast competent cells were suspended in freshly prepared 1.5 ml of 1X TE/LiAc solution. 0.1 ml of yeast competent cells was added to 0.1 µg of the plasmid DNA to be transformed, along with 0.1mg salmon testes carrier DNA (Sigma). 0.6 ml of PEG/ LiAc solution (40% PEG 4000, 1X TE buffer, 1X LiAc) was then added and vortexed to mix. The mixture of DNA and yeast was incubated at 30^{o}C for 30 minutes with shaking at 200 rpm. After incubation, 70 µl of DMSO was added, and the cells were heat-shocked for 15 minutes in a 42^{o}C water bath, which allowed DNA to enter the cells. The cells were then chilled on ice for 1-2 minutes before being plated on appropriate SD plates to select for transformants carrying the introduced plasmid(s). This LiAc/PEG yeast transformation

protocol was scaled up or down depending on the number of transformations performed.

3.1.5. Plasmid isolation from yeast

The fresh yeast colony was re-suspended in 0.5 ml of S-Buffer (10 mM KPO_4 pH7.2, 10 mM EDTA, 50 mM β-mercaptoethanol, 100 U/ml zymolase) and incubated at 37^{o}C for 30 minutes. After that, 50 µl of lysing solution (0.25M Tris-HCl pH 7.5, 25 mM EDTA, 2.5% SDS) was added and the sample was incubated at 65^{o}C for 30 minutes. 88 µl of 3 M potassium acetate was added and the sample was chilled on ice for 10 minutes. After centrifugation at 14,000 rpm for 10 minutes, the aqueous phase was transferred to a fresh tube 1 ml of 100% ethanol. The sample was placed at -80^{o}C for 1 hour before centrifugation at 14,000 rpm for 10 minutes. The supernatant was discarded and the pellet dried and re-suspended in 20 µl of distilled water.

3.1.6. Transforming E.coli with yeast plasmids

1-2 µl of yeast plasmid solution was added to 40 µl of electrocompetent *E.coli* cells on ice, and the samples transferred to a pre-chilled cuvette with a 0.1 cm gap. Electroporation was carried out using the Electro Cell Manipulator 630 (BTX, Genetronics) with the parameters set to 25 µF, 2,500 V and 200 Ω.

3.2. Making of electrocompetent *E.coli* cells

Electrocompetent cells were generated from TOP10 *E.coli* cells (Invitrogen), with genotype F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G. A single colony was picked and cultured overnight in 10 ml of LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl, pH 7.0), at 37^{o}C in a shaking incubator. 400 ml of LB medium was then inoculated with the 10 ml of fresh overnight culture and grown shaking at 37^{o}C until the OD_{600} is 0.5 to 0.6. The culture was chilled on ice for 30 minutes. After centrifugation at 5,000 rpm for 15 minutes at 4^{o}C, the cells were re-suspended in 400 ml of ice cold sterile water. The cells were spun down again at 5,000 rpm for 15 minutes at 4^{o}C, and re-suspended in 200 ml of ice cold sterile water. The third round of centrifugation was also carried out at 5,000 rpm for 15 minutes at 4^{o}C, and the cells were re-suspended in 8 ml of sterile 10% glycerol. The final round of centrifugation was performed at 10,000 rpm for 15 minutes at 4^{o}C, and the cells were finally re-suspended in 1 ml of sterile 10% glycerol. The cell concentration was at least 3 x 10^{10} cells/ml. This suspension was then frozen in 40 µl aliquots and stored at -70^{o}C.

3.3. Plasmids construction

3.3.1. Plasmids expressing Rasd1 and Rasd1 mutants

pHisHA-Rasd1 was generated by cloning the PCR generated 843 bp coding region of *Rasd1* into *Kpn*I and *Eco*RI digested pcDNA4/HisMax©B (Stratagene) vector. Rasd1 was tagged in frame with the 5' polyHis tag and Xpress epitope present in pcDNA4/HisMax©B. In addition, a hemagglutinin (HA) tag was added in frame with the coding region of *Rasd1* at its 3' terminal, by incorporating sequences encoding for a HA tag in the reverse PCR primer. Similarly, Rasd1[ΔCAAX] was also cloned into *Kpn*I and *Eco*RI digested pcDNA4/HisMax©B. Site-directed mutagenesis based on overlap extension PCR (186) was employed to generate the Rasd1 mutant constructs A178V, G81A and T38N. Two common flanking primers along with specific internal overlapping mutagenic primers were designed. Two fragments of the target sequence were amplified in separate PCRs, each with one flanking primer and one internal overlapping mutagenic primer that contained the mismatched bases. The two PCR fragments that contained the mutation were then used as templates in a subsequent PCR that included the addition of flanking primers. The denatured fragments annealed at the overlaps and were extended and amplified by PCR to form the desired mutant fusion product. The desired mutant fusion PCR products were then cloned into *Kpn*I and *Eco*RI digested pcDNA4/HisMax©B.

3.3.2. Plasmids expressing Ear2 and Ear2 truncated constructs

pGST-Ear2 construct was generated by cloning the PCR generated 1173 bp *Ear2* coding region into *Not*I digested pxJGST vector, tagged in frame with a 5' glutathione-S-transferase (GST) tag. GST-Ear2 truncated constructs carrying DNA sequences corresponding to amino acids 1-193, 1-130, 1-53, 54-390, 131-390 and 194-390 of Ear2 were also cloned into *Not*I digested pxJGST vector. Cloning was carried out using the inFusion cloning kit (Clontech), which employs homologous recombination strategy to fuse the ends of the PCR fragments to the homologous ends of linearized pxJGST vector. To generate the 3' and 5' regions of homology, both forward and reverse PCR primers were designed to include the addition of 15 bp extensions that precisely match the ends of the *Not*I linearized pxJGST vector. The vector was combined with the inserts in a reaction, according to manufacturer's instructions. The infusion enzyme converts the double-stranded extensions into single-stranded DNA and fuses these homologous regions of the inserts to the corresponding ends of the linearized pxJGST vector. pxJGST vector was derived from pxJ-FLAG-S vector (187).

3.3.3. Reporter plasmids used in luciferase assays

p4.1-Luc was generated by cloning PCR generated 4.1 kb of renin 5'-flanking sequence into *Nhe*I and *Hind*III digested pGL3-Basic vector (Promega). The

template used for PCR was BAC clone rp23-240p23 from mus musculus strain C57B2/6J, chromosome 1 (accession number: AC_068906.14) (Invitrogen).

p3XRARE-117P-Luc was generated by first cloning PCR generated 117 bp renin minimal promoter into *Xho*I and *Hind*III digested pGL3-Basic vector (Promega). 1XRARE was then cloned using *Nhe*I and *Xho*I sites and 2XRARE was cloned using *Kpn*I and *Nhe*I sites. Oligonucleotides for 1XRARE and 2XRARE were annealed in annealing buffer (100 mM NaCl, 50 mM HEPES, pH 7.4) at 95^{o}C for 5 minutes, then ramp cooled to 25^{o}C over a period of 45 minutes on a thermal cycler (iCycler, BioRad).

3.3.4. General cloning procedures

PCR reactions were carried out with FastStart Taq DNA polymerase (Roche). Restriction enzyme digestions were carried out according to respective manufacturer's protocol. Restriction enzyme digested DNA was electrophoresed in agarose gels and purified by QIAquick GEL Extraction Kit (Qiagen) or directly purified by QIAquick PCR Purification kit (Qiagen). DNA ligation was carried out with T4 DNA ligase (Invitrogen) according to manufacturer's protocol. Ligation reactions were carried out at a 3:1 ratio of insert to vector and incubated at 4^{o}C overnight. The standard chemical transformation protocol was used to transform TOP10 *E.coli* cells (Invitrogen). Plasmid mini-, midi- and maxi-preparations were performed with QIAprep Spin

Mininprep Kit, QIAGEN plasmid Midi kit and QIAGEN Plasmid Maxi Kit respectively, according to manufacturer's protocol. All clones were checked by restriction analysis and verified by sequencing.

3.4. DNA extraction from bacterial artificial chromosome (BAC) clone

A single BAC colony was inoculated in LB medium and cultured overnight in a 37^{o}C shaking incubator at 250 rpm. Bacteria were harvested by centrifugation at 6000 rpm for 15 minutes at 4^{o}C, and the BAC DNA was extracted using QIAGEN Plasmid Miniprep following manufacturer's instructions with minor modifications. To ensure complete lysis of bacteria cells, two times as much buffer P1, P2 and P3 were used for each sample.

3.5. Mammalian cell cultures

COS-7 cells (American Type Culture Collection, ATCC CRL-1651) were maintained in RPMI1640 (HyClone). As4.1 cells (ATCC CRL-2193) were maintained in DMEM (ATCC). Neuro2a cells were maintained in MEM (Hyclone). HEK293T cells were maintained in DMEM (Hyclone). All media was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were grown in a 37^{o}C incubator, with 95% air and 5% CO_2.

3.6. Mammalian cell transfection

Cells (3.0-3.5 x 10^5) were cultured in each well of 6-well culture plates 24 hours before transfection.

For protein binding assays and interaction studies, COS-7 cells were transfected with pGST-Ear2 (2.5 μg) or pxJGST vector (2.5 μg) or pHisHA-Rasd1 (3.5 μg). In co-transfection experiments, pHisHA-Rasd1 (3.5 μg) was transfected together with pGST-Ear2 (or its truncated constructs) (2.0 μg) or with the respective carrier vectors as negative controls. Total amount of transfected DNA in all transfections were held constant with the appropriate amounts of respective DNA carrier plasmids

For luciferase assays, COS-7, Neuro2a or As4.1 cells were co-transfected with p4.1-Luc or pGL3-basic (2.0 μg), pGST-Ear2 or plasmids expressing Ear2 truncated constructs (0.5- 1.5 μg), pHisHA-Rasd1 or plasmids expressing Rasd1 mutant constructs (0.1- 1.5 μg), and pSV-β-gal (0.5 μg) in the combinations as indicated in the figures. Total DNA concentration was held constant with respective carrier plasmid DNA. Cells were treated with 1μM all-trans retinoic acid 24 hours post-transfection where applicable.

For experiments that measure the endogenous renin levels, As4.1 cells were transfected with pGST-Ear2 (2.5 μg), pHisHA-Rasd1 (3.5 μg) or both.

For chromatin immunoprecipitation assays, 1.5 µg of pHisHa-Rasd1 was transfected.

For immunofluorescence staining and confocal studies, COS-7 cells were transfected with pGST-Ear2 (1.5 µg) or with pHisHA-Rasd1 (2.0 µg). In co-transfection experiments, pHisHA-Rasd1 or its indicated mutant constructs (2.0 µg) was transfected together with pGST-Ear2 (1.5 µg).

Transfection was carried out with DNA (µg) to Lipofectamine 2000 (µl) (Invitrogen) ratio of 1:2, according to manufacturer's instructions. The above was scaled according in 12-well culture plates.

3.7. Protein extraction from mouse brain

Entire mouse brain from wild type mice was gently ground using plastic pestles, resuspended in 1 ml of cold lysis buffer (1% Triton-X 100, 15% Glycerol, 1 mM PMSF, 150 mM NaCl, 100 mM Tris, pH 7.4, protease inhibitor (Roche)) and tip sonicated (X520 CAT) on ice for cycles of 1 minute each, until the brain tissues were completely homogenized. After sonication, the samples were vortexed for 5 min at and pelleted by centrifugation at 13,000 rpm for 20 minutes at 4 °C. The supernatant, containing the protein lysates, was collected and stored at -80^{o}C until required.

3.8. Protein binding assays and Western blotting

3.8.1. GST pull-down assay

COS-7 cells were transfected separately with pxJGST, pGST-Ear2 or pHisHA-Rasd1. Cells were harvested 40 to 48 hours after transfection and lysed with lysis buffer (1% Triton-X 100, 15% Glycerol, 1 mM PMSF, 150 mM NaCl, 100 mM Tris, pH 7.4, protease inhibitor (Roche)) for 20 minutes to 1 hour, on a rotating platform, at 4^{o}C. For cell lysis, 100 µl of lysis buffer was added into each well of a 6-well plate. Crude cell lysate was cleared by centrifugation at 13,000 rpm, 4^{o}C for 20 minutes. GST fusion proteins were immobilized on magnetic glutathione (GSH)-linked beads (Promega) by incubating 200 µl of the respective crude cell lysate with GSH-linked beads on a rotating platform for 30 minutes at room temperature. The beads were then washed 3 times with binding/washing buffer (Promega) and re-suspended in 40 µl of the same binding/washing buffer. Cellular lysate (100 µl) from pHisHA-Rasd1 transfected cells was then incubated with the GST-Ear2 proteins immobilized on magnetic GSH-linked beads on a rotating platform for 1 hour, at room temperature, with 1% bovine serum albumin (BSA) as the blocking reagent. After incubation, the beads were vortexed once, then washed five times with binding/washing buffer, and bound proteins were eluted from the GSH-linked beads by heating in Laemmli buffer at 95^{o}C for 10 minutes.

3.8.2. Co-precipitation assay

COS-7 cells were co-transfected with pHisHA-Rasd1 and pGST-Ear2. Cells were harvested as described above. GST fusion proteins were immobilized on magnetic GSH-linked beads by incubating 200 µl of the cleared crude cell lysate with 30 µl of GSH-linked beads, with 1% BSA, on a rotating platform for 1 hour at room temperature. After incubation, the beads were washed four times with binding/washing buffer, and bound proteins were eluted from the GSH-linked beads by heating in Laemmli buffer at 95^{o}C for 10 minutes.

3.8.3. Co-immunoprecipitation (coIP)

Co-immunoprecipitations were performed by first pre-clearing 200 µl of crude cell lysate with 4 µg of mouse monoclonal IgG_1 antibody (anti-c-Myc antibody, Santa Cruz), for 1 hour, on ice. The cell lysate was then incubated with 20 µl of protein G agarose resin beads (Invitrogen), for 30 minutes at 4^{o}C on a rotating platform, followed by centrifugation at 14,000xg for 10 minutes at 4^{o}C. The supernatant was incubated with 4 µg of anti-GST antibody overnight, followed by 20 µl of resin beads for 1.5 hours, both on a rotating platform at 4^{o}C. The resin was recovered after centrifugation at 13,000 rpm for 10 minutes at 4^{o}C, washed once with PBS buffer and bound proteins were finally eluted from the resin beads by heating in Laemmli buffer at 95^{o}C for 10 minutes.

Immunoprecipitation of endogenous Rasd1-Ear2 complexes was performed using crude lysate from untransfected HEK293T cells, as well as wild type mouse brain crude lysate. Procedure was similar to coIP protocol except that the crude cell lysate was pre-cleared with 80 µl of protein-G agarose resin beads for 30 minutes at 4^{o}C. Goat polyclonal IgG anti-Ear2 antibody (Santa Cruz) was used to carry out immunoprecipitation, and a non-relevant goat polyclonal IgG anti-Tdg antibody (Santa Cruz) served as negative control.

3.8.4. Ni-NTA pull-down

Immobilization of HisHA-Rasd1 was carried out using nickel-nitrilotriacetic acid (Ni-NTA) Magnetic agarose beads (Qiagen) according to manufacturer's protocol. Proteins were eluted from bead by heating in Laemmeli buffer at 95^{o}C for 10 minutes. The Ni-NTA magnetic beads target the 5´-polyHis tag present on our HisHA-Rasd1 construct.

3.8.5. Western blotting

Proteins were analyzed using Western blots. Protein samples mixed with 1X Laemmli buffer were heated at 95^{o}C for 10 minutes, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, CA, USA). Blots were blocked in 5% non-fat milk in TBST (0.1% Tween-20), overnight at 4^{o}C. Primary antibodies used included anti-GST mouse monoclonal IgG_1 (1:5000) (Santa Cruz), anti-

HA mouse monoclonal IgG_{2a} (1:250) (Santa Cruz), anti-Ear2 goat polyclonal IgG (1:250) (Santa Cruz) and goat polyclonal anti-Rasd1 (1:1000) (Abcam). Secondary antibodies used included sheep anti-mouse horseradish peroxidase (HRP)-linked IgG (1:5000) (Amersham, GE Healthcare, UK) and rabbit anti-goat HRP-linked IgG (Abcam). Both primary and secondary antibodies were in TBST (0.1% Tween-20) and were incubated for 1 hour, at 37^{o}C. Detection was performed with Western blot ECL kit detection reagents (Amersham, GE Healthcare, UK). All washes in between incubations were carried out with TBST (0.1% Tween-20), for three times of 10 minutes each, at room temperature.

3.9. Indirect immunofluorescence staining

Indirect immunofluorescence staining protocol was adapted from (39). Transfected COS-7 cells were cultured on glass cover-slips and fixed in 4% paraformaldehyde for 10 minutes. After permeabilization with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes and blocked in blocking solution (5% FBS in PBS) for 1 hour, cells were incubated on a rotating platform for 1 hour with primary antibody diluted in blocking solution, followed by another 1 hour incubation in the dark on a rotating platform with secondary antibody. HisHA-Rasd1 was detected with anti-HA antibody and visualized with goat anti-mouse IgG AlexaFluor 568 (Invitrogen). GST-Ear2 was detected and visualized with anti-GST AlexaFluor 488 (Santa Cruz).

Nuclei were stained by 4´,6-diamidino-2-phenylindole (DAPI) (Sigma). Images were viewed and captured with laser scanning confocal microscope (Carl Zeiss LSM 510 Meta).

3.10. RNA interference experiments

3.10.1. Design of shRNA expressing plasmids

Rasd1 knockdown was achieved by RNA interference (RNAi) using a vector based shRNA approach (188). Oligonucleotides that consisted of a unique 19 bp sequence specific stretch that targeted the open reading frame (ORF) of Rasd1 in both sense and antisense direction, separated by a 9 nucleotide spacer, were designed and synthesized. Restriction sites were added at the 5' and 3' ends to facilitate directional cloning into *Bgl*II and *Hind*III digested pSUPER.puro (Oligoengine) vector. The oligonucleotides were annealed in annealing buffer (100 mM NaCl and 50 mM HEPES, pH 7.4) and incubated at 90°C for 4 min, then at 70°C for 10 minutes. The annealed oligonucleotides were then slowly cooled to 10^{o}C and ligated into *Bgl*II and *Hind*III digested pSUPER.puro vector. When transfected into mammalian cells, the resulting transcript of the recombinant vector was predicted to fold back on itself to form a 19–base pair stem-loop structure, and this stem-loop precursor transcript would be quickly cleaved in the cell to produce a functional siRNA (Oligoengine). Control shRNA was generated by inserting randomly jumbled sequences of the 19 bp sequence specific stretch that targeted the ORF of Rasd1

in both sense and antisense direction into *Bgl*II and *Hind*III digested pSUPER.puro. All clones were checked by restriction analysis and verified by sequencing.

3.10.2. shRNA Knockdown

shRNA knockdown experiments were performed using As4.1 cells, by transfecting Rasd1 targeting shRNA expressing vector or non-targeting shRNA control vector (2 μg/ml). Transfection was carried out with DNA (μg) to Lipofectamine 2000 (μl) (Invitrogen) ratio of 1:2, according to manufacturer's instructions. Selection with puromycin (2 μg/ml) and dexamethasone (100 nM) treatment was carried out 24 hours post transfection. Cells were harvested 48 hours post transfection. Knockdown efficiency was determined by semi-quantitative RT-PCR and Western blotting. Quantification of immunoblots was measured using the GS-800 calibrated densitometer (Bio-Rad).

3.11. Luciferase reporter assay

Luciferase assays were performed using a Luciferase Assay System (Promega) kit according to manufacturer's protocol. Cells were harvested in 1X reporter lysis buffer (Promega) 48 hours post transfection. The firefly luciferase activity was measured by a 20/20^{n} Luminometer (Turner Biosystems). β-gal levels were measured using β-Galactosidase Enzyme Assay System (Promega) according to manufacturer's protocol. Luciferase activity was normalized against β-gal

activity. Basal activity of promoterless enhancerless pGL3-basic was set to 100. Relative luciferase activities for all constructs were obtained from dividing normalized values against values obtained from promoter-less pGL3-basic. At least three sets of biological triplicates were conducted for each experiment. For all luciferase reporter assays, statistical analyses were performed using ANOVA or unpaired t-tests. Error bars shown are standard deviations.

3.12. Real time RT-PCR

Total RNA was harvested from As4.1 cells 24 hours or 48 hours post transfection with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Extracted RNA was used as template for reverse transcription reaction using iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with iTaq SYBR green supermix (Bio-Rad). The cycling conditions were initial denaturation at 95^{o}C for 15 seconds, amplification with cycles of 60^{o}C for 30 seconds (for renin and G3PDH) or 66.5^{o}C for 30 seconds (for Rasd1), 72^{o}C for 40 seconds, and a final extension at 72^{o}C for 10 minutes. The amount of transcribed cDNA was normalized to G3PDH expression with the 7500 Real-time PCR system (Applied Biosystem). The C_T value for each gene was determined in the linear phase of the amplification, and normalized to the C_T value of G3PDH to obtain the ΔC_T. The fold change for each gene was obtained using $2^{-(\text{mean } \Delta CT(gene1) - \text{mean } \Delta CT(gene2))}$. Three independent experiments were performed in biological duplicates.

