NUCLEAR RECEPTORS OF THE ADRENAL CORTEX: GENOMIC TARGETS OF STEROIDOGENIC FACTOR 1 AND EVIDENCE OF ADRENAL FAILURE IN DAX1-DEFICIENT MICE

by

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DEDICATION

To my wife, Shara.

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LIST OF ABBREVIATIONS

Adcy	Adenlyl cyclase
ACTH	Adrenocorticotropic Hormone
AF	Activation Function
AGP	Adrenogonadal Primordium
AHC	Adrenal Hypoplasia Congenita
AVP	Arginine Vasopressin
BrdU	5-bromo-2-deoxyuridine
CRH	Corticotropin Releasing Hormone
CYP	Cytochrome P450
DAX1	Dosage-sensitive sex reversal, Adrenal Hypoplasia Congenita critical region on the X chromosome, gene 1
DBD	DNA Binding Domain
GR	Glucocorticoid Receptor
HPA	Hypothlamic-pituitary-adrenal
HPG	Hypothalic-pituitary-gonadal
LBD	Ligand Binding Domain
LRH-1	Liver Receptor Homologue-1
MC2R	Melanocortin Receptor, type 2
PCNA	Proliferating Cell Nuclear Antigen
POMC	Pro-opiomelanocortin
SCC	Side Chain Cleavage
SHP	Short Heterodimer Partner
SF1	Steroidogenic Factor 1
StAR	Steroidogenic Acute Regulatory Enzyme

VMH	Ventromedial Hypothalamus
zG	Zona Glomerulosa
zF	Zona Fasciculata
zR	Zona Reticularis

ABSTRACT

Steroidogenic Factor 1 (SF1) and DAX1 are nuclear receptors that play an integral role in the adrenal cortex. SF1 is a transcription factor that regulates expression of myriad genes, including those involved in steroidogenesis, production of various endocrine hormones, and development of the adrenals and gonads. DAX1 is an orphan nuclear receptor that inhibits SF1 and, when mutated, is responsible for X-linked Adrenal Hypoplasia Congenita (AHC). In the adrenal cortex, adrenocorticotropic hormone (ACTH) activates SF1-mediated transcription of the genes involved in cholesterol transport and steroid production. However, SF1 is also implicated in adrenal proliferation and differentiation; therefore, it is likely that several different signaling pathways regulate SF1-dependent transcription by altering cofactor availability or by feedback mechanisms that modulate signaling molecules that impinge on SF1 transactivation. This thesis describes three genomic targets of SF1 that provide feedback upon and affect subsequent SF1 transcriptional activity. First, I describe the identification of Edg5, the sphingosine 1-phosphate receptor, as a target of SF1 using chromatin immunoprecipitation-chip microarray (ChIP-chip). Activation of Edg5 may alter levels of intracellular sphingosine 1-phosphate, a ligand of SF1 that activates steroidogenesis and stimulates release of adrenal hormones. I also describe association of SF1 on the adenylyl cyclase 4 (Adcy4) promoter. Though usually an activator, SF1 represses transcription of *Adcy4*, which may alter cAMP-dependent activation of SF1.

Finally, I describe an interaction between SF1 and GR to activate expression of *Dax1*, which is abrogated by ACTH. In all three cases, the genomic target of SF1 encodes a factor that can modulate SF1-dependent transcription. While loss of DAX1 in humans results in AHC, Dax1 deficient-mice have a mild adrenal phenotype. To reconcile these disparate phenotypes, I analyzed Dax1-deficient mice across their lifespan. Young Dax1-deficent mice have increased steroidogenic capacity, expression of steroidogenic enzymes, and proliferation. However, after 60 weeks all three measures are dramatically reduced, suggesting that loss of Dax1 may result in premature differentiation resulting in ultimate exhaustion of the adrenal cortex, which has implications for AHC. Overall, this dissertation contributes to the understanding of both SF1-dependent gene regulation and the significance of DAX1 in homeostasis of the adrenal cortex.

CHAPTER 1: INTRODUCTION

Nearly three hundred years after the first mention of adrenals in an anatomy text, Thomas Addison made the first substantial observations regarding the function of this obscure organ, to which he referred as "supra-renal capsules" (1). In 1855, he presented ten clinical cases from patients suffering with what would become Addison's Disease, whose condition he described as such:

> *The leading and characteristic features of the morbid state to which I would direct attention, are, anæmia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach, and a peculiar change of colour in the skin, occurring in connexion with a diseased condition of the "supra-renal capsules."*

Prior to his work, the importance of these organs was a subject of great debate. The first report of a gland superior to the kidney by Bartolomeo Eustachius directly contradicted the conventional wisdom of the day that adrenals were nonfunctional tissues, not to mention a seminal anatomy volume by Andreas Vesalius, which completely lacked any mention of such a gland. At the time, these structures were believed to be a congenital outgrowth of the kidney or simply vestigial organs, and their function remained enigmatic for many years. In fact, a mere ten years before Addison's publication, the adrenal was declared to be a non-secreting gland (2).