3.13. Semi-quantitative RT-PCR

Total RNA was harvested from As4.1 cells 24 hours or 48 hours post transfection with Trizol reagent (Invitrogen) according to the manufacturer's protocol. 2 µg of extracted RNA was used as template for reverse transcription reaction, using iScript cDNA synthesis kit (Bio-Rad). Subsequently, 1 µl of the generated cDNA was used as template for each PCR reaction using FastStart Taq Polymerase (Roche). The cycling conditions were initial denaturation at 95^{o}C for 15 seconds, amplification with cycles of 60^{o}C for 30 seconds (for renin and G3PDH) or 66.5^{o}C for 30 seconds (for Rasd1), 72^{o}C for 40 seconds, and a final extension at 72^{o}C for 10 minutes. The number of PCR cycles for semi-quantitative real time RT-PCR was optimized to ensure that the reactions were in the linear range of amplification (G3PDH for 15 cycles, renin for 19 cycles and Rasd1 for 21 cycles).

3.14. Chromatin immunoprecipitation (ChIP)

As4.1 cells (1 X 10^{7}) were crossed linked with 1% formaldehyde in tissue culture media for 10 minutes at room temperature. Cross-linking was quenched with 125 mM glycine for 5 minutes at room temperature. Cells were then rinsed twice with 1X PBS. The cells were lysed in SDS lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS). Sonication was carried out on ice, in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl

pH 8, 167 mM NaCl, PMSF protease inhibitor). The cells were sonicated (Sonics and Materials VC750) for 5 times at 20% power for 30 seconds with 1 minute breaks in between. Sonicated DNA was analysed on 1.5% agarose gel to ensure that DNA was sheared to between 100 bp and 1000 bp, with an average around 350 bp. A sample of sheared chromatin was removed for quantification. Reverse cross-linking was performed by adding 3.2 µl of 5 M NaCl to 80 µl of sample and heating at 65^{o}C for 4 hours. DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA was quantified with a spectrophotometer (Nanodrop 1000, Thermo Scientific).

10 µg of sheared DNA was pre-cleared with 40 µl Protein-G Agarose (Invitrogen) resin beads for 1 hour at 4^{o}C. The supernatant was collected and incubated with 2 µg anti-Ear2 antibody (Santa Cruz), or 2 µg non-relevant IgG antibody as a control, overnight at 4^{o}C on a rotating platform, and subsequently incubated with Protein-G Agarose beads for 1 hour at 4^{o}C. The beads were collected and washed once with low salt wash buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% Triton X-100), once with high salt wash buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 500 mM NaCl, 1% Triton X-100), once with LiCl wash buffer (10 mM Tris-HCl pH 8, 0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA) and twice with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Each wash was carried out for 10 minutes, at 4^{o}C on a rotating platform. Elution was performed twice by adding 225 µl of

elution buffer (1% SDS and 0.1 M $NaHCO_3$), and rotating at room temperature for 15 minutes each time. The supernatant from the elutions were combined and reverse cross-linking was performed. The DNA was recovered by phenol-chloroform extraction, ethanol precipitated and PCR amplified with primers specific for the RARE sites on the renin enhancer. Cycling conditions were 10 minutes at 95^{o}C, then 35 cycles of 30 seconds at 95^{o}C, 30 seconds at 65^{o}C, and 15 seconds at 72^{o}C, and a final extension at 72^{o}C for 10 minutes.

3.15. Electromobility Shift Assay (EMSA)

Two pairs of oligonucleotides were used as probes, one pair labeled with Cy3 at the 5' end (1st base) and the other pair unlabeled. Probes were obtained by annealing oligonucleotides in annealing buffer (100 mM NaCl, 50 mM HEPES, pH 7.4) at 95^{o}C for 5 minutes, then ramp cooled to 25^{o}C over a period of 45 minutes on a thermal cycler (iCycler, BioRad). 50 ng of probe was incubated with 5 µg of the indicated purified proteins or BSA in EMSA binding buffer (50 mM Tris-HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol, 1 mM DTT) at 50^{o}C for 20 minutes. For supershift, 1.0 µg of anti-Ear2 antibody was added to the reaction. For competition with unlabeled probe, a 200-fold molar excess of unlabeled probe was added. The final volume of the binding reaction was kept at 20 µl. The mixture was resolved by 6% non-denaturing PAGE gel in 0.5X TBE at 180 V for 40 minutes. The gel was

scanned with the Typhoon Trio scanner (Amersham Biosciences) for visualization of labeled probes.

CHAPTER 4: RESULTS

4.1. Yeast two-hybrid

In order to identify proteins that interact with Rasd1, I performed a yeast two-hybrid screen using Rasd1 as bait against a mouse brain cDNA library. Two rounds of screening yielded thirteen unique positive clones which were subjected to sequencing. Their identities were determined using NCBI BLAST searches against nucleotide and EST databases. Out of the thirteen clones, eight contained in frame fusion of Gal4-AD with known cDNA open reading frame (ORF) (summarized in Table 2). Three clones carried out of frame fusions with the Gal4-AD, and the sequences of another two clones did not match any known genes.

No	Gene	Gene Name	Accession No.	No. of independent fusions	Cellular localizations
1	*Cenpb*	*Mus musculus* centromere protein B	NM_007682.2	1	Nucleus, chromosome, centromeric region
2	*Gnb1*	*Mus musculus* guanine nucleotide binding protein, beta 1	NM_008142.3	1	Plasma membrane

3	*Nr2f6*	*Mus musculus* nuclear receptor subfamily 2, group F, member 6	NM_010150.2	3	Nucleus
4	*Plscr1*	*Mus musculus* phospholipid scramblase 1	NM_011636.2	1	Membrane
5	*Sh3gl2*	*Mus musculus* SH3-domain GRB2-like 2	NM_019535.2	1	Cytoplasm, cytosol, membrane, plasma membrane
6	*Supt16h*	*Mus musculus* suppressor of Ty 16 homolog	NM_033618.2	1	Nucleus, nucleoplasm
7	*Trp53bp2*	*Mus musculus* transformation related protein 53 binding protein 2	NM_173378.2	1	Cytoplasm, nucleus
8	*Ywhah*	*Mus musculus* tyrosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein, eta polypeptide	NM_011738.1	1	Cytoplasm

Table 2. Proteins that interact with Rasd1, as identified from yeast two-hybrid.

Guanine nucleotide binding protein, beta 1 (Gnb1) and phospholipid scramblase 1 (Plscr1) have been previously identified to interact with Rasd1 (189,190), and

this adds credence to the yeast two-hybrid screen. However, it was decided to focus subsequent research to further explore the significance of the interaction between Rasd1 and mouse nuclear receptor subfamily 2, group F, member 6 (Nr2f6), also known as Erb-A related protein 2 (Ear2), because Ear2 was the only positive clone from the yeast two-hybrid assay that presented multiple hits. Furthermore, similar to Rasd1, Ear2 has been shown to be involved in neurological developments and in the regulation of circadian clock mechanisms.

4.2. Ear2 interacts with Rasd1

4.2.1 Rasd1 and Ear2 interact in vitro

To confirm the specificity of biochemical interaction between Rasd1 and Ear2, an *in vitro* binding study was conducted. Rasd1 and Ear2 proteins were overexpressed in COS-7 cells. COS-7 cells are derived from kidney cells of the African green monkey and expresses the SV40 T antigen (191). Both mammalian expression vectors for Rasd1 and Ear2, pcDNA4/HisMax©B and pxJGST respectively, contain SV40 promoters which facilitated the ease of expression of these proteins in COS-7 cells.

Transfected COS-7 crude lysates were checked for the expression of HisHA-Rasd1 and GST-Ear2 proteins (Figure 7, inputs). GST or GST-Ear2 were immobilized on GSH-linked beads and incubated with HisHA-Rasd1 from transfected COS-7 cell lysates. Bound proteins were eluted, resolved by SDS-

PAGE and any interacting HisHA-Rasd1 was detected by Western blot with anti-HA antibody (Figure 7, pull downs). The results showed that GST-Ear2 interacted with HisHA-Rasd1 from the lysate of transfected COS-7 cells (Figure 7, lane 1), whereas GST alone did not (Figure 7, lane 2).

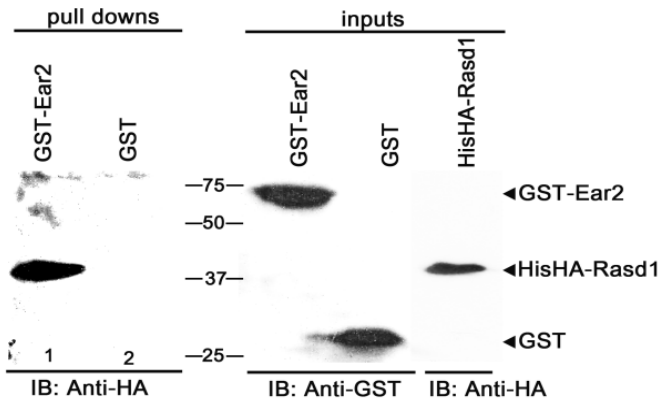


Figure 7. *In vitro* binding of GST-Ear2 and HisHA-Rasd1.
COS-7 cells were transfected with pGST-Ear2 (2.5 µg), pxJGST (2.5 µg) or pHisHA-Rasd1 (3.5 µg) as indicated (inputs). Cell lysates containing HisHA-Rasd1 was incubated with GST-Ear2 or GST immobilized on GSH-linked beads. Specifically bound HisHA-Rasd1 was eluted from the beads by heating in Laemmeli buffer at 95^{o}C for 10 minutes, resolved on 10% SDS-PAGE and detected by Western blotting with anti-HA antibody (pull downs). IB, immunoblot.

4.2.2. Rasd1 and Ear2 interact in living cells

To test whether Rasd1 and Ear2 interact in intact mammalian cells, co-transfection and co-precipitation experiments were performed. pHisHA-Rasd1 was co-transfected with pGST-Ear2 into COS-7 cells. As a control, pHisHA-Rasd1 was co-transfected with pxJGST into COS-7 cells. GST-Ear2 or GST

from transfected COS-7 cell lysates was pulled down using GSH-linked magnetic particles and any interacting HisHA-Rasd1 proteins were subsequently eluted and resolved on SDS-PAGE, and detected by Western blotting with anti-HA antibody. Precipitation of GST-Ear2 resulted in the co-precipitation of HisHA-Rasd1 (Figure 8A, lane 1), whereas precipitation of GST did not co-precipitate HisHA-Rasd1 (Figure 8A, lane 2).

In addition, a co-immunoprecipitation assay was carried out using lysate derived from COS-7 cells which were co-transfected with pHisHA-Rasd1 and pGST-Ear2. Similarly, as a control, experiments were also conducted using lysates derived from COS-7 cells which were co-transfected with pHisHA-Rasd1 and pxJGST. Immunoprecipitation of GST-Ear2 with anti-GST antibody resulted in the co-precipitation of HisHA-Rasd1, as detected by anti-HA antibody using Western blot (Figure 8B, lane 1). There was no non-specific interaction between the GST tag and HisHA-Rasd1 (Figure 8B, lane 2). This demonstrates that Rasd1 and Ear2 form a physiologic complex in cultured cells.

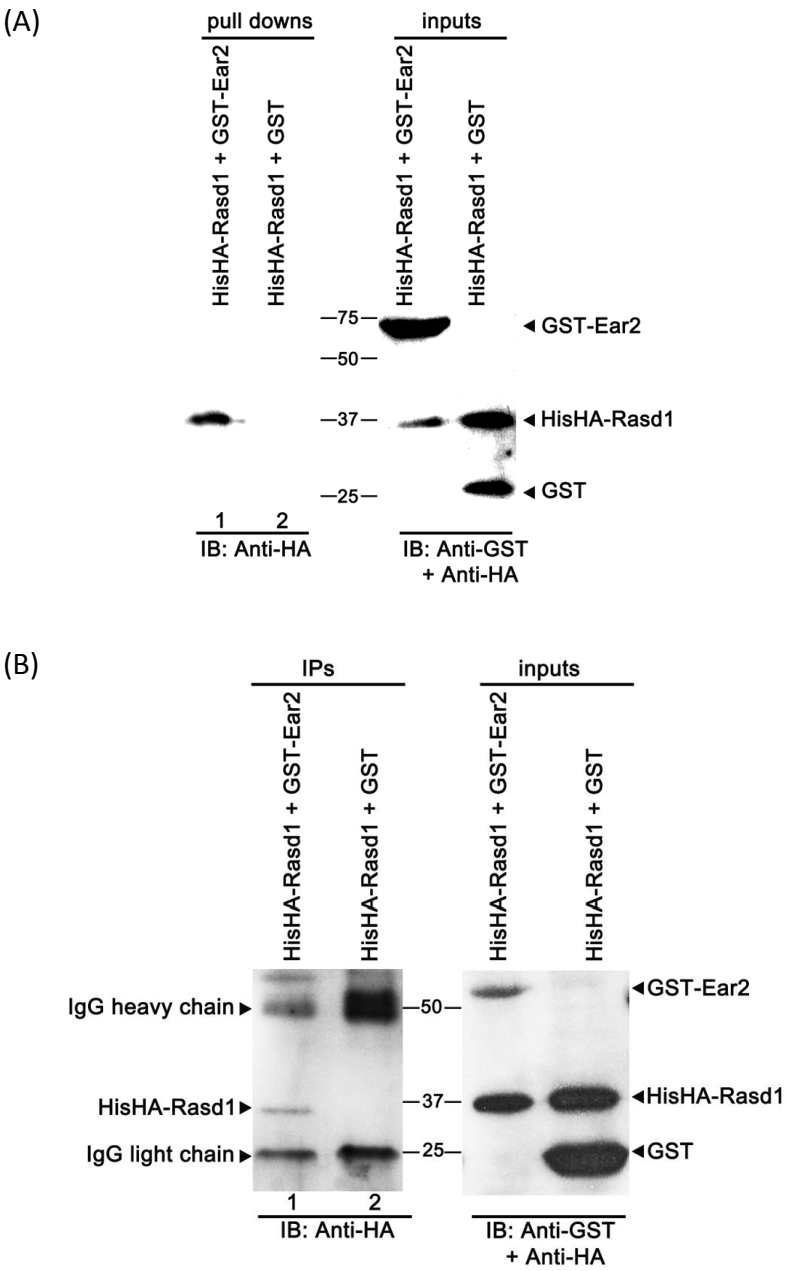
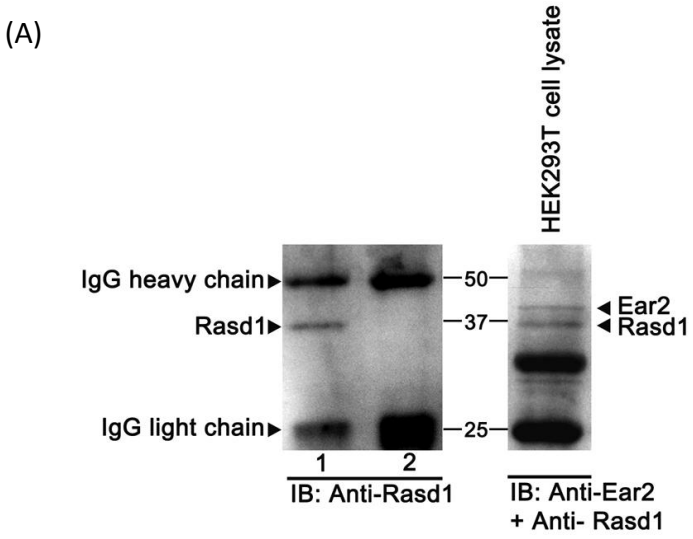


Figure 8. Rasd1 and Ear2 interact in cultured cells.
(A) HisHA-Rasd1 and GST-Ear2 interact in intact mammalian cells. pHisHA-Rasd1 (3.5 µg) was co-transfected with either pGST-Ear2 or pxJGST (2.0 µg) into COS-7 cells (inputs). GST and GST-Ear2 were captured from the cell lysates by GSH-linked beads and any interacting HisHA-Rasd1 was eluted and detected using western blotting with Anti-HA antibody (pull downs).
(B) COS-7 cells were co-transfected with pHisHA-Rasd1 (3.5 µg) and pGST-

Ear2 or pxJGST (2.0 µg) (inputs). Immunoprecipitations were performed with anti-GST antibody. Co-immunoprecipitated HisHA-Rasd1 was detected using Western blotting with Anti-HA antibody (IPs). IB, immunoblot.

4.2.3. Rasd1 and Ear2 form an endogenous complex

The existence of an endogenous Rasd1-Ear2 complex was further demonstrated by co-immunoprecipitation of the protein complex from crude lysates derived from both HEK293T cells (Figure 9A) and wild type mouse brain (Figure 9B). Immunoprecipitation of endogenous Ear2 by polyclonal anti-Ear2 IgG antibody and protein G agarose resin beads co-immunoprecipitated Rasd1, as detected by Western blotting with anti-Rasd1 antibody (Figure 9A, lane 1 and Figure 9B, lane 1). As a negative control, immunoprecipitation performed with a non-relevant goat polyclonal IgG antibody did not co-immunoprecipitate Rasd1 (Figure 9A, lane 2 and Figure 9B, lane 2). These experiments confirm the specificity of the endogenous interaction between Rasd1 and Ear2.



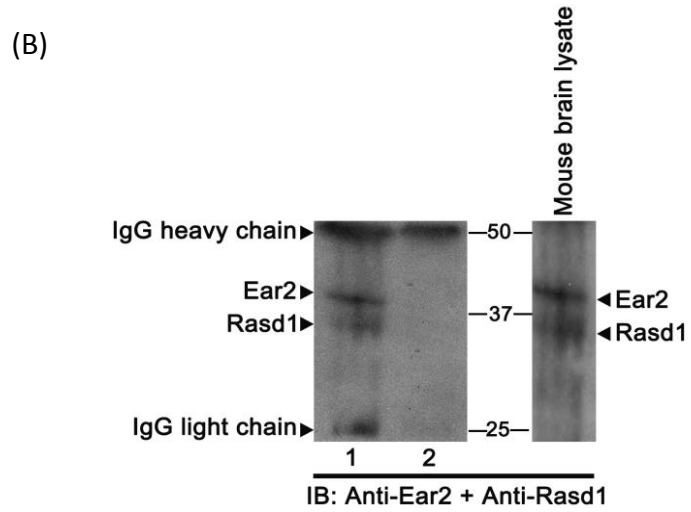


Figure 9. Rasd1 and Ear2 form an endogenous complex in living cells.
(A and B) Endogenous Rasd1-Ear2 complexes were detected by immunoprecipitating Ear2 from HEK293T crude cell lysates (A) or mouse brain crude lysates (B) with goat polyclonal IgG anti-Ear2 antibody (A and B, lanes 1). coIP was simultaneously performed with a non-relevant goat polyclonal IgG antibody as a negative control (A and B, lanes 2). The reactions were resolved on 10% SDS-PAGE and presence of Rasd1 was probed for with anti-Rasd1 antibody. IB, immunoblot.

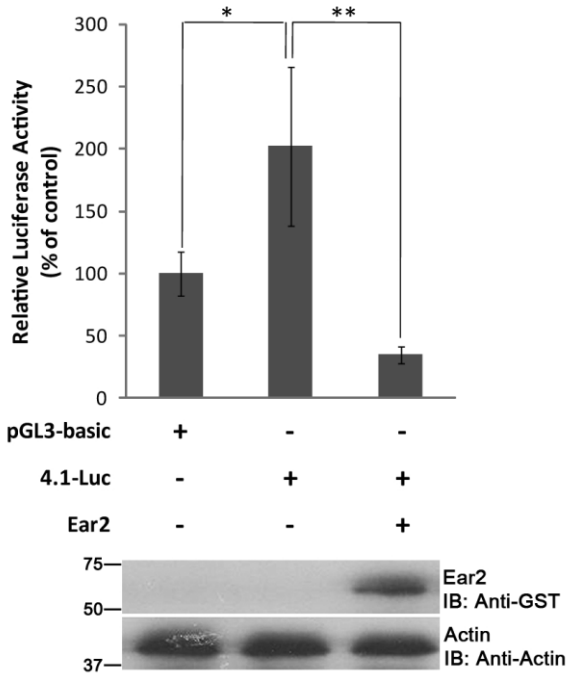
4.3. Rasd1 alleviates Ear2-mediated repression of renin transcription

4.3.1. Rasd1 alleviates Ear2-mediated repression of renin transcription in COS-7

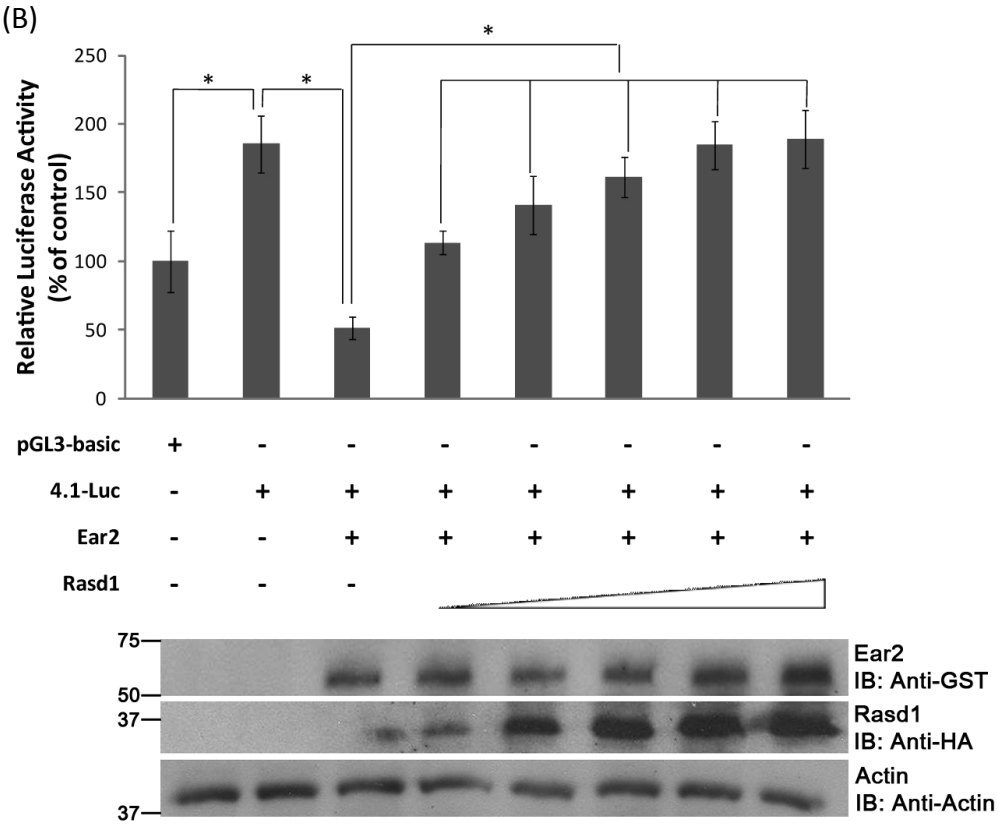
Ear2 binds to the RARE on the renin enhancer and negatively regulates renin gene transcription (132). The most important regulatory regions of the mouse renin gene reside in 4.1 kb of the renin 5´ flanking sequence (121,179). To explore the possibility that Rasd1 modulates Ear2-mediated transrepression of renin expression, luciferase reporter construct p4.1-Luc was generated by cloning 4.1 kb of renin 5´ flanking sequence containing the renin promoter and enhancer into the promoterless, enhancerless luciferase pGL3-Basic vector.

Ear2 has been shown to act as a negative regulator on renin gene transcription (132). COS-7 cells were co-transfected with either p4.1-Luc or pGL3-Basic and pGST-Ear2 and it was shown that Ear2 repressed renin transcriptional activity in COS-7 cells (Figure 10A). I went on to test if Rasd1 is able to modulate this Ear2-mediated repression of renin transcription. The results showed that Rasd1 alleviated Ear2-mediated transcriptional repression of renin promoter activity in a dosage-dependent manner (Figure 10B). To ensure that Rasd1 alone does not have an effect on the renin promoter, I transfected 1.0 µg of pHisHA-Rasd1 into COS-7 cells and observed its effects on the 4.1-Luc luciferase reporter. The results showed that Rasd1 alone did not produce any significant effect, stimulatory or inhibitory, on the activity of the renin promoter (Figure 10C). This indicates that Rasd1 does not have a direct influence on the regulation of renin transcription; instead it mediates the activity of the renin promoter through its physical interaction with Ear2.

(A)


*
**
300
250
200
150
100
50
0
Relative Luciferase Activity
(% of control)
pGL3-basic + - -
4.1-Luc - + +
Ear2 - - +
75
50
37
Ear2
IB: Anti-GST
Actin
IB: Anti-Actin

(B)


*
*
*
250
200
150
100
50
0
Relative Luciferase Activity
(% of control)
pGL3-basic + - - - - - - -
4.1-Luc - + + + + + + +
Ear2 - - + + + + + +
Rasd1 - - -
75
50
37
37
Ear2
IB: Anti-GST
Rasd1
IB: Anti-HA
Actin
IB: Anti-Actin

(C)

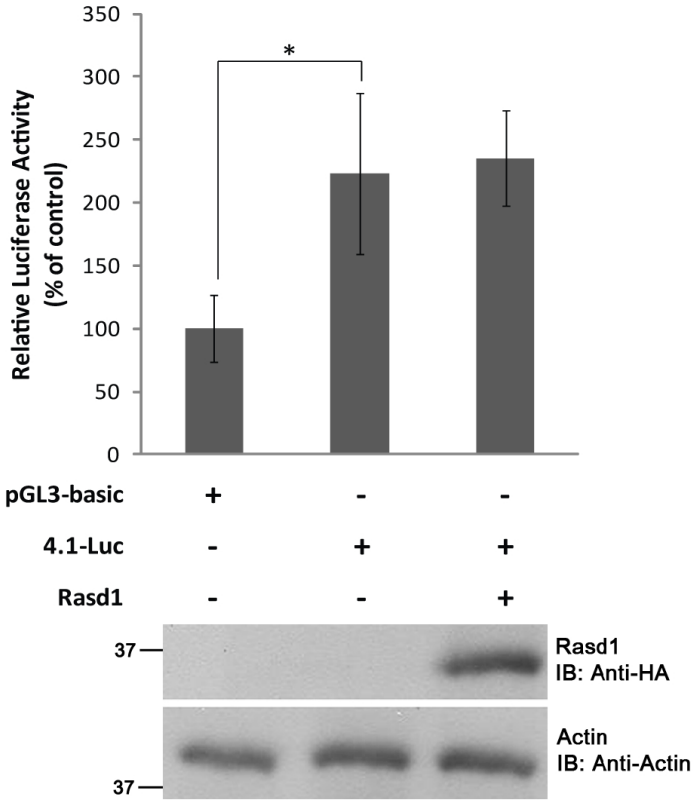


Figure 10. Rasd1 alleviates Ear2-mediated repression of renin transcription in COS-7.

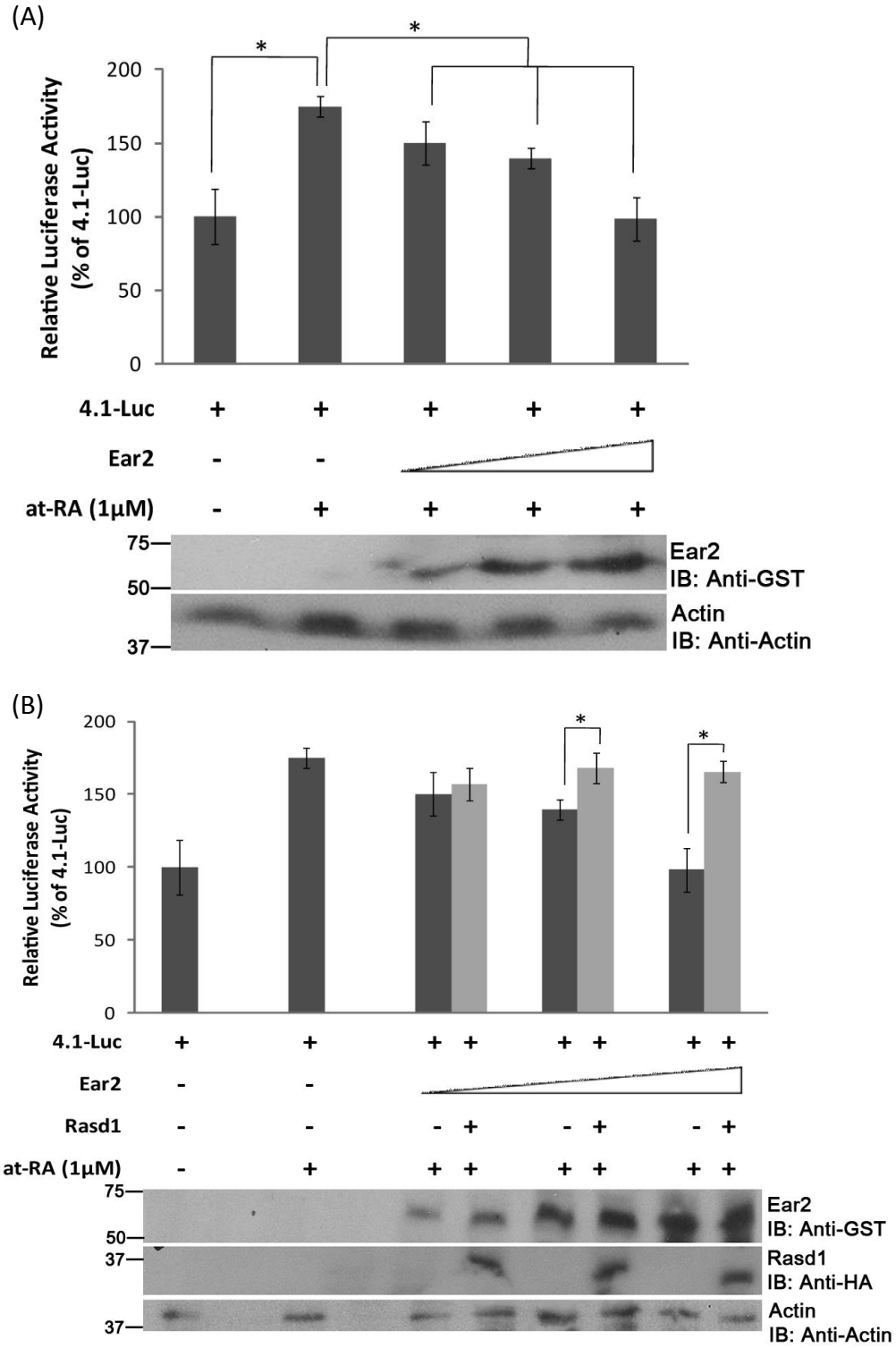
(A) Ear2 represses renin transcription. COS-7 cells were transfected with p4.1-Luc (2.0 µg), pSV-β-gal (0.5 µg) and pGST-Ear2 (1.5 µg) as indicated. Controls were transfected with pGL3-basic (2.0 µg) and pSV-β-gal (0.5 µg). Appropriate amounts of the respective carrier vectors were transfected to keep total amount of transfected DNA constant. Effects on the renin promoter were determined by measuring the relative luciferase activities, normalized against β-gal activity. Representative immunoblots show the expression of transfected proteins.

(B) Rasd1 alleviates Ear2-mediated repression of renin transcription in a dosage dependent manner. COS-7 cells were transfected with a constant amount of p4.1-Luc (2.0 µg), pSV-β-gal (0.5 µg) and pGST-Ear2 (1.5 µg), and with an increasing amount of pHisHA-Rasd1 as indicated. The amounts of pHisHA-Rasd1 transfected were 0.1, 0.2, 0.5, 1.0 and 1.5 µg. Controls were transfected with pGL3-basic (2.0 µg) and pSV-β-gal (0.5 µg). Total amount of DNA transfected was kept constant in all transfections with appropriate amounts of the respective carrier vectors. Relative luciferase activity was normalized against β-gal activity. Immunoblots are representative of the expression of

transfected proteins.
(C) Rasd1 alone does not have an effect on the renin promoter. COS-7 cells were transfected with p4.1-Luc (2.0 µg), pSV-β-gal (0.5 µg) and pHisHA-Rasd1 (1.5 µg) as indicated. Controls were transfected with pGL3-basic (2.0 µg) and pSV-β-gal (0.5 µg). In all transfections, total amount of transfected DNA was kept constant with appropriate amounts of the respective carrier vectors. Western blots are representative of the expression of transfected proteins.
* $p<0.05$; ** $p<0.01$; IB, immunoblot.

4.3.2. Rasd1 alleviates Ear2-mediated repression of retinoic acid-induced renin transcription in a dosage dependent manner in COS-7

The RARE site on the renin enhancer consists of two TGACCT motifs separated by a 10 bp spacing (128). RAR/RXR binds to this RARE site to mediate retinoic acid-induced renin transcription (128). It has been reported that Ear2 down-regulates retinoic acid-induced renin transcription (132). COS-7 cells were transfected with p4.1-Luc and the renin promoter was induced with all-trans retinoic acid (at-RA) 24 hours post transfection. It was validated that Ear2 was able to repress retinoic acid-induced renin transcription in a dosage dependent manner in COS-7 cells (Figure 11A). I went on to show that Rasd1 was able to alleviate Ear2-mediated transcriptional repression of retinoic acid-induced renin expression (Figure 11B). Interestingly, Rasd1 alleviates Ear2-mediated transcriptional repression of retinoic acid induced renin promoter activity in a dosage dependent manner (Figure 11C).


(A)
Relative Luciferase Activity (% of 4.1-Luc)
200
150
100
50
0
*
*
4.1-Luc + + + + +
Ear2 - -
at-RA (1μM) - + + + +
75
50
37
Ear2
IB: Anti-GST
Actin
IB: Anti-Actin
(B)
Relative Luciferase Activity (% of 4.1-Luc)
200
150
100
50
0
*
*
4.1-Luc + + + + + + + +
Ear2 - -
Rasd1 - - - + - + - +
at-RA (1μM) - + + + + + + +
75
50
37
37
Ear2
IB: Anti-GST
Rasd1
IB: Anti-HA
Actin
IB: Anti-Actin

(C)

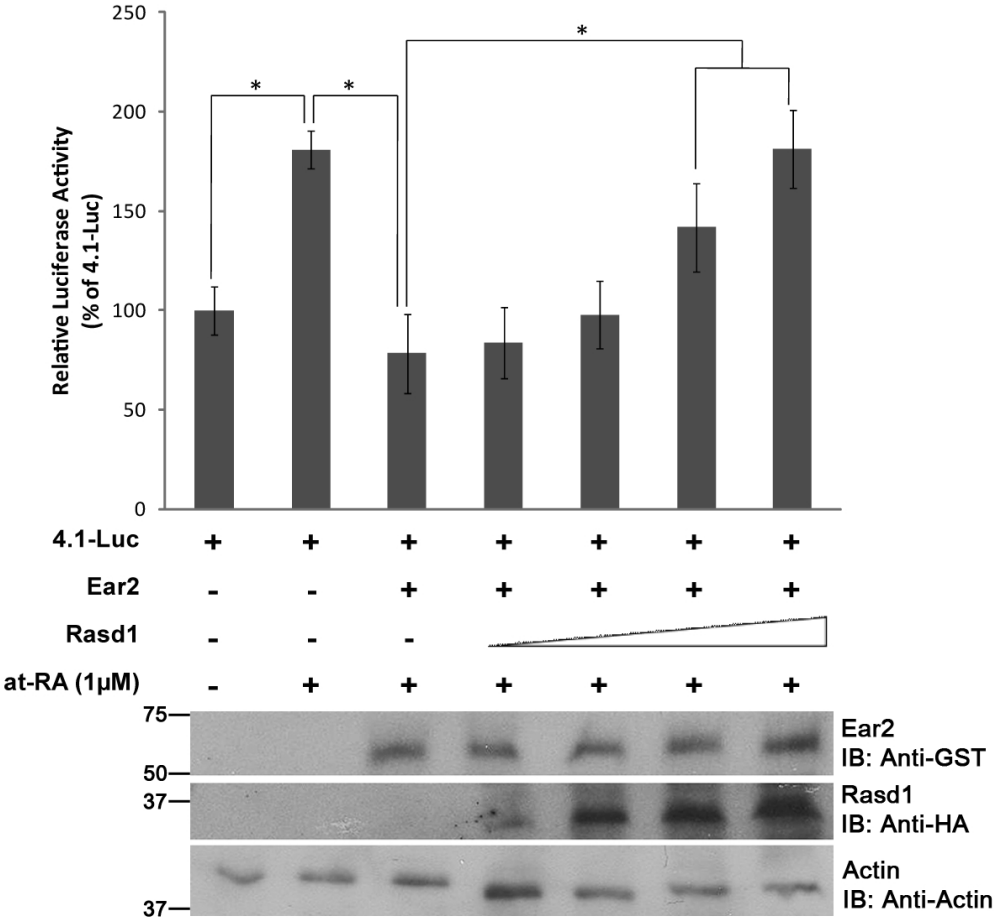


Figure 11. Rasd1 alleviates Ear2-mediated repression of retinoic acid-induced renin transcription in a dosage dependent manner in COS-7.
(A) Ear2 attenuates the renin promoter activity induced by retinoic acid in a dosage dependent manner. COS-7 cells were transfected with a constant amount of p4.1-Luc (2.0 µg), pSV-β-gal (0.5 µg) and pHisHA-Rasd1 (1.5 µg), and with an increasing amount of pGST-Ear2. The amounts of pGST-Ear2 transfected were 0.5, 1.0 and 1.5 µg. Total amount of DNA transfected was kept constant with the respective carrier plasmids. Renin transcription was induced by all-trans retinoic acid 24 hours post transfection. Cells were harvested 48 hours post transfection and relative luciferase activity, normalized against β-gal activity, was determined. Immunoblots show the expression of the transfected proteins.
(B and C) Rasd1 alleviates Ear2-mediated repression of retinoic acid induced renin transcription in a dosage dependent manner. COS-7 cells were transfected

with p4.1-Luc (2.0 µg) and pSV-β-gal (0.5 µg). The effects of Rasd1 and Ear2 on retinoic acid-induced renin transcription were tested by measuring the luciferase activity in varying amounts of either Ear2 (B) or Rasd1 (C). The amounts of pGST-Ear2 transfected in *B* were 0.5, 1.0 and 1.5 µg; the amounts of pHisHA-Rasd1 transfected in *C* were 0.2, 0.5, 1.0 and 2.0 µg. Immunoblots are representative of the expression of the transfected proteins.
* $p<0.05$; ** $p<0.01$; IB, immunoblot.

4.4. Generation of Rasd1 mutant constructs

Rasd1 is a brain enriched G protein that belongs to the Ras superfamily (2). Ras superfamily members consists of GTPases with high conservation in sequence and structural organization, especially within their GTP binding pockets – the G1, G3, G4 and G5 boxes (2). These G boxes are essential for the basic biochemical activity- GTP binding and hydrolysis of Ras proteins (2). Most Ras subfamily proteins also possess a C-terminus CAAX box, which undergoes post-translational isoprenylation to regulate the subcellular localization and function of the proteins (192). The DNA corresponding to the domains present was mutagenized, and four Rasd1 mutants were generated - Rasd1[A178V], Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] (Figure 12), to investigate whether GTPase activity, GDP-GTP exchange by GEF and post-translational isoprenylation of *Rasd1* are required for its alleviation of Ear2-mediated repression of renin transcription.

- *Rasd1[A178V]*

  Rasd1[A178V] (39) is a constitutively active mutant that contains a single nucleotide mutation in its G5 domain. This mutation disrupts its

guanyl nucleotide binding pocket (34), resulting in the mutant having a decreased affinity for both GDP and GTP, and an increased exchange rate of GDP for GTP, but with normal GTPase activity (193,194). The exchange of GDP for GTP is a slow step and is often the rate limiting step in the activation of G proteins (6). Due to the presence of a high cellular ratio of GTP to GDP (7), the increased exchange rate of GDP for GTP of the Rasd1[A178V] mutant ensures a higher chance of it being in an active GTP-bound state, and Rasd1 [A178V] behaves functionally as a constitutively active signal transducer, even in the absence of upstream signals that lead to the increased guanyl nucleotide exchange under normal physiological conditions (34,193).

- *Rasd1[G81A]*

  Rasd1[G81A] possesses a point mutation that lies on a highly conserved glycine residue in G3 box (12). Rasd1[G81A] is also believed to be locked in a constitutively active GTP-bound state. Substitution of the residue 81 from glycine to alanine disrupts the biological and regulatory functions of GTPases. In HRas, the corresponding G60A mutation does not significantly affect its GDP/GTP binding affinity but drastically reduces its GTPase activity by perturbing GTP-induced conformational changes (195).

- *Rasd1[T38N]*

  Rasd1[T38N] contains a mutation in the G1 box, corresponding to the mutation in HRas[S17N]. HRas[S17N] is a dominant negative mutant that binds GEFs unproductively. It is defective in the final step of exchange process of displacement of GEF by GTP (196). HRas[S17N] removes GEF activity from cells by sequestering GEFs into dead end complexes and does not effectively catalyze the release of GDP, thus effectively blocking the activation of endogenous Ras (197,198).

- *Rasd1[ΔCAAX]*

  To explore the potential role of isoprenylation and plasma membrane localization in the functional properties of Rasd1, we generated Rasd1[ΔCAAX]. This mutant contains a premature termination codon that gives rise to a truncated Rasd1 devoid of the CAAX box. The CAAX box is a consensus sequence that is post-translationally isoprenylated and required for the regulation of subcellular localizations and functions of GTP-binding proteins (14).

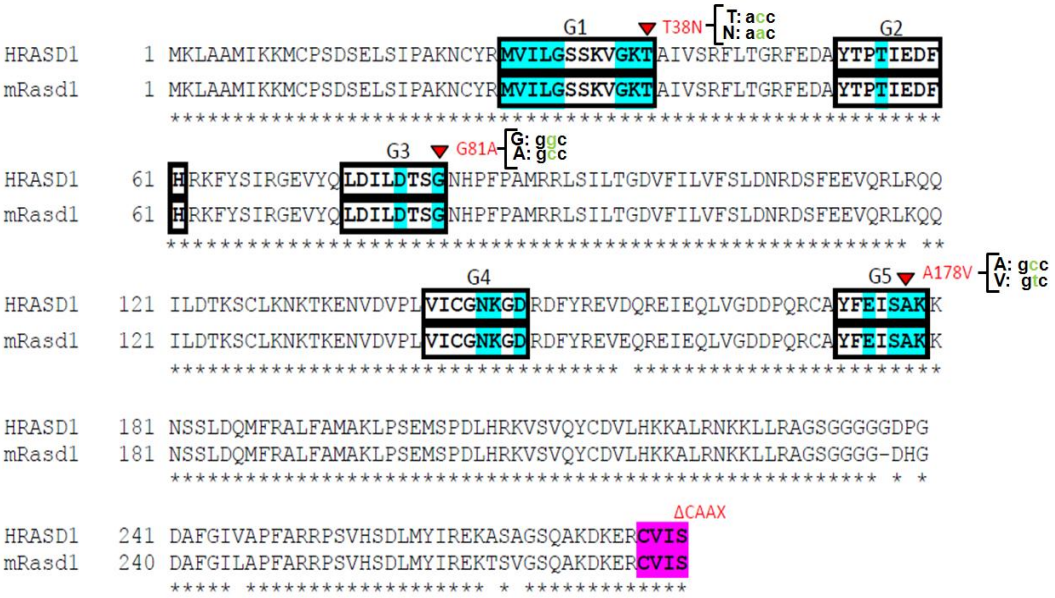


Figure 12. Amino acid sequence alignment comparing human RASD1 (HRASD1) and mouse Rasd1 (mRasd1).
Sequence alignment was carried out using blastp, NCBI. Sequences that align with perfect match are denoted by asterisks. G box residues are in bold and boxed. G box consensus residues are highlighted in blue. C-terminus CAAX box is highlighted in pink. Rasd1 mutant constructs generated are as annotated. Specific residues that are mutated indicated by red arrowheads and the corresponding DNA sequence changes are indicated in green.

4.5. Rasd1- and Ear2- mediated transcriptional regulation of renin is cell type dependent

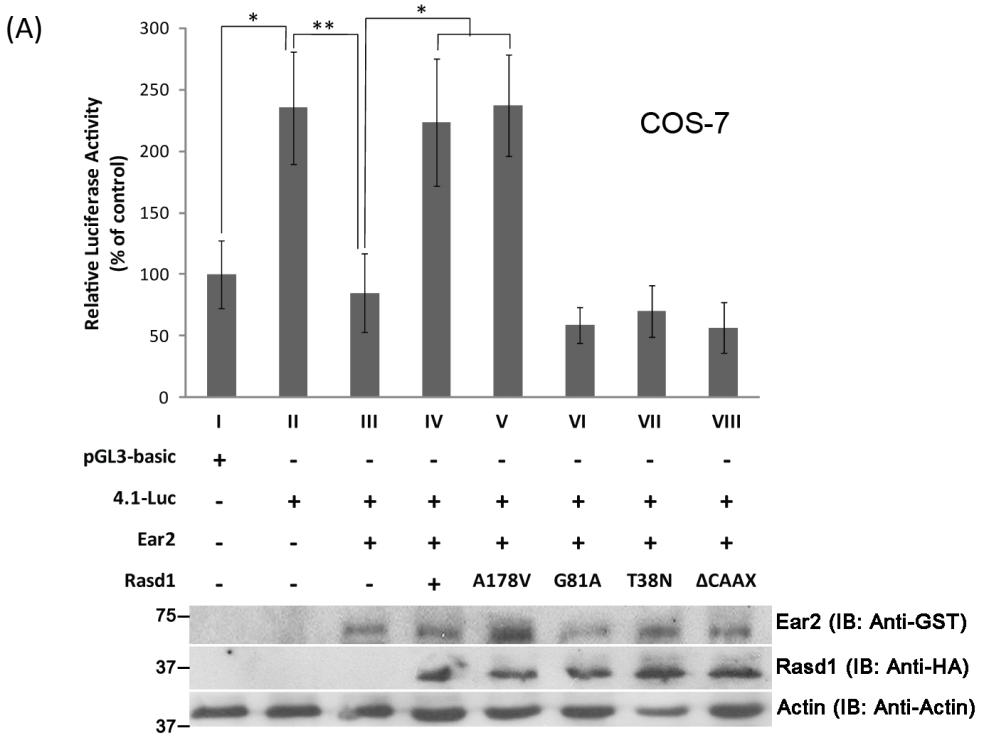
All my previous experiments conducted to investigate the effects of wild-type Rasd1 compared to that of the several mutant Rasd1 proteins on Ear2-mediated renin promoter activity have been carried out using the COS-7 cell line thus far. COS-7 has proven to be an excellent cell line exhibiting high levels of expression of the transfected plasmids. No endogenous expression of both Ear2 and Rasd1 was detected in COS-7 cells, and thus all observed effects on the

luciferase reporter can generally be attributed to the effects and actions of the transfected plasmids and their respective over-expressed proteins. However, it is known that COS-7 cells do not express endogenous renin (123) and this may raise caveats and pose limitations in the interpretation of data. To address this issue, luciferase assays were carried out in three cell lines, including COS-7 (monkey kidney fibroblast cell line), As4.1 (mouse juxtaglomerular cell line) and Neuro2a (mouse neuroblastoma cell line). While no endogenous expression of renin is observed in COS-7 cells (123), both As4.1 and Neuro2a cells are known to express endogenous renin (199,200). In fact, As4.1, a kidney-derived tumoral cell line, which maintains expression of its renin gene over long-term culture (199), currently represents the best model of a renal juxtaglomerular cell in culture. Interestingly, the 4.1-Luc reporter construct exhibited an eleven fold and four fold higher luciferase reporter activity in As4.1 and Neuro2a cells than in COS-7 cells, respectively (compare Figure 13 bars: BII and AII; CII and AII). Despite this, Rasd1 and Ear2 displayed similar effects on renin transcription in all three cell lines tested (Figure 13).

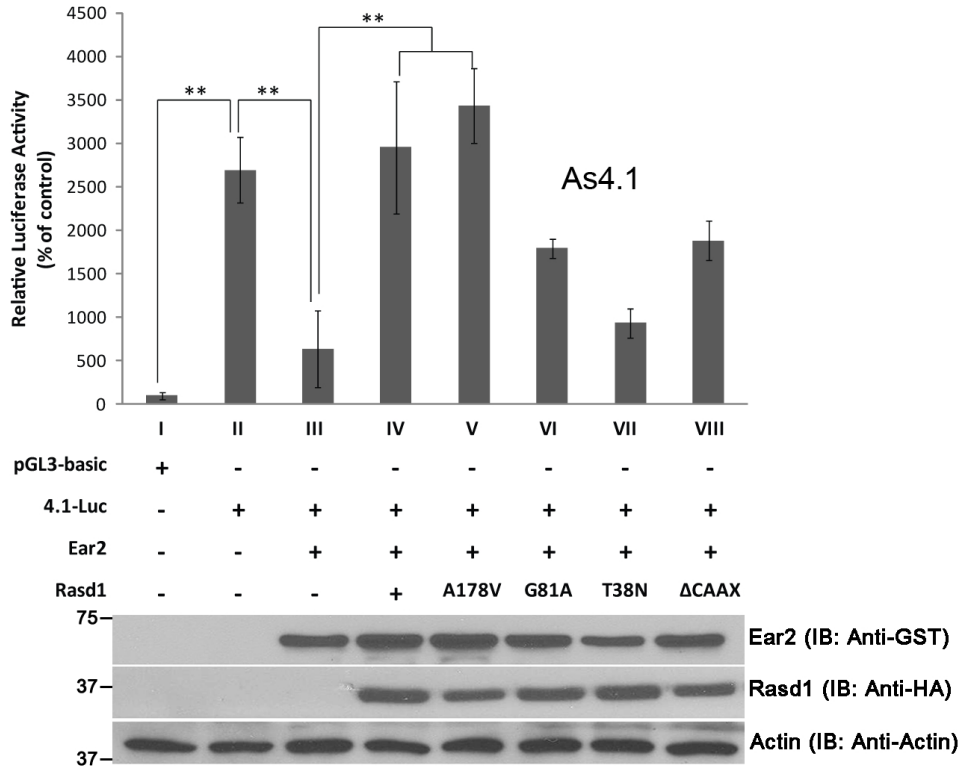
4.6. Point mutations in Rasd1 abolish its effects on the alleviation of Ear2-mediated repression of renin transcription in COS-7, Neuro2a and As4.1

Luciferase reporter assay showed that the over-expression of Ear2 was able to suppress renin promoter activity in all three cell lines (compare Figure 13, bars: AII and AIII; BII and BIII; CII and CIII), and the over-expression of Rasd1

alleviated Ear2-mediated transcriptional repression of the renin promoter (compare Figure 13, bars: AIII and AIV; BIII and BIV; CIII and CIV). Rasd1[A178V], a constitutively active mutant, alleviated Ear2-mediated down-regulation of renin promoter in a magnitude comparable to that of wild type Rasd1 (Figure 13, compare bars: AIII and AIV; BIII and BIV; CIII and CIV). In contrast, Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] did not significantly alleviate Ear2-mediated down-regulation of renin transcription (Figure 13, compare bars III, IV and VI-VIII), indicating that the GTP hydrolysis activity of Rasd1, GDP-GTP exchange by GEF and isoprenylation of Rasd1 are required for Rasd1's activity on the alleviation of Ear2-mediated transcriptional repression of the renin promoter.



(B)


As4.1
Relative Luciferase Activity (% of control)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
**
**
I II III IV V VI VII VIII
pGL3-basic + - - - - - - -
4.1-Luc - + + + + + + +
Ear2 - - + + + + + +
Rasd1 - - - + A178V G81A T38N ΔCAAX
75
37
37
Ear2 (IB: Anti-GST)
Rasd1 (IB: Anti-HA)
Actin (IB: Anti-Actin)

(C)

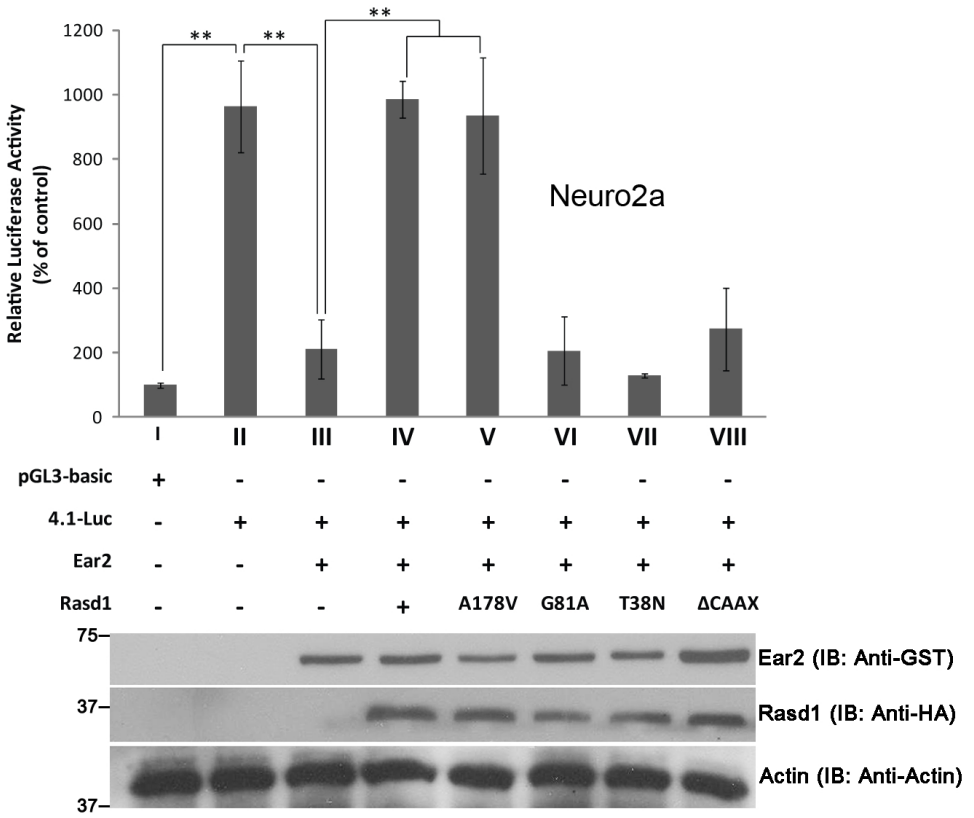

Neuro2a
Relative Luciferase Activity (% of control)
1200
1000
800
600
400
200
0
**
**
**
I II III IV V VI VII VIII
pGL3-basic + - - - - - - -
4.1-Luc - + + + + + + +
Ear2 - - + + + + + +
Rasd1 - - - + A178V G81A T38N ΔCAAX
75
37
37
Ear2 (IB: Anti-GST)
Rasd1 (IB: Anti-HA)
Actin (IB: Anti-Actin)

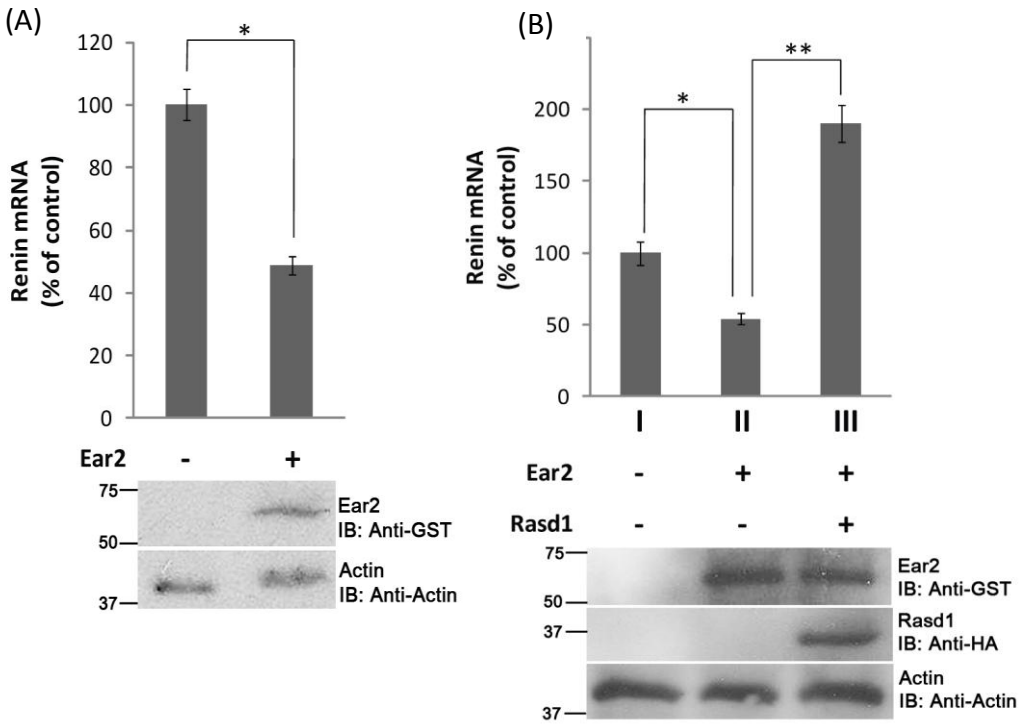
Figure 13. Rasd1 mutations G81A, T38N and ΔCAAX abolish its activity to alleviate Ear2-mediated renin transcription in A, COS-7; B, As4.1; and C, Neuro2a.
(A-C) Cells were transfected with p4.1-Luc (2.0 μg), pSV-β-gal (0.5 μg), pGST-Ear2 (1.5 μg), and pHisHA-Rasd1 or Rasd1 mutants expressing plasmids (1.5 μg) as indicated. Controls were transfected with pGL3-basic (2.0 μg) and pSV-β-gal (0.5 μg). Total amount of transfected plasmids were kept constant with the respective carrier vectors. The effects of wild type Rasd1 or Rasd1 mutant constructs on Ear2-mediated renin transcriptional activity was determined using luciferase assays, normalized against β-gal activity. Lower panels: Western blots of Ear2, Rasd1 and actin with anti-GST, anti-HA and anti-actin antibodies respectively demonstrating transgene expressions 48 hours after transfection.
* $p<0.05$; ** $p<0.01$; IB, immunoblot.

4.7. Rasd1 modulates endogenous Ear2-mediated renin transcription in As4.1

Next, the effects of Ear2 and Rasd1 on the endogenous renin gene expression in As4.1 cells were investigated. As4.1 cells express endogenous renin (199), as well as Rasd1 and Ear2. As4.1 cells were transiently transfected with pGST-Ear2 or with pxJGST (2.0 μg) as control. The transfection efficiency was at least 70%, as visualized by immunostaining and confocal microscopy. Real-time RT-PCR showed that endogenous renin mRNA levels were lower in cells that were over-expressing Ear2. Endogenous renin mRNA levels in As4.1 cells over-expressing Ear2 was reduced to about 48% of the control (Figure 14A). When pHisHA-Rasd1 (3.5 μg) was co-transfected together with pGST-Ear2 (2.0 μg) into As4.1 cells, the repression of renin transcription mediated by Ear2 was alleviated. In fact, renin mRNA levels as detected by real-time RT-PCR, was increased to almost twice that of control when Rasd1 was over-expressed

(Figure 14B, compare bars I and III). This spike in renin gene expression when Rasd1 was over-expressed might be attributed to the presence of abundant Rasd1 proteins, which might have removed the repression of renin gene expression by both endogenous and transfected Ear2.

Semi-quantitative RT-PCR was also conducted with the number of PCR cycles optimized at the linear range of amplification. Similarly, it was observed that the over-expression of Ear2 repressed endogenous renin transcription and that the over-expression of Rasd1, together with Ear2, restored endogenous renin transcript levels (Figure 14C).



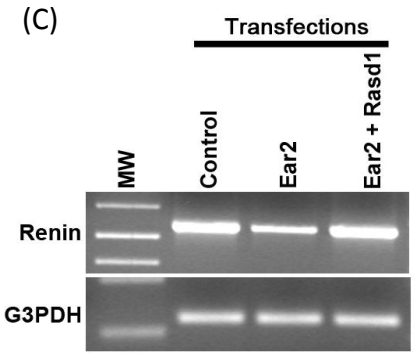


Figure 14. Ear2 and Rasd1 affect endogenous renin expression in As4.1 cells.
(A) Over-expression of Ear2 represses endogenous renin mRNA expression. As4.1 cells were transiently transfected with pGST-Ear2 or pxJGST (2.0 µg) as indicated. Real-time RT-PCR, normalized against G3PDH expression, showed that endogenous renin expression was reduced to 48% of the control when Ear2 was over-expressed. Immunoblot is representative of transgene expression. * $p<0.05$; IB, immunoblot.
(B) Over-expression of Rasd1 alleviates Ear2-mediated repression of endogenous renin transcription. As4.1 cells were transiently transfected with pGST-Ear2 or pxJGST (2.0 µg), and pHisHA-Rasd1 or pcDNA4.0 (3.5 µg) as indicated. Real-time RT-PCR, normalized against G3PDH expression, showed that endogenous renin expression was repressed when Ear2 was over-expressed. The co-expression of Ear2 and Rasd1 alleviated Ear2-mediated repression of endogenous renin expression, and renin expression levels were increased to almost twice that of the control. A representative immunoblot demonstrates transgene expression 48 hours after transfection (lower panels). * $p<0.05$; ** $P<0.01$; IB, immunoblot.
(C) Ear2 and Rasd1 mediate endogenous renin transcription. Semi-quantitative RT-PCR was performed with primers specific to renin mRNA. G3PDH was used as an internal control. When pGST-Ear2 (2.0 µg) was transfected, endogenous renin expression was repressed. When pHisHA-Rasd1 (3.5 µg) was co-transfected together with pGST-Ear2 (2.0 µg), endogenous renin expression was restored. MW, molecular weight ladder.

4.8. Rasd1 knockdown suppresses Ear2-mediated repression of renin expression

To study if alteration of endogenous Rasd1 levels in As4.1 cells is capable of modulating Ear2-mediated repression of renin transcription, I altered the

endogenous Rasd1 level using RNAi knockdown and dexamethasone induction. I first determined that Rasd1 shRNA, but not control shRNA, effectively knock-downed the endogenous expression of both Rasd1 mRNA (Figure 15A, upper panels) and protein (Figure 15A, lower panels) levels in As4.1 cells. Quantifications with the densitometer indicated that Rasd1 protein levels were effectively knocked down by more than 48% in Rasd1 shRNA transfected cells.

I went on to demonstrate that shRNA-mediated knockdown of Rasd1 resulted in a further repression of Ear2-mediated renin transcription (Figure 15B, compare bars V and VI). Since dexamethasone is known to induce Rasd1 expression (30), I next examined whether dexamethasone treatment could alleviate the effects of shRNA-mediated Rasd1 knockdown. After treatment with dexamethasone, Ear2-mediated repression of renin transcription was alleviated (Figure 15B, compare bars V and VII), which corresponds to the results that were observed from the over-expression of Rasd1 (Figure 15B, compare bars III and IV). Treatment with dexamethasone reversed the effects of Rasd1 shRNA on the Ear2-mediated repression of renin transcription (Figure 15B, compare bars VI and VIII); however, renin transcription levels were not restored to the levels as high as treatment with dexamethasone alone (Figure 15B, bar VII). These experiments suggest that transcriptional repression of renin by Ear2 can be modulated by altering the level of endogenous Rasd1 in As4.1 cells.

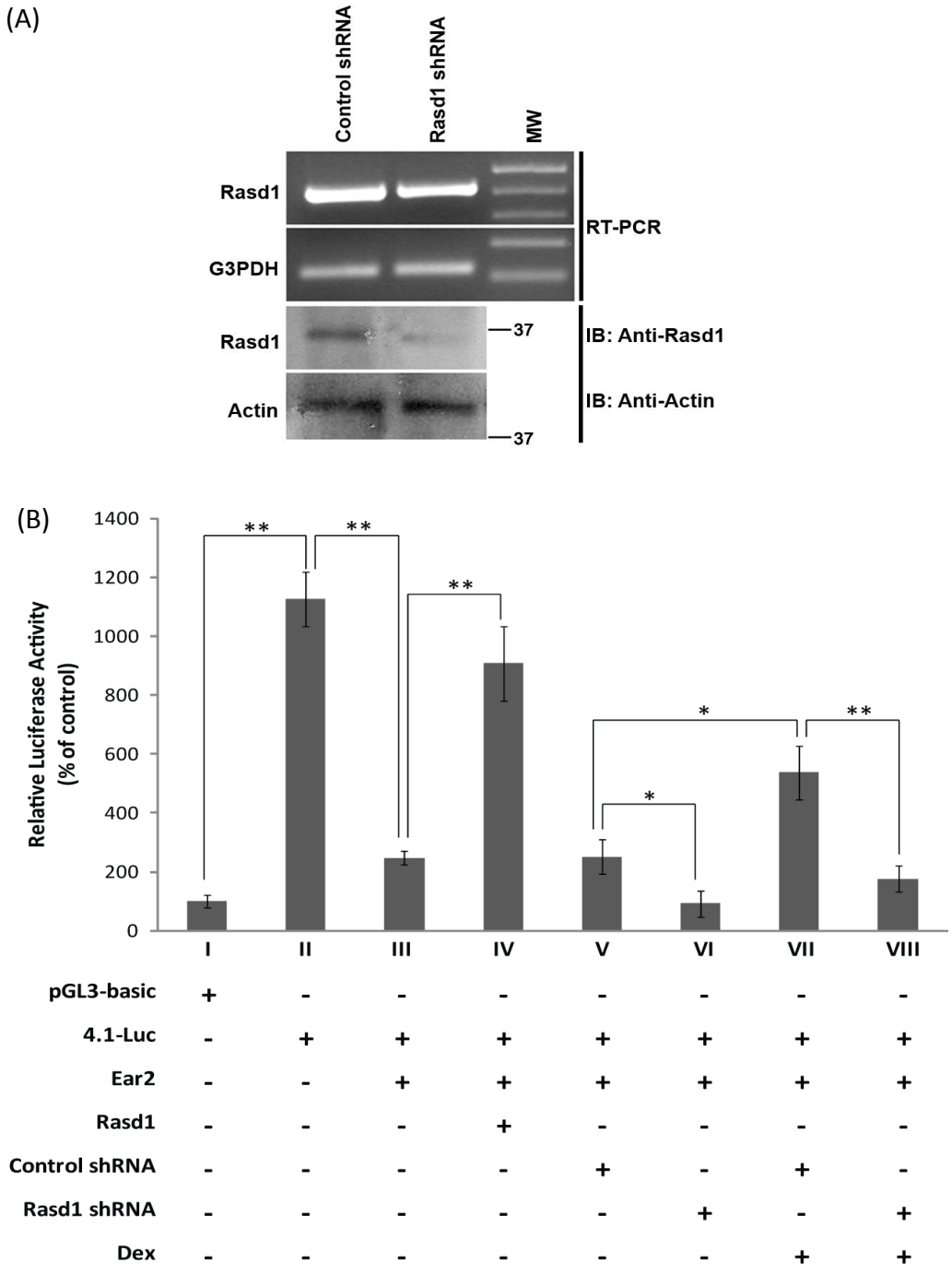


Figure 15. shRNA knockdown of Rasd1 results in a further repression of Ear2-mediated renin transcription in As4.1 cells.
(A) Rasd1 shRNA or control shRNA (2.0 µg/ml) were transfected into As4.1

cells. Cells were selected by puromycin 24 hours post transfection and harvested 48 hours post transfection. Semi-quantitative RT-PCR, with G3PDH as an internal control, showed that Rasd1 shRNA, but not control shRNA, effectively knocked down endogenous Rasd1 mRNA levels (upper panel). Endogenous Rasd1 protein levels were determined by Western blotting with anti-Rasd1 antibody. Quantification of the immunoblots by densitometer revealed that endogenous Rasd1 protein levels, normalized against endogenous actin protein levels, were knocked down by more than 48% (lower panel). IB, immunoblot; MW, molecular weight ladder.
(B) Modulation of endogenous Rasd1 levels affects Ear2-mediated repression of renin transcriptional activity. As4.1 cells were transfected with p4.1-Luc (2.0 µg), pGST-Ear2 (1.5 µg), pHisHA-Rasd1 (1.5 µg), Rasd1 shRNA or control shRNA (2.0 µg/ml) as indicated, together with pSV-β-gal (0.5 µg). Controls were transfected with pGL3-basic (2.0 µg) and pSV-β-gal (0.5 µg). Appropriate amounts of the respective carrier vectors were co-transfected to keep the total amount of transfected DNA constant. Selection with puromycin (2.0 µg/ml) and dexamethasone (100 nM) treatment was carried out 24 hours post transfection, and cells were harvested 48 hours post transfection. Relative luciferase activity was normalized against β-gal activity. * $p<0.05$; ** $p<0.01$.

4.9. Rasd1 and Ear2 modulate renin transcriptional activity by acting through RARE sites on the renin enhancer

4.9.1. Rasd1 and Ear2 mediate renin promoter activity through RARE sites

To study if Ear2 represses renin promoter activity by directly binding to the RARE sites on the renin enhancer, luciferase construct p3XRARE-117P-Luc was generated. p3XRARE-117P-Luc consists of three tandem copies of RARE fused to a 117 bp minimal renin promoter driving the expression of the luciferase reporter gene. When transfected in As4.1 cells, p3XRARE-117P-Luc resulted in approximately 100-fold increase in renin promoter activity (Figure 16, bars I and III). This was comparable to the 120-fold increase in renin promoter activity when p4.1-Luc was transfected (Figure 16, bars I-III). When co-transfected, Ear2 caused a 3-fold decrease in renin promoter activity (Figure

16, bars III and IV), confirming a previous study which showed that Ear2 represses renin promoter activity via RARE sites (132). When Rasd1 was co-transfected with Ear2, Ear2-mediated repression of renin promoter activity was alleviated, and promoter activity as indicated by luciferase activity was restored (Figure 16, bars IV and V).

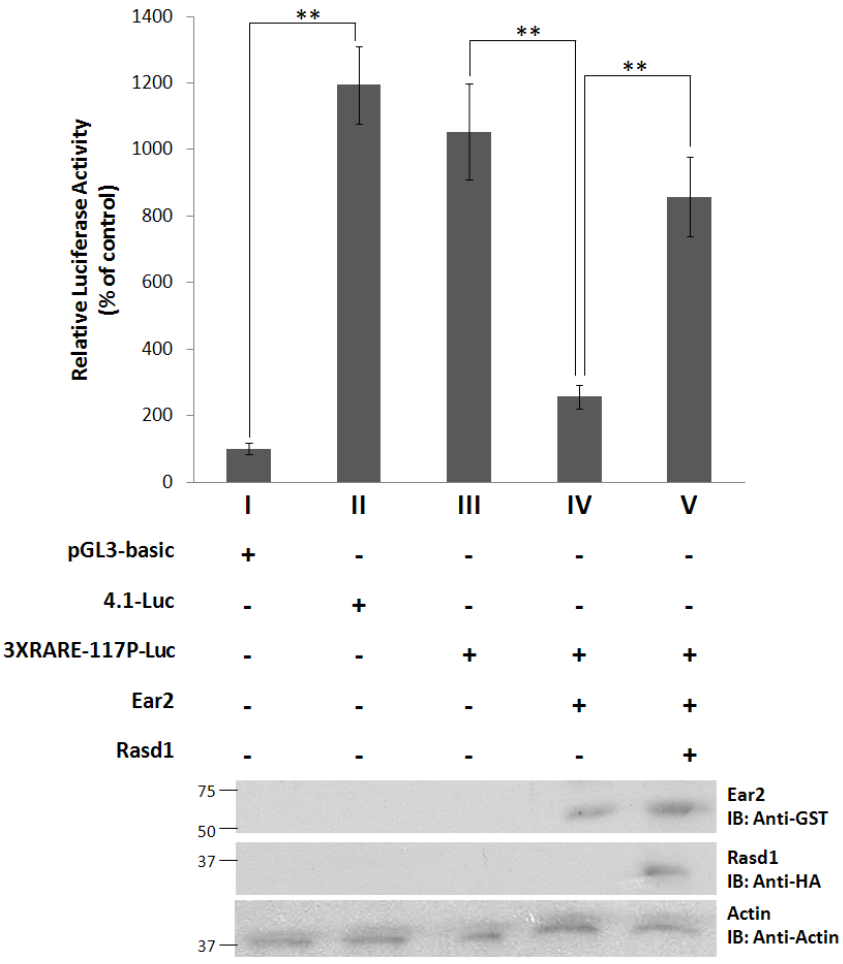


Figure 16. Rasd1 and Ear2 mediate renin promoter activity through RARE sites.

As4.1 cells were transiently transfected with pSV-β-gal (0.3 µg), pGL3-basic, p4.1-Luc or p3XRARE-117P-Luc (2.0 µg). Effects of Ear2 and Rasd1 on p3XRARE-117P-Luc were determined by co-transfecting 1.5 µg of the

respective expressing plasmids. Total DNA concentration was kept constant with the appropriate carrier plasmids. ** $p<0.01$.

4.9.2. Rasd1 removes physical binding of Ear2 to RARE on the renin enhancer

In addition, ChIP assay was performed using As4.1 cells to confirm the physical localization of Ear2 on the renin enhancer. Figure 17A depicts the primer pairs used for PCR after immunoprecipitation with anti-Ear2 antibody. Immunoprecipitation of Ear2 with anti-Ear2 antibody enriched the minimal enhancer region of the renin gene (Figure 17B, lane V). When Rasd1 was transiently transfected, the amount of enriched chromatin by anti-Ear2 antibody was reduced (Figure 17B, lane VI), suggesting that Rasd1 removes the binding of Ear2 from the renin enhancer. To determine that Ear2 binds specifically to the RARE site of the renin enhancer, another primer- ChIP-3R (Figure 17A) was designed. Primer pair ChIP-1F and ChIP-3R amplifies region -2866 to -2674 on the renin enhancer that excludes the RARE site. Immunoprecipitation with anti-Ear2 antibody and subsequent amplification by PCR with primer pair ChIP-1F and ChIP-3R did not produce any PCR product (Figure 17B, lane VII), suggesting that Ear2 binds specifically to the RARE site of the renin enhancer. Immunoprecipitation with a non-relevant IgG antibody and subsequent PCR with primer pair ChIP-1F and ChIP-2R also did not produce any PCR products (Figure 17B, lane VIII).

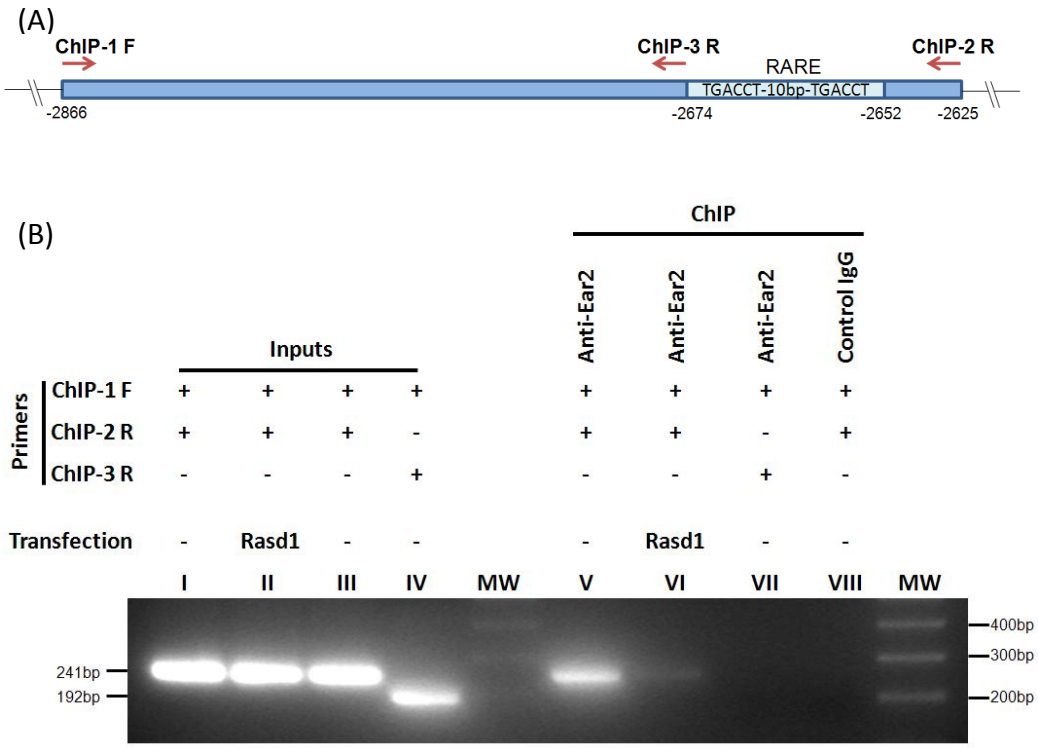


Figure 17. Rasd1 prevents Ear2 from binding to the RARE site.
(A) Schematic representation of the renin minimal enhancer with the RARE site marked out. Primers used for ChIP are annotated as ChIP-1F, ChIP-2R and ChIP-3R.
(B) As4.1 cells were used for ChIP assay with anti-Ear2 antibody. Lanes I-IV are the inputs for lanes V-VIII respectively, amplified using PCR with primer pairs ChIP-1F + ChIP-2R or ChIP-1F + ChIP-3R as indicated. Primer pair ChIP-1F + ChIP-2R amplifies the minimal renin enhancer (-2866 to -2625) and produces a PCR product of 241 bp, while primer pair ChIP-1F + ChIP-3R amplifies region -2866 to -2674 of the renin enhancer and produces a 192 bp PCR product. Immunoprecipitation with anti-Ear2 antibody enriched the renin enhancer (lane V). When 1.5 µg pHisHA-Rasd1 was transiently transfected and ChIP was performed, less renin enhancer region was enriched (lane VI). Amplification with primer pair ChIP-1F + ChIP-3R after ChIP with anti-Ear2 antibody did not produce any product (lane VII). ChIP performed with a non-relevant IgG antibody also did not produce any PCR product (lane VIII). MW, 1 Kb plus DNA ladder (Invitrogen).

4.9.3. Rasd1 does not bind to RARE; Rasd1 and Ear2 do not complex at RARE

EMSA was performed with Cy3-labeled RARE sequences as probes. EMSA results confirmed that Ear2 binds physically to RARE, as indicated by the

presence of protein-DNA complexes (Figure 18, lane II). Binding of Ear2 to RARE is specific because when incubated with BSA, no protein-DNA complexes were detected (Figure 18, lane III), anti-Ear2 antibody generated a supershifted complex (Figure 18, lane IV), and labeled Ear2-RARE complexes were efficiently competed by unlabeled competitor RARE oligonucleotides (Figure 18, lane VII).

In contrast, Rasd1 alone does not bind to RARE (Figure 18, lane I). No protein-DNA complex bands were observed when Rasd1 was incubated with Ear2, suggesting that Rasd1 does not complex with Ear2 at RARE (Figure 18, lane V). Instead, Rasd1 prevents Ear2 from binding to RARE, as indicated by the absence of protein-DNA complexes when Rasd1 was incubated together with Ear2 (Figure 18, lane V). Incubation of Ear2 with BSA control did not result in the disappearance of the protein-DNA complex band (Figure 18, lane VI).

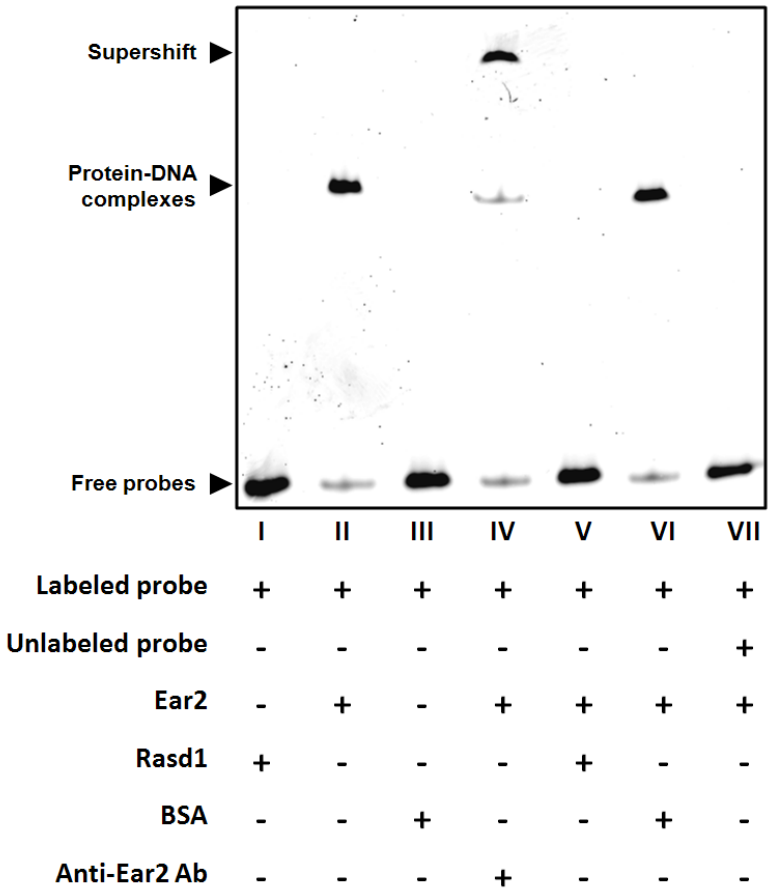


Figure 18. Rasd1 does not localize at RARE and does not complex with Ear2 at RARE.
Supershift EMSA of Ear2 and Rasd1 binding activity to RARE is shown. Cy3-labeled double stranded RARE oligonucleotides (50 ng) were used as probes, and incubated with the indicated purified proteins (5 µg) or BSA in EMSA binding buffer. For supershift, 1 µg of anti-Ear2 antibody was added to the binding reaction. Unlabeled RARE sequences were used as competitors and were present in 200-fold molar excess. The reactions were resolved on 6% TBE non-denaturing gel and Cy3 fluorescence was visualized. EMSA results are indicative that while Ear2 forms a protein-DNA complex with RARE (lanes II – IV, and VII), Rasd1 does not (lane 1). Rasd1-Ear2 complexes also do not bind to RARE, but Rasd1 removes the physical binding of Ear2 to RARE (lane V).

4.10. Ear2 and Rasd1 colocalise in the nucleus

It is only logical that both Rasd1 and Ear2 are physically present in the same cellular compartment for them to be able to form an endogenous physiological complex *in vivo*. I therefore proceeded to examine the localization of Rasd1 and Ear2 using transfected COS-7 cells. Expression of transfected Rasd1 and Ear2 proteins in the cells were detected using indirect immunofluorescence staining with anti-HA and anti-GST primary antibodies respectively and fluorescence-tagged secondary antibodies, followed by confocal microscopy.

When transfected alone, HisHA-Rasd1 was expressed in both the cytoplasm and the nucleus (Figure 19, A1-3), This is in line with sequence analysis of Rasd1 which revealed that Rasd1 contains a bipartite nuclear localization signal from amino acids 207 to 224 that is required for the targeting of proteins to the nucleus (39). When transfected alone, GST-Ear2 was mainly present in the nucleus (Figure 19, B1-3). When GST-Ear2 and HisHA-Rasd1 were co-expressed, it was observed that HisHA-Rasd1 and GST-Ear2 colocalized in both the cytoplasm and the nucleus (Figure 19, C1-4).

4.11. Rasd1 translocates and retains Ear2 in the cytoplasm

Interestingly, it was observed that the amount of GST-Ear2 present in the cytoplasm was significantly increased in the presence of HisHA-Rasd1

(compare Figure 19, B1 with C2). There was no noticeable change in the distribution of HisHA-Rasd1 in the presence or absence of GST-Ear2 (compare Figure 19, A1 with C1). It appears that Rasd1 is involved in the translocation of Ear2 from the nucleus to the cytoplasm, and the retention of Ear2 in the cytoplasm.

4.12. GTP hydrolysis activity, GDP-GTP exchange by GEF and isoprenylation of Rasd1 are involved in the translocation and retention of Ear2 in the cytoplasm

The Rasd1 mutant constructs were co-transfected with Ear2, and their cellular localizations were examined. Rasd1[A178V] and Rasd1[T38N] exhibited a similar distribution to that of wild type Rasd1, and were present in both the nucleus and cytoplasm (Figure 19, A1, D1 and F1). Rasd1[G81A] and Rasd1[ΔCAAX] were mainly located only in the nucleus (Figure 19, E1 and G1). When co-expressed with Ear2, the amount of Ear2 present in the cytoplasm was visibly increased in the presence of Rasd1[A178V] (compare Figure 19, B1 and D2). On the other hand, Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] did not alter the distribution of Ear2. Ear2 was still mostly located in the nucleus in the presence of these Rasd1 mutants (Figure 19, E2, F2, and G2).

The results show that except for wild type Rasd1 and the constitutively active Rasd1[A178V], the other Rasd1 mutants- Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], were ineffective in the translocation and retaining of Ear2 in the cytoplasm. This observation is consistent with the findings from luciferase assays where it was observed that while wild type Rasd1 and Rasd1[A178V] alleviated Ear2-mediated repression of renin transcription, mutants Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] did not show a similar effect, and Ear2-mediated renin transcription remained in a repressed state in the presence of these three Rasd1 mutants (Figure 13).

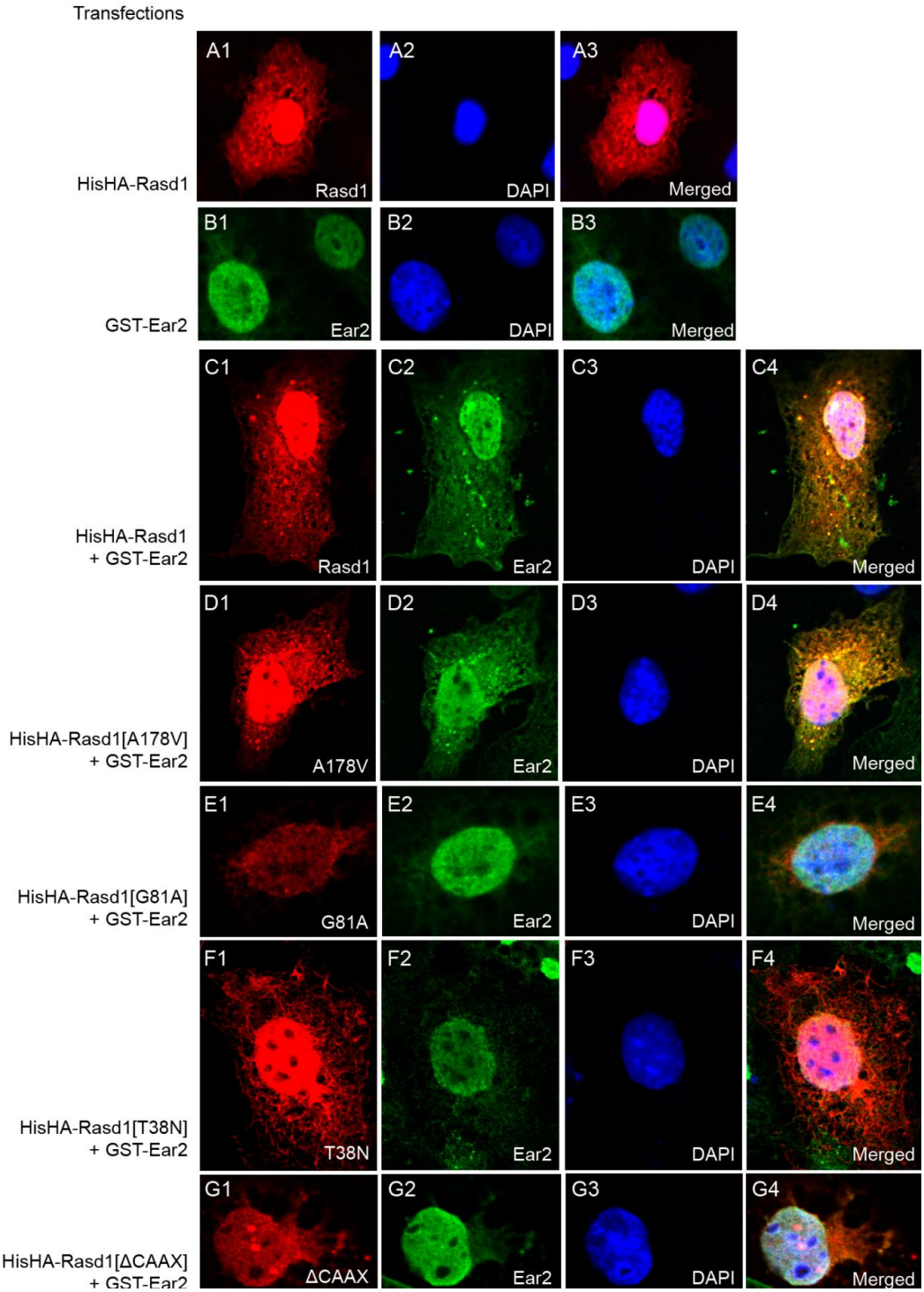


Figure 19. Wild-type Rasd1 and Rasd1[A178V] affect the nuclear-cytoplasmic distribution of Ear2, while Rasd1[G81A], Rasd1[T38N] and

Rasd1[ΔCAAX] do not.
(A-G) Rasd1 and Rasd1[A178V], but not Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], translocates and retains Ear2 in the cytoplasm. Immunofluorescence staining of COS-7 cells transfected with either pHisHA-Rasd1 (2.0 µg) (A1-3) or pGST-Ear2 (1.5 µg) (B1-3), or pHisHA-Rasd1 (wild type or mutants) + pGST-Ear2 (C1 to G4). HisHARasd1 (wild type or mutants) were detected with anti-HA antibody and visualized with AlexaFluor 568 (red); GST-Ear2 was labeled with anti-GST AlexaFluor 488 (green); nucleus was labeled with 4´,6-diamidino-2-phenylindole (DAPI) (blue). Cells were viewed under confocal microscope. Confocal imaging showed that HisHA-Rasd1 was present in both the nucleus and cytoplasm (A1-3). GST-Ear2 was located mainly in the nucleus (B1-3), but when co-transfected together with HisHA-Rasd1, GST-Ear2 was detected in both the nucleus and cytoplasm (C2). HisHA-Rasd1 co-localized with GST-Ear2 in both the cytoplasm and nucleus in co-transfected cells (C4). Similar to wild type HisHA-Rasd1, HisHA-Rasd1[A178V] (D1) and HisHA-Rasd1[T38N] (F1) were present in both the nucleus and cytoplasm. On the other hand, HisHA-Rasd1[G81A] (E1) and HisHA-Rasd1[ΔCAAX] (G1) were located mainly in the nucleus. In co-transfections, only HisHA-Rasd1[A178V] co-localized with GST-Ear2 in the cytoplasm and nucleus (D4); the other three Rasd1 mutant constructs did not alter Ear2 nuclear-cytoplasmic distribution, and GST-Ear2 remained mainly in the nucleus. (E4, F4 and G4).

4.13. GTP hydrolysis activity, GDP-GTP exchange by GEF and isoprenylation of Rasd1 affects its binding ability to Ear2

Having determined that the Rasd1 mutants- Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], were ineffective in the translocation and retaining of Ear2 in the cytoplasm, I wanted to investigate whether GTP hydrolysis activity, GDP-GTP exchange and isoprenylation of Rasd1 are essential for its interaction with Ear2. Interaction studies between Ear2 and Rasd1 and its four mutants were carried out.

The precipitation of GST-Ear2 by GSH-linked magnetic beads co-precipitated wild type Rasd1 and all four Rasd1 mutants (Figure 20, panels a and b). However, significantly less Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] compared to wild type Rasd1 and Rasd1[A178V] were co-precipitated with GST-Ear2 (compare Figure 20, panel a, lanes I and II with lanes III-V). Similarly, precipitation of HisHA-Rasd1 and the four HisHA-Rasd1 mutants with magnetic Ni-NTA beads co-precipitated GST-Ear2 (Figure 20, panels c and d), with noticeably less GST-Ear2 co-precipitated with Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] compared to that of wild type Rasd1 and Rasd1[A178V] (compare Figure 20, panel c, lanes I and II with lanes III-V). This indicates that GTP hydrolysis, GDP-GTP exchange by GEF and isoprenylation of Rasd1 affects its binding ability to Ear2.

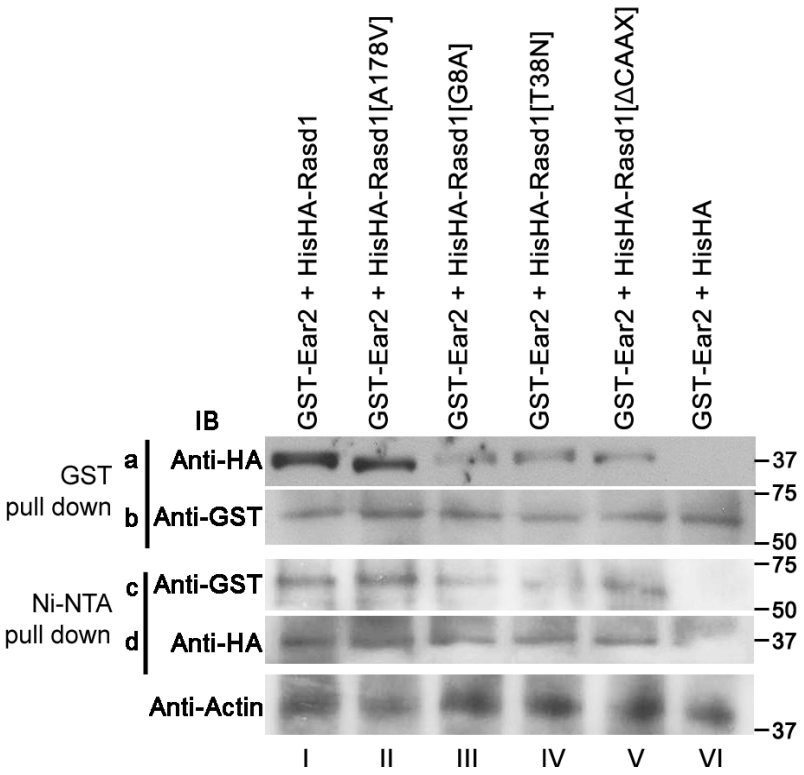


Figure 20. Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] have weakened interaction with Ear2.
COS-7 cells were co-transfected with pGST-Ear2 (2.0 µg) and the respective plasmids expressing the several Rasd1 constructs (3.5 µg) or the empty expression vectors as indicated. Precipitation of GST-Ear2 with GSH-linked magnetic beads co-precipitated all five Rasd1 constructs (panel a, lanes I-V). However, the amount of HisHA-Rasd1[G81A], HisHA-Rasd1[T38N] and HisHA-Rasd1[ΔCAAX] co-precipitated by Ear2 were significantly less than that of HisHA-Rasd1 and HisHA-Rasd1[A178V] (compare panel a, lanes I and II with lanes III-V). Similarly, co-precipitation assays using magnetic Ni-NTA beads revealed that the amount of GST-Ear2 co-precipitated with HisHA-Rasd1[G81A], HisHA-Rasd1[T38N] and HisHA-Rasd1[ΔCAAX] were significantly less than that of HisHA-Rasd1 and HisHA-Rasd1[A178V] (compare panel c, lanes I and II with lanes III-V). IB, immunoblot.

4.14. Mapping the region on Ear2 to which Rasd1 binds

4.14.1. Generation of Ear2 truncated constructs

Ear2 is a 390 amino acid nuclear hormone receptor and is known to contain several domains, including an activator function I site (residues 1-53), a DNA-binding domain (residues 54-130), a linker region consisting of a zinc finger domain (residues 131-193) and a ligand binding domain (residues 194-376). To identify the domain in Ear2 to which Rasd1 binds to, six additional GST-Ear2 truncated constructs were generated- Ear2-N193, Ear2-N130, Ear2-N53, Ear2-C54, Ear2-C131 and Ear2-C194 (Figure 21).

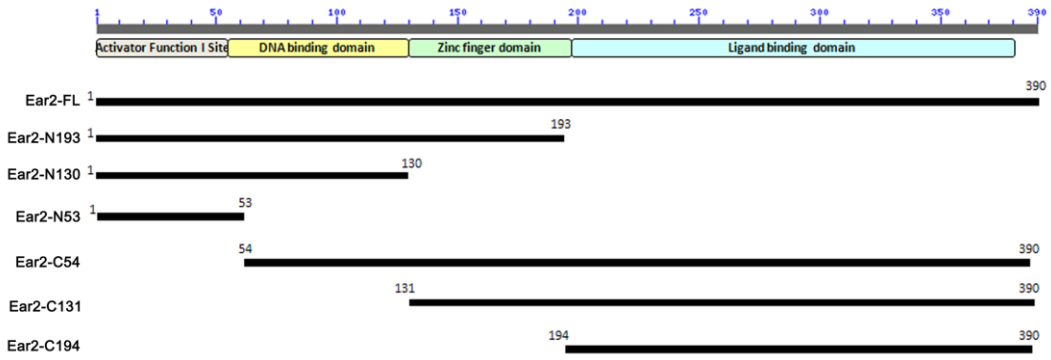


Figure 21. Schematic diagram showing the truncated constructs of Ear2. Ear2 contains 4 main domains: an activator function I site (residues 1-53), a DNA binding domain (residues 54-130), a zinc finger domain (residues 131-193) and a ligand binding domain (residues 194-376). Full length (FL) Ear2 has 390 amino acids and each of the six truncated Ear2 constructs are annotated by their amino acid numbers.

4.14.2. The ligand binding domain of Ear2 interacts with Rasd1

These six GST-tagged Ear2 truncated constructs were used to carry out Ni-NTA pull-down assays against full-length HisHA-Rasd1. It was found that only full-length Ear2 (Figure 22, Ear2-FL) and Ear2 truncated constructs that contain the ligand binding domain (Figure 22, Ear2-C54, Ear2-C131 and Ear2-C194) co-precipitated with HisHA-Rasd1 (Figure 22, lane 1 and lanes 5-7). This indicates that the C-terminus ligand binding domain of Ear2, containing amino acids 194-390, is required for it to bind to Rasd1.

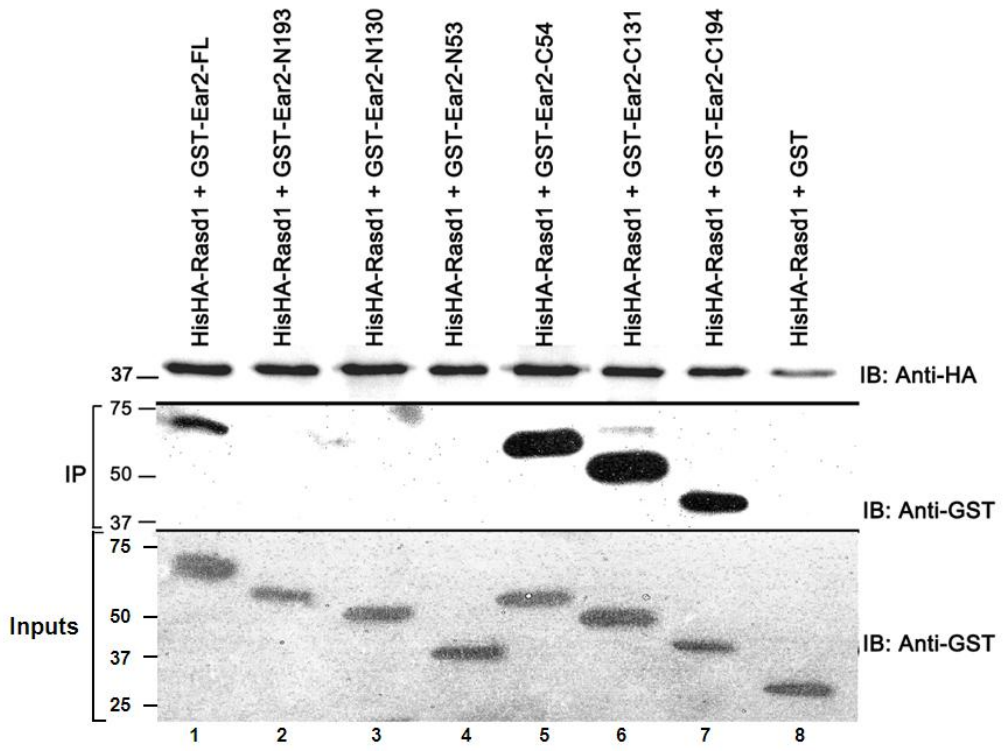


Figure 22. Rasd1 binds to Ear2 ligand binding domain.
COS-7 cells were co-transfected with pHisHA-Rasd1 (3.5 µg) and the indicated pGST-Ear2 constructs (2.0 µg). HisHA-Rasd1 from the cell lysates was

immobilized on Ni-NTA beads and GST-Ear2 constructs bound to the complexes were eluted from the beads by heating in Lammaeli buffer at 95^{o}C for 10 minutes, and detected by immunoblotting with anti-GST (lanes 1 and 5-7). Inputs for GST-Ear2 constructs are shown (lanes 1-8). IB, immunoblot; IP, immunoprecipitation.

4.15. Mapping of the domains on Ear2 that is critical for its regulation of renin transcriptional activity

4.15.1. The DNA binding domain of Ear2 is critical for its repression of renin transcription

To further investigate the binding of Ear2 on the renin enhancer (132), luciferase assay was performed using the six truncated constructs of Ear2. Ear2 truncated constructs devoid of their DNA binding domains markedly lost their ability to repress renin transcription (Figure 23, bars with Ear2-N53, Ear2-C131 and Ear2-C194). This is in accordance with published data where Ear2 with mutated DNA binding domain was unable to bind RARE, and was ineffective in inhibiting the activity of renin promoter (132). These findings confirmed that the DNA binding domain of Ear2 is essential for its binding to the renin enhancer, and that the DNA binding domain of Ear2 plays a critical role in its regulation of renin transcriptional activity.

4.15.2. Ear2 ligand binding domain is required for Rasd1 to alleviate Ear2-mediated transcriptional repression of renin

When Rasd1 was added, it was observed that Rasd1 alleviated Ear2-mediated transcriptional repression of renin promoter activity only in Ear2 constructs

with intact DNA and ligand binding domains (Figure 23, bars with Ear2-FL and Ear2-C54). This further confirmed the previous observations from pull-down assays that Rasd1 interacted with the ligand binding domain of Ear2 (Figure 22), and also showed that the specific binding of Rasd1 to Ear2 is essential for Rasd1 to alleviate Ear2-mediated transcriptional repression of renin expression. In addition, it was observed that the activator function I domain (Figure 21, Ear2-N53) of Ear2 was not required for its repression of renin promoter activity. Deletion of the activator function I domain had no influence on either the Ear2-mediated repression or the Rasd1-mediated alleviation of renin promoter activity (Figure 23, lanes with Ear2-FL and Ear2-C54).

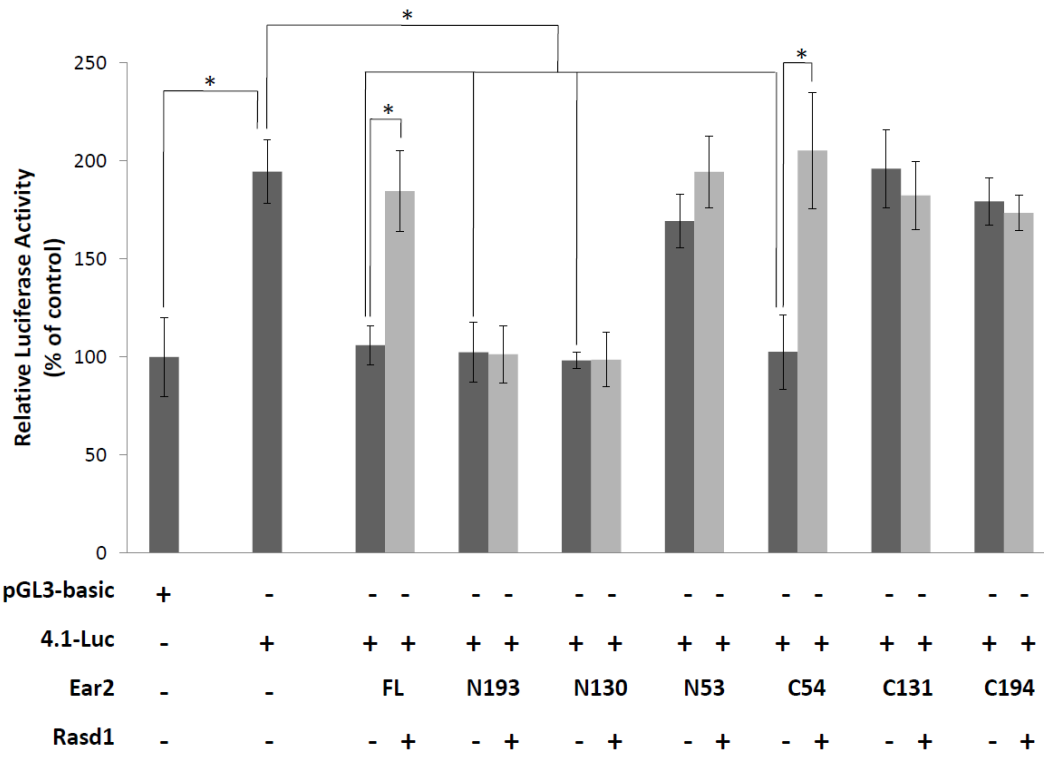


Figure 23. Ear2 ligand binding domain and DNA binding domain are required for Rasd1 and Ear2-mediated regulation of renin transcription.
COS-7 cells were transfected with p4.1-Luc (2.0 µg), pGST-Ear2 or plasmids expressing Ear2 truncated constructs (1.5 µg), together with pSV-β-gal (0.5 µg), with or without pHisHA-Rasd1 (1.5 µg) as indicated. Total DNA transfected was held constant with the respective carrier vector plasmids. Controls were transfected with pGL3-basic (2.0 µg), pSV-β-gal (0.5 µg) and appropriate amounts of the respective carrier vectors. Effects of the various Ear2 constructs on renin transcription were determined by luciferase assay. Relative luciferase activity was normalized against β-gal activity. Ear2 constructs that were missing their DNA binding domains (Ear2-N53, Ear2-C131, Ear2-C194) did not significantly repress renin transcription. Rasd1 alleviated Ear2-repressed renin transcription only if Ear2 ligand binding domain is present (Ear2-FL, Ear2-C54). * $p<0.05$.

CHAPTER 5: CONCLUSIONS AND DISCUSSION

5.1. Summary of results

In this study, Ear2 is identified as a direct interacting target of Rasd1. The interaction between Ear2 and Rasd1 *in vitro* was confirmed using pull-down assays. The existence of endogenous Ear2 and Rasd1 complexes in living cells was also confirmed by performing coIP with lysates from HEK293T cells and mouse brain. Ear2 interacts with Rasd1 via its ligand binding domain, and both the DNA binding and ligand binding domains of Ear2 are critical in Rasd1- and Ear2-mediated renin gene transcription.

In addition, it was shown that Rasd1 is able to alleviate retinoic acid-dependent and independent Ear2-mediated transcriptional repression of both endogenous renin promoter activity and transfected renin promoter constructs. Knockdown of Rasd1 by RNAi resulted in a further suppression of Ear2-mediated repression of renin transcription and the effects on the renin promoter caused by the knockdown of Rasd1 could be partially alleviated by inducing the expression of Rasd1 with dexamethasone.

Furthermore, the Rasd1 mutants- Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], which showed reduced physical interaction with Ear2, also showed a significant reduction in the alleviation Ear2-mediated repression of

renin transcriptional activity. Indirect immunofluorescence and confocal studies revealed that these Rasd1 mutants with defects in GTPase activity, GDP-GTP exchange or isoprenylation and membrane localization also possessed a markedly reduced ability to translocate Ear2 from the nucleus to the cytoplasm in co-transfection experiments. Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX], when co-transfected with Ear2, resulted in a significantly less amount of Ear2 translocated and retained in the nucleus, as compared to wild type Rasd1 or its constitutively active mutant Rasd1[A178V]. This study demonstrates a novel interaction between Rasd1 and Ear2, and a novel regulatory role of Rasd1 in the mediation of renin transcription.

5.2. Proposed mechanism of the regulation of the renin promoter activity by Rasd1 and Ear2

Renin is the rate-limiting enzyme in the renin-angiotensin enzymatic cascade that leads to the production of the bioactive product Ang-II (95,192). However, despite the efforts aimed at characterizing the renin enhancer, its functional relevance *in vivo* has yet to be determined. In this study, Rasd1 was discovered as a novel interacting protein of Ear2, which prompted further investigation of the possible roles of Rasd1 in Ear2-mediated renin gene transcription, since Ear2 has been found to be involved in the negative regulation of renin gene transcription (132).

The renin transcriptional enhancer contains both positive and negative regulatory elements (123,128-130). Among them include an unusual RARE with TGACCT tandem repeats spacing by 10 nucleotides- TGACCT DR10 TGACCT, which is required for both retinoic acid-mediated and retinoic acid-independent activity of the enhancer (128). Ear2 has been shown to negatively mediate the regulation of renin transcription by competing with RAR/RXR for its binding to RARE (128,132). Interestingly, I found that Rasd1 alleviated Ear2-mediated repression of renin transcription in a dosage-dependent manner. My experiments also showed that Rasd1 is able to relieve Ear2-mediated transcriptional repression of renin via direct interaction with Ear2 both in the presence or absence of retinoic acid. This novel regulatory function of Rasd1 required specific interaction of Rasd1 with the ligand binding domain of Ear2. Furthermore, it was demonstrated that both the DNA and ligand binding domains of Ear2 are critical in Ear2- and Rasd1-mediated regulation of renin promoter activity. All of these data suggest that Ear2 binds to the RARE on the renin enhancer via its DNA binding domain to repress renin promoter activity; and that in the presence of excess Rasd1 from the overexpression of transfected constructs, Rasd1 binds to the ligand binding domain of Ear2 to remove the Ear2-mediated repression on the renin promoter.

I hypothesize that Rasd1 acts by binding directly to Ear2 and removing it from binding to RARE on the renin enhancer, subsequently sequestering Ear2 from

the nucleus to the cytoplasm and thus effectively removing the inhibitory effect of Ear2 on the renin enhancer by allowing other stimulatory factors, like RAR/RXR, to bind to the renin enhancer. Immunofluorescence and confocal studies showed that a significant amount of Rasd1 is localized in the nuclei. In accordance, Rasd1 contains a bipartite nuclear localization signal that is typically required for the transport of proteins into the nucleus (39), and has been shown to be present in both the cytosol and membrane (35). Interestingly, when Rasd1 was co-transfected with Ear2, it resulted in a significant movement of Ear2 from the nucleus into the cytosol. This suggests that Rasd1 plays a role in the regulation Ear2 nucleus-cytosol distribution.

My current data suggest that physical binding of Rasd1 to Ear2 facilitates the translocation of Ear2 from the nucleus to the cytoplasm or results in an increased retention of Ear2 in the cytoplasm, thereby removing the binding of Ear2 on the RARE site of the renin enhancer and leads to the alleviation of Ear2-mediated repression of the renin promoter. However, it cannot be ruled out that Rasd1 may act as a co-activator of the renin promoter, and its binding to Ear2 impairs the repression activity of Ear2 on renin gene transcription through a currently unknown mechanism.

5.3. Novel regulatory roles of Rasd1 in transcription and translocation

Despite extensive similarities to members of the Ras superfamily, Rasd1 also possesses several differences, including an extended carboxyl terminus variable cationic domain, a high basic net isoelectric point, and a higher molecular weight compared to typical members of the Ras family (34). In addition, while most Ras proteins are located at the membranes, Rasd1 has been localized to the nucleus, cytoplasm and membranes (35). These differences suggest that Rasd1 may play diverse biological roles which are not in common with other members of the Ras family.

Previously, Rasd1 is mainly thought of as a Ras-like protein for nucleotide binding and hydrolysis, implicated in G protein signaling (33,36). Like other members of the Ras family, Rasd1 was involved in cell growth, proliferation and cell transformation (38). Later when it was discovered that the expression of Rasd1 exhibits a circadian pattern in the SCN (41,42), Rasd1 was uncovered to be involved in the regulation of circadian timing mechanisms (44,45).

Although the notion of the involvement of Ras-like proteins in transcriptional regulation is not new, it is worthwhile to mention that this study reports a novel regulatory mechanism of renin transcription by Rasd1. Rasd1 has been previously implicated in transcriptional regulation and has been reported to interact with FE65 to suppress FE65-amyloid precursor protein (APP)-

dependent transcription (39). FE65 is an adaptor protein that binds to APP to form a transcriptionally active complex. APP is a type I integral transmembrane protein with functions still largely unknown. However, APP is a known precursor of β-amyloid protein, which can aggregate to form neurotic plagues in brain leading to the pathogenesis of Alzheimer's disease (201). FE65 has also been shown to modulate the production of β-amyloid proteins (202,203). In the study of the interaction between Rasd1 and FE65, a co-transfection of FE65 and Rasd1 resulted in a marked increase in proportion of nuclear Rasd1 (39), suggesting that FE65 mediates the translocation of Rasd1 from the cytosol to nucleus. Interestingly, my immunostaining and confocal studies showed that when Rasd1 and Ear2 were co-expressed in cells, Ear2 was shifted from the nucleus to cytosol. In contrast to it being a passive protein that is being translocated, Rasd1 is able to act as the active modulator in the mediation of the translocation of Ear2 from the nucleus to the cytosol in the transcriptional control of renin gene expression.

5.4. Elucidating the biochemical activities of Rasd1 involved in its regulation of Ear2-mediated transcriptional repression of renin promoter

Rasd1 belongs to the RAS superfamily. Members of the RAS superfamily are highly conserved in sequence and show high homology across species. Similar to other members of the RAS superfamily, Rasd1 possesses five highly conserved motifs for GTP binding and hydrolysis (G1-G5), an effector loop that

mediates protein-protein interactions, and a C-terminus CAAX box which serves as a consensus site for isoprenylation required for membrane localization (2).

In view of the highly conserved sequences and functions of the members of the RAS superfamily, I attempted to elucidate the involvement of GTP binding and GTP hydrolysis of Rasd1 in the alleviation of Ear2-mediated repression of renin transcription by generating and studying several Rasd1 mutants.

As expected, Rasd1[A178V], a constitutively active mutant of Rasd1, alleviates Ear2-mediated transcriptional repression of renin promoter activity in a magnitude comparable to wild type Rasd1. In contrast, Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] did not alleviate Ear2-mediated repression of renin transcription, suggesting that GTPase activity, GDP-GTP exchange by GEF, isoprenylation and possibly targeting of Rasd1 to the cell membrane is involved in this function. When interaction studies were conducted, it was discovered that Rasd1[G81A], Rasd1[T38N], Rasd1[ΔCAAX] had visibly weaker interactions with Ear2 compared to wild type Rasd1 and Rasd1[A178V]. Immunofluorescence and confocal studies further showed that Rasd1[G81A], Rasd1[T38N] and Rasd1[ΔCAAX] were ineffective in translocating and retaining Ear2 in the cytoplasm. It is also interesting to note that while wild type Rasd1, Rasd1[A178V] and Rasd1[T38N] were present in both the nucleus

and cytoplasm, Rasd1[G81A] and Rasd1[ΔCAAX] were localized mainly in the nucleus, suggesting that GTPase activity and isoprenylation of Rasd1 affects the nuclear-cytoplasmic distribution of Rasd1 itself.

Mutant Hras[S17N], a corresponding mutation in humans to the mouse Rasd1[T38N], was shown to have a much higher affinity for GDP than GTP, and inhibits Ras dependent pathways (198). Thus it was expected when it was observed that the Rasd1[T38N] mutant was not able to upregulate renin transcription. This result suggests that the transcriptional regulation of renin by Rasd1 is dependent on GEFs. On a side note, there exist Ras-dependent pathways that are not GEF-dependent. For example, Ras may be activated by inhibiting GAP actively without the need for GEFs (204,205), a pathway that will thus be insensitive to the mutation of residue T38.

Likewise, it was observed that mutant Rasd1[G81A], which binds with reduced affinity to GAPs and possesses attenuated GTPase activity (195), did not alleviate Ear2-mediated repression of renin transcription. I propose a couple of plausible explanations for this observation. The binding of Rasd1 to Ear2, and subsequent translocation and retention of Ear2 in the cytosol by Rasd1 may require the GTP hydrolysis activity of Rasd1. Thus, Rasd1[G81A], with reduced GTPase activity, does not effectively bind to Ear2 and translocate it out of the nucleus to remove the repression of Ear2 on renin promoter.

Alternatively, the lack of functional activity of Rasd1[G81A] may possibly be due to a reduced affinity of Rasd1[G81A] to Ear2. In fact it has been reported that in Hras, the corresponding G60A mutation perturbs the GTP-induced conformational change and abolishes its biological activity (206). In another example, a corresponding mutant of EF-Tu, EF-Tu[G83A], showed increased GTPase activity and a reduced binding affinity to aa-tRNA (207).

It is very interesting to note that although both the Rasd1 mutants, Rasd1[A178V] and Rasd1[G81A], are constitutively active mutants in a perpetual GTP-bound states, they appear to display conflicting results with respect to their effects in the regulation of Ear2-mediated renin transcription- while Rasd1[A178V] alleviates Ear2-mediated repression of renin gene transcription, Rasd1[G81A] does not; Rasd1[A178V] translocates and retains Ear2 in the cytoplasm but Rasd1[G81A] does not; Rasd1[A178V] has stronger interactions with Ear2 than Rasd1[G81A]. It may seem perplexing, but upon closer inspection, it becomes obvious that although both of these mutations theoretically render the Rasd1 mutants as constitutively active, Rasd1[A178V] possesses normal GTPase activity, while Rasd1[G81A] has drastically attenuated GTPase activity.

Taken together, the data suggests that the intrinsic Rasd1 GTPase activity, GDP-GTP exchange by GEF and isoprenylation and membrane localization are

required for its functional activity in the mediation of Ear2-mediated repression of renin gene transcription. Rasd1 mutants that possess these defects were rendered ineffective in the alleviation of Ear2-mediated repression of renin promoter activity, possibly due to a weakened interaction with Ear2, a reduced effectiveness in the translocation of Ear2 from the nucleus to the cytoplasm, a decreased in its ability in the retention of Ear2 in the cytoplasm or a combination of any of these effects.

5.5. Implications of Rasd1's involvement in Ear2-mediated renin gene transcription on the circadian control of blood pressure

5.5.1. Overview of the circadian system

Many biochemical and physiological functions, such as our daily pattern of blood pressure, levels of circulating hormones, sleep-wake cycle, temperature regulation, and water and food intake exhibit endogenous rhythmic variability corresponding to a day-night cycle of close to 24 hours. A disrupted circadian function can adversely disrupt metabolism, reproduction and even longevity (208). In the brain, altered clock functions have been attributed to neurological, behavioral and psychiatric deterioration (209).

The endogenous biological clock is driven by the circadian system consisting of central oscillators in the SCN and peripheral oscillators in organs like the heart, pancreas, kidneys and liver (210-212). The 24 hour oscillations of the circadian

system are generated by a molecular mechanism entrained by external and internal cues which in turn generate rhythmic outputs. In the absence of external time cues, the master clock in the SCN oscillates with an approximately 24 hour period. Photic cues are transmitted to the SCN via the retino-hypothalamic tract to keep the external and internal timings synchronized. Peripheral clocks are entrained by the SCN clock through internal synchronizers. Central and peripheral oscillators can communicate via metabolic, hormonal and neuronal pathways (213).

5.5.2. Rasd1 and Ear2 are involved in the regulation of the RAS and in the control of the circadian clock

I have demonstrated the involvement of Rasd1 and Ear2 in the regulation of renin transcription. In addition to its involvement in the RAS, *Rasd1*'s expression is regulated by clock genes, and in the SCN its expression pattern exhibits a circadian rhythm (41). In fact, Rasd1 knockout mice displayed circadian defects, suggesting that Rasd1 is involved in the responsiveness of the circadian clock to external environmental photic cues (44,45). This leads to the postulation about the relevance of the involvement of Rasd1 in the regulation of Ear2-mediated renin transcription. I theorize that Rasd1 might be a player in the regulation of the daily circadian pattern of blood pressure through its physical interaction with Ear2 to affect the transcriptional activity of the renin gene. Interestingly, Ear2 knockout mice displayed several circadian defects, including

a reduced efficiency to the entrainment to photic and feeding time cues (139). Ear2 knockout mice also lack about 70% of the LC, whose firing pattern displays a circadian rhythm (139). The LC also possesses important neuronal connections to the central circadian center in the SCN and other regions of the brain. Ear2 is also required for the normal rhythmic expression of *Per1* and *Per2 in* the forebrain (139,214). All these are strong evidence that points to the involvement of both Rasd1 and Ear2 in the intrinsic regulation of the circadian clock.

5.5.3. TGR(mREN-2)27 rats and disturbed circadian system: Are Rasd1 and Ear2 involved in the maintenance of daily blood pressure?

A daily pattern of blood pressure is a physiological condition persisting in constant conditions. The RAS regulates blood pressure via several mechanisms, including vasoconstriction, sodium reabsorption and increased water intake. The RAS is in turn modulated by several mechanisms- vascular pressure, sodium concentration, and the sympathetic nervous system. The RAS can also be regulated by the circadian system (215).

TGR(mREN-2)27 rats, established by introducing the murine renin-2 gene into the genome of the rat (216), represent a genetic model for hypertension. Interestingly, this manipulation has the capability to influence the function of the circadian system. Transgenic hypertensive TGR(mREN-2)27 rats display a

disturbed blood pressure profile. In addition to severe high blood pressure, they possess inverse circadian blood pressure profiles (217). Circadian rhythm in the heart rate and locomotor activity retains their original phase (218). TGR rats also have increased activity of the RAS, with generally higher plasma renin activity and higher plasma Ang II levels (219,220).

The daily pattern of blood pressure is maintained by the interplay of at least three systems- the autonomic nervous system, the RAS, and the circadian system. Recently, it was hypothesized that the inverted blood pressure in TGR rats develops as a result of reciprocal interactions between central circadian system in the SCN, structures in caudal brainstem involved in blood pressure control, and renal and extra-renal renin-angiotensin system, both systemic and peripheral (221).

In spite of uncertainty about actual mechanism of blood pressure inversion in TGR rats, it is clear that the additional renin-2 gene is the reason for the modified circadian phenotype in this strain. At least part of the reason for the inverted blood pressure profile in TGR rats can be attributed to Ang II, since the administration of ACE inhibitors or Ang II receptor antagonists relieved hypertension (222) and normalized the daily pattern of blood pressure in TGR rats (223). This indicates the involvement of a disturbed RAS in the manifestation of the inverse blood pressure profile in TGR rats. It is known that

neither Ang II nor ACE expression pattern exhibits a rhythmic pattern. *Rasd1*, however exhibits circadian rhythmic gene expression in the SCN (41). Ear2 itself is also a regulator of clock genes, and in Ear2 knockout mutant mice, *per* gene expressions are disturbed. Might Rasd1 then be the elusive key player in the maintenance of a constant blood pressure via its regulation of renin through Ear2, the missing link between the central and peripheral oscillators? Might Ear2 be more directly involved in the control of daily blood pressure pattern through its regulation of clock genes? It is no doubt that a lot more work has to be done to solve the mystery.

5.5.4. Prorenin receptors

A piece of evidence that supports the notion that Rasd1 and Ear2 play a role in the regulation of blood pressure through their regulation of renin transcriptional activity is the recent discovery of prorenin receptors. Prorenin receptors are highly expressed in the human brain, in levels comparable to that observed in the heart and placenta, and in levels greater than the levels observed in the kidney, liver and pancreas (224). Prorenin receptors are functional receptors that trigger signaling cascades involving MAPK, leading to the production of Ang I and Ang II, synonymous to the effects of AT1 receptors activation (225). Binding studies have suggested that prorenin is the likely natural agonist for prorenin receptors (226). The hyperactivation of prorenin receptors, both centrally and peripherally, has been attributed to hypertension. Prorenin levels

were also significantly increased in TGR rats (227). The discovery of prorenin receptors in the brain suggests an alternative regulatory pathway for Rasd1 and Ear2 in the modulation of blood pressure. Through their mediation of renin gene expression, Rasd1 and Ear2 may modulate blood pressure via prorenin receptors.

5.6. Renin angiotensin system in the central nervous system- Implications of Ear2 and Rasd1 in the progression of neurological diseases

5.6.1. The renin-angiotensin system in the central nervous system

Since the processing of AGT to its bioactive products would require substantial amounts of physiological renin or renin-like activity, the existence of a complete RAS in the brain has previously been questioned due to the poor expression of renin in the brain (110). The discovery of prorenin receptors resolved the conundrum and may help elucidate the mechanism of a brain localized RAS (227). Detailed analysis of the distribution and functions of the components of the RAS has now provided substantial evidence supporting the existence of a complete and functionally competent RAS in the brain (94,95,110,228). The brain RAS is distinctly separate from the peripheral RAS and contains all the necessary precursor peptides and enzymes required for the biosynthesis and formation of all bioactive forms of angiotensins (94). The brain RAS is however not completely independent from the peripheral RAS. In certain areas of the brain, for example at the circumventricular organs that lack

the blood brain barrier, angiotensins generated in the periphery can interact and exert effects on the brain RAS (229).

5.6.2. Functions of the neuronal renin angiotensin system

Within the brain, angiotensins can act as neuropeptides and exert neuromodulatory functions. In addition to the classical roles of peripheral angiotensins that include regulation of blood pressure, water and salt homeostasis, various studies have shown that central angiotensins are also involved in less conventional functions such as sexual behavior (98,230), stress (101,102,231), and learning and memory (98).

There is evidence that drugs acting on the brain RAS exert anxiolytic effects and are effective antidepressants. The anxiolytic effects of Ang II has been proven (232), and it has also been reported that ACE inhibitors facilitate mental functioning and well being in human patients (233). With regards to learning and memory, it was found that single doses of captopril, an ACE inhibitor, improved short term memory (234). This naturally implies that a reduced availability of Ang II facilitates cognitive processes (235). Indeed, Ang II, via its actions on AT1 receptors, impairs learning and memory (236,237). Interestingly, intracerebroventricular infusion of renin produced a deficit in performance of a passive avoidance response in rats in a dosage dependent manner (237). It is widely accepted that long-term potentiation, a specific form

of synaptic plasticity, and memory and learning function share the same mechanism (238), and it has also been shown that Ang II blocks the induction of long-term potentiation via AT1 receptors in the hippocampus (239) and amygdala (240). Ang II also regulates cerebral blood flow by stimulating cerebral vasoconstriction by acting on AT1 receptors (241). An effective blockade of brain AT1 receptors has neuroprotective effects against damage induced by cerebral ischemia and improves the neurological outcome of focal brain ischemia (242).

5.6.3. Involvement of the central renin angiotensin system in neurodegenerative disorders

There are also studies showing that the vascular homeostasis is tightly linked to neurodegenerative disorders. For example, hypertensive subjects show an increased risk for Alzheimer's dementia and elderly treated with hypotensive drugs show a decreased risk of dementia (243). As a major player in the regulation of vascular homeostasis, the RAS is thus naturally involved in neurodegenerative diseases. There is evidence to suggest that the RAS is involved in neurological disorders such as Alzheimer's (106,107,243,244) and Parkinson's (109,244) diseases. ACE activity in the cerebrospinal fluid of humans has been shown to be decreased in Alzheimer's and Parkinson's patients with moderate degrees of dementia (245), and in addition, ACE density is increased in the temporal cortex of patients with Alzheimer's disease (106).

An association between ACE genotype and Alzheimer's disease has been found in females (246), and an association between genetic polymorphism of the ACE gene and Parkinson's disease has also been found (109).

Apart from these observations that unequivocally link the RAS to the neurodegenerative diseases, morphological modifications in neuronal tissues through the progression of these diseases are linked to the RAS. The AT2 receptor recognition sites levels were significantly increased in patients with Alzheimer's disease compared to control patients (108). In another study, ACE, Ang II and AT1 staining intensities in the neurons of the parietal cortex were increased in patients with Alzheimer's dementia compared to controls, indicating an enhanced activity of the brain RAS in the disease progression (107). AT1 receptor recognition site levels and angiotensin AT2 receptor levels were significantly reduced in the brains of patients with Parkinson's disease relative to matched controls (108). Thus it appears that the brain RAS is involved and is affected by neurological disorders like Alzheimer's and Parkinson's diseases.

5.6.4. Rasd1 and Ear2 mediation of renin gene transcription: New candidate genes for treatments?

Although it is of no doubt that additional research efforts are needed to clarify the association between the RAS and neurological diseases, it does appear that the brain RAS is involved and is affected in Alzheimer's and Parkinson's

diseases. This leads to the speculation of the implications of potential roles that Rasd1 and Ear2 may have through their regulation of renin transcription. In this study, the identification of this novel regulatory transcriptional control of renin gene expression, one of the key components of the RAS, opens exciting possibilities for future research, and will be no doubt be of considerable interest for clinical applications. It is apparent that the brain RAS is a very complex system, with numerous enzymes involved and several alternative biosynthesis pathways. Targeting the brain RAS may be a viable option for the future treatment of mood disorders, cognitive dysfunctions and even neurodegenerative diseases. Better understanding of the regulation of the RAS may eventually lead to more effective drugs, not only in treatments for hypertension, but possibly in the management of neurodegenerative disorders.

5.7. Perspectives: Novel interaction between Rasd1 and Ear2 in the regulation of renin gene transcription

The RAS is classically known for its involvement in the regulation of blood pressure. As a major player in the regulation of vascular homeostasis, the RAS has been a favorite target in the development of drugs for the management and treatment of hypertension. Clinically, inhibitors of the RAS are observed to be effective and excellent antihypertensive treatment options (247-249). Hypertension is in turn a potent risk factor for cardiovascular diseases such as stroke, atherosclerosis, and heart attacks (250-252).

The identification of an independent RAS in the central nervous system demonstrates that angiotensins are involved in a large variety of physiological processes. In addition to classical functions such as blood, water and salt homeostasis, the RAS in the brain is involved in the regulation of multiple brain functions like learning and memory, stress and emotional responses. The RAS thus not only provides excellent targets in treatments for hypertension but also constitutes an interesting source of candidate genes which may be involved in neuromodulatory functions and even in neurodegenerative disorders. Thus efforts aimed at the detailed understanding of the interplay between the various bioactive components of the RAS and the regulation of the biosynthesis of these peptides, as well as the identification of further functions and regulatory mechanisms are extremely important and cannot be undermined.

My identification of the involvement Rasd1 and Ear2 in the regulation of renin gene transcription contributes to the further understanding of the regulatory mechanism of the RAS, and another weapon in the arsenal against diseases and conditions associated with the RAS. In addition, since renin is the enzyme responsible for the rate determining step in the conversion of AGT to Ang I, the catalytic role of renin is crucial and critical. The fact that Ear2 and Rasd1 are expressed endogenously in the brain, and are involved in the circadian control mechanism makes the discovery even more exciting.

One recent breakthrough made in the RAS is the discovery of prorenin receptors. This discovery has given way to new answers with regards to formerly unexpected findings in the search of treatments of hypertension and its associated disorders. It suggests that renin itself can act as a regulator through its binding to prorenin receptors, to affect brain development and blood pressure modulation. Thus the understanding of the biosynthesis of renin becomes increasingly urgent and important. Certainly, better understanding of the pathway to renin production and regulation of renin biosynthesis is a definitive step towards providing us a clearer picture on the complex regulatory network of the RAS.

Despite the availability of many effective drugs such as ACE inhibitors and Ang receptors blockers, the blockade of RAS is incomplete in many patients, resulting in uncontrolled high blood pressure and ineffective cardiovascular and renal protection (253,254). About fifty years ago, scientists have already identified renin inhibition as the preferred pharmacologic approach to the blockade of the RAS. There are several theoretical reasons to suggest that renin inhibitors may have renoprotective actions superior to those of ACE inhibitors and Ang II receptor blockers, one of them being the fact that targeting renin blocks the RAS at its origin (255). The identification of an effective renin inhibitor however remained elusive. Early renin inhibitors were monoclonal

antibodies that were unsuitable for medication purposes as they were immunogenic (256). It was only until very recently in 2008 that a direct renin inhibitor, Aliskiren, was developed and approved for use as an antihypertensive drug (257,258). Today, Aliskiren is the only available renin inhibitor drug in the market against hypertension. Aliskiren has proven to be a safe and effective antihypertensive agent- it is effective in the inhibition of plasma renin activity and the reduction of blood pressure, as well as in the prevention of renal damage in diabetic patients with nephropathy (253). Aliskiren can also be used in combination with other drugs to improve blood pressure control (253). The identification of the involvement of Rasd1 and Ear2 in the regulation of renin gene expression provides alternative targets in the desperate search for renin inhibitors. It is possible that renin expression could be modulated by targeting Rasd1 or Ear2. Rational understanding and exploitation of the regulation of renin biosynthesis and its related pathways will allow us to ascertain the definitive roles of renin and reap the true advantages of renin blockade.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

7.1. Lists of oligonucleotides used

7.1.1. Primers used in yeast two-hybrid cloning and sequencing

Primer name	Sequence	Description
Rasd1 F	5'- cga*catatg*tatgaaactggccgcgatg -3'	Forward primer for PCR of Rasd1 (*Nde*I)
Rasd1 R	5'- ggc*gaattc*actgatgacacagcgctcc -3'	Reverse primer for Rasd1 PCR (*Eco*RI)
MATCH MAKER 5' AD LD-Insert screening amplimer	5'- ctattcgatgatgaagataccccaccaaaccc-3'	Forward primer for screening positive clones
MATCH MAKER 3' AD LD-Insert screening amplimer	5'-gtgaacttgcggggtttttcagtatctacgat-3'	Reverse primer for screening positive clones
GAL4 AD sequencing primer	5'- gggtttggaatcactacaggggta -3'	Sequencing primer for identifying positive clones

7.1.2. Primers used in generating the wild-type and mutant Rasd1

Primer name	Sequence	Description
HisHA-Rasd1 F	5'-cga*ggtacc*tatgaaactggccgcgatg-3'	Forward primer for PCR of Rasd1 (*Kpn*I)
HisHA-Rasd1 R	5'-ggc*gaattc*agcgtagtctgggacgtcgtatgggtaactgatgacacagcg-3'	Reverse primer for PCR of Rasd1 (*Eco*RI)
Rasd1[CAAX] F	5'-cga*ggtacc*tatgaaactggccgcgatg-3'	Forward primer for generating Rasd1[CAAX] (*Kpn*I)
Rasd1[CAAX] R	5'-ggc*gaattc*agcgtagtctgggacgtcgtatgggtagcgctccttgtccttagc-3'	Reverse primer for generating Rasd1[CAAX] (*Eco*RI)
Rasd1 flank F	5'-cga*ggtacc*tatgaaactggccgcgatg-3'	Forward flanking primer for generating Rasd1 mutants (*Kpn*I)
Rasd1 flank R	5'-ggc*gaattc*agcgtagtctgggacgtcgtatgggtaactgatgacacagcg-3'	Reverse flanking primer for generating Rasd1 mutants (*Eco*RI)
Rasd1[A178V] F	5'-tcgagatctca**gtc**aagaagaacag-3'	Forward mutagenic primer for generating Rasd1[A178V]
Rasd1[A178V] R	5'-ctgttcttctt**gac**tgagatctcga-3'	Reverse mutagenic primer for generating Rasd1[A178V]

Rasd1[G81A] F	5'-tggacacatcc**gcc**aatcatccgttt-3'	Forward mutagenic primer for generating Rasd1[G81A]
Rasd1[G81A] R	5'-aaacggatgatt**ggc**ggatgtgtcca-3'	Reverse mutagenic primer for generating Rasd1[G81A]
Rasd1[T38N] F	5'-aagtgggcaag**aac**gccattgtgtc-3'	Forward mutagenic primer for generating Rasd1[T38N]
Rasd1 [T38N] R	5'-gacacaatggc**gtt**cttgcccactt-3'	Reverse mutagenic primer for generating Rasd1[T38N]

Restriction sites are in italics, sequences for HA tag is underlined and bold font indicates mutated nucleotides.

7.1.3. Primers used in the generating full-length and truncated Ear2

Primer name	Sequence	Description
Ear2 F	5'-cttctcgag*gcggccgc*tatggccatggtgaccggt-3'	Forward primer for PCR of Ear2 (*Not*I)
Ear2 R	5'-cagcccggg*gcggccgc*tccagatacccatgacacca-3'	Reverse primer for PCR of Ear2 (*Not*I)
Ear2 N193 F	5'-cttctcgag*gcggccgc*tatggccatggtgaccggt-3'	Forward primer for PCR of Ear2 N193 (*Not*I)
Ear2 N193 R	5'-cagcccggg*gcggccgc*caactcgcacacgttgt-3'	Reverse primer for PCR of Ear2 N193 (*Not*I)
Ear2 N130 F	5'-cttctcgag*gcggccgc*tatggccatggtgaccggt-3'	Forward primer for PCR of Ear2 N130 (*Not*I)
Ear2 N130 R	5'-cagcccggg*gcggccgc*gcctcgctgcacggcctcct-3'	Reverse primer for PCR of Ear2 N130 (*Not*I)
Ear2 N53 F	5'-cttctcgag*gcggccgc*tatggccatggtgaccggt-3'	Forward primer for PCR of Ear2 N53 (*Not*I)
Ear2 N53 R	5'-gagcccggg*gcggccgc*caaccccggacgctcctcgt-3'	Reverse primer for PCR of Ear2 N53 (*Not*I)
Ear2 C54 F	5'-cttctcgag*gcggccgc*gcaggtggactgcgtggtgt-3'	Forward primer for PCR of Ear2 C54 (*Not*I)
Ear2 C54 R	5'-cagcccggg*gcggccgc*tccagatacccatgacacca-3'	Reverse primer for PCR of Ear2 C54 (*Not*I)

Ear2 C131 F	5'-cttctcgag*gcggccgc*cccgcatcccgcatgagcgcc-3'	Forward primer for PCR of Ear2 C131 (*Not*I)
Ear2 C131 R	5'-cagcccggg*gcggccgc*tccagatacccatgacacca-3'	Reverse primer for PCR of Ear2 C131 (*Not*I)
Ear2 C194 F	5'-cttctcgag*gcggccgc*ggcacgcctgctgttcagca-3'	Forward primer for PCR of Ear2 C194 (*Not*I)
Ear2 C194 R	5'-cagcccggg*gcggccgc*tccagatacccatgacacca-3'	Reverse primer for PCR of Ear2 C194 (*Not*I)

Restriction sites are in italics

7.1.4. Primers used in generating the luciferase reporter constructs

Primer name	Sequence	Description
4.1 F	5'-gga*gctagc*agccctcttctggcctct-3'	Forward primer for PCR of 4.1kb of renin 5'-flanking sequences (*Nhe*I)
4.1 R	5'-aaa*aagctt*tagcccagacccctgag-3'	Reverse primer for PCR of 4.1kb of renin 5'-flanking sequences (*Hind*III)
117P F	5'-tat*ctcgag*gggtccttggccagaaaa-3'	Forward primer for PCR of 117 kb of renin minimal promoter (*Xho*I)
117P R	5'-ttt*aagctt*gctgtgtagcccagaccc-3'	Reverse primer for PCR of 117 kb of renin minimal promoter (*Hind*III)

Restriction sites are in italics.

7.1.5. Oligonucleotides used in generating RARE sites, for ChIP and for EMSA

Primer name	Sequence	Description
1X RARE F	5'-*ctagc*cagatggtgacctggctgtactctgacctctg*c*-3'	Oligonucleotide sequences of forward strand for generation of 1X RARE site with 5' *Nhe*I and 3' *Xho*I restriction sites
1X RARE R	5'-*tcgag*cagaggtcagagtacagccaggtcaccatct g*g*-3'	Oligonucleotide sequences of reverse strand for generation of 1X RARE site with 5' *Nhe*I and 3' *Xho*I restriction sites
2X RARE F	5'-*c*cagatggtgacctggctgtactctgacctctgcagat ggtgacctggctgtactctgacctctg*g*-3'	Oligonucleotide sequences of forward strand for generation of 2X RARE site with 5' *Kpn*I and 3' *Nhe*I restriction sites
2X RARE R	5'-*ctagc*cagaggtcagagtacagccaggtcaccatct gcagaggtcagagtacagccaggtcaccatctg*ggt ac*-3'	Oligonucleotide sequences of reverse strand for generation of 2X RARE site with 5' *Kpn*I and 3' *Nhe*I restriction sites
ChIP-1 F	5'-tagacaccaggagatgac-3'	Forward PCR primer for ChIP (-2866)
ChIP-2 R	5'-catgcgctatcacaacca-3'	Reverse PCR primer for ChIP (-2625)

ChIP-3 R	5'-ccatctgcgtggttagtg-3'	Reverse PCR primer for ChIP (-2674)
EMSACy3-F	5'-/Cy3/tcactaaccacgcagatggtgacctggctgtactctgacctctgagtggctggttgtgat-3'	Oligonucleotide sequences of forward strand used as probe in EMSA, labeled with Cy3 at 5' end
EMSA-F	5'-tcactaaccacgcagatggtgacctggctgtactctgacctctgagtggctggttgtgat-3'	Oligonucleotide sequences of unlabeled forward strand used as probe in EMSA
EMSA-R	5'-atcacaaccagccactcagaggtcagagtacagccaggtcaccatctgcgtggttagtga-3'	Oligonucleotide sequences of reverse strand used as probe in EMSA

Restriction sites are in italics.

7.1.6. Oligonucleotides used in generating the shRNA knockdown constructs

Primer name	Sequence	Description
Rasd1 shRNA F	5'-*gatc*cccctgaaactggccgcgatgatttcaagagaatcatcgcggccagtttcattttta-3'	Oilgonucleotide sequences for generating Rasd1 shRNA (*Bgl*II)
Rasd1 shRNA R	5'-*agctt*aaaaatgaaactggccgcgatgattctcttgaaatcatcgcggccagtttcaggg-3'	Oilgonucleotide sequences for generating Rasd1 shRNA (*Hind*III)
Control shRNA F	5'-*gatc*cccgtcgaacggattgcacgtattcaaagagatacgtgcaatccgttcgacttttta-3'	Oilgonucleotide sequences for generating control shRNA (*Bgl*II)
Control shRNA R	5'-*agctt*aaaaagtcgaacggattgcacgtatctcttgaatacgtgcaatccgttcgacggg-3'	Oilgonucleotide sequences for generating control shRNA (*Hind*III)

Restriction sites are in italics.

7.1.7. Primers used in real time PCR

Primer name	Sequence	Description
Renin F	5'-aggtttcctcagccaggactcgg-3'	Forward primer against renin mRNA
Renin R	5'-ggccctgcctcccaggtcaa-3'	Reverse primer against renin mRNA
Rasd1 F	5'-cgatccgcggcgaagtctac-3'	Forward primer against Rasd1 mRNA
Rasd1 R	5'-gcggtgcaagtcggggctcatct-3'	Reverse primer against Rasd1 mRNA
G3PDH F	5'-catccactggtgctgccaaggctgt-3'	Forward primer against G3PDH mRNA
G3PDH R	5'- acaacctggtcctcagtgtagccca-3'	Reverse primer against G3PDH mRNA

7.2. My publications

1. Jen Jen Tan, Shufen Angeline Ong and Ken-Shiung Chen. (2011) Rasd1 interacts with Ear2 (Nr2f6) to regulate renin transcription. *BMC Mol Biol*, **12**:4.
2. Shufen Angeline Ong, Jen Jen Tan and Ken-Shiung Chen. (2011) Rasd1 modulates the coactivator function of NonO in the cyclic AMP pathway. *pLoS ONE*, **6**(9):e24401.
3. Wai Loon Tew, Ignasius Aditya Jappar, Shufen Angeline Ong, Jen Jen Tan and Ken-Shiung Chen. Interaction between Rai1 and Tdg links DNA repair to Smith-Magenis Syndrome. *(submission in progress).*
4. Shufen Angeline Ong, Yuk Kien Chong, Daren Low, Jen Jen Tan, and Ken-Shiung Chen. Characterisation of *Rasd1* promoter and its novel regulation by Dbp, linking circadian rhythm regulation of *Rasd1* with Dbp and GRE. *(manuscript under preparation).*