Addison's work ignited study of this obscure gland, which culminated in the awarding of the 1950 Nobel Prize in Physiology or Medicine to Edward Kendall, Tadeus

Reichstein, and Phillip Hench "for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects" (3). Amazingly, a mere five years later, thousands of patients could attribute a prolonged life to adrenal hormone replacement therapy (4).

It is now understood that, just as he suspected, Addison's patients were suffering from primary adrenal failure, which manifests with hyponatremia, hyperkalemia, and acidosis. If left untreated, death results from hypoglycemia and shock. All of these symptoms are a result of insufficient production of the adrenal hormones aldosterone and cortisol. Rather than being the build-up of toxic substances within the blood, the darker skin complexion is a result of excess circulating ACTH, which occurs when the endocrine axis in which the adrenal resides is disrupted. While adrenal function and the etiologies of adrenal-specific diseases are quite well understood today, current endeavors now focus our understanding of this organ at the molecular level. These data will provide critical answers to questions regarding the contributions of specific proteins to adrenocortical development, as well as leading to therapies for adrenal-specific conditions. This type of intimate knowledge will also lead to the ability to understand how cancers form in the adrenal, which will ultimately allow for a more targeted approach for their treatment.

Adrenal Development and Function

The adrenal glands reside superior to the kidneys and are responsible for coordinating the mammalian stress response. The adrenal consists of two developmentally distinct organs; the medulla and the cortex. The adrenal medulla is a

neuroendocrine gland that produces catecholamine in the classic 'fight or flight' immediate stress response. The adrenal cortex regulates the long-term stress response by modulating salt, pH, and glucose homeostasis. This is accomplished mainly through the hypothalamic-pituitary-adrenal (HPA) axis, of which the adrenal is the terminal organ. A wide variety of signals, or stressors, result in release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypopthalamus. CRH and AVP travel through the hypothalamic-pituitary portal vessels to the anterior pituitary where they stimulate corticotropes in the anterior pituitary to release adrenocorticotropic hormone (ACTH), which is the cleavage product of pro-opiomelanocortin (POMC), into the systemic blood. ACTH is released from the anterior pituitary and travels through the bloodstream to the adrenal where it is bound by melanocortin 2 receptors (MC2R). Signaling downstream of MC2R results in immediate release of glucocorticoids, cortisol (in primates) or corticosterone (in rodents), which then travel through the blood to exert their effects throughout the body. Glucocorticoids are bound by the Glucocorticoid Receptor (GR), which is nearly ubiquitously expressed and has diverse effects, such as immune suppression and modulation of glucose homeostasis. Glucocorticoids also provide negative feedback at the hypothalamus and pituitary to modulate the release of ACTH, in a classical endocrine long feedback loop.

There are three concentric functional zones of the adult adrenal cortex that are defined by their steroid product as well as the hormone required for activation. **(Figure 1.1)** All of the hormones produced by the adrenal cortex are cholesterol-derived and exhibit only subtle differences from each other based on the nature and location of side modifications of the main cholesterol backbone. Enzymes required for steroidogenesis,

which include Cytochrome P450 (Cyp450) and cholesterol transport enzymes, are uniquely expressed within the zones of the adrenal cortex. **(Figure 1.2)** Enzymes commonly expressed in all three zones include steroidogenic acute regulatory protein, (StAR), cholesterol side-chain cleavage enzyme (SCC), 3β-hydroxysteroid dehydrogenase (3βHSD), and steroid 21-hydroxylase (21-OH). The outer-most zone of the cortex, the zona glomerulosa (zG), uniquely expresses the enzyme CYP11B2, which converts corticosterone into aldosterone, as well as the angiotensin II receptor, which allows these cells to be activated by the renin- angiotensin pathway. The zona fasciculata (zF) expresses the enzyme CYP11B1, which is required for the hydroxylation of deoxycortisol into cortisol. CYP17 is expressed in the fasciculata and zona reticularis (zR) and is required for production of cortisol as well as androgens. Mice do not express Cyp17 and, therefore produce neither of these hormones. The fasciculata and reticularis also express MC2R, which enables a response to circulating ACTH (5).

While there is a degree of interspecies variability, both functional and histological, among various mammalian species, a common developmental pathway is mediated by distinct genetic signals (5, 6). The adrenogonadal primordium (AGP) is first formed by proliferation of mesoderm-derived coelomic epithelia of the urogenital ridge (7). Coincident to invasion by primordial germ cells, the AGP divides into the adrenal primordia and gonadal primordia (8). Proliferation of the adrenal primordia results in formation of the fetal adrenal gland, which is marked by large eosinophilic cells expressing 17α-hydroxylase. Production of dihydorepiandrosterone (DHEA) in the fetal adrenal is converted to estradiol in the placenta and is required for the maintenance of early pregnancy (9). At approximately the same time as formation of the capsule from

surrounding mesenchymal cells, the definitive zone begins to form. This definitive zone is what eventually develops into the zonated functional adult cortex. It is not yet clear whether the definitive zone arises from precursors within the fetal adrenal or from within the surrounding capsule (10). Also coincident with encapsulation is invagination by neural crest cells that eventually form the adrenal medulla following parturition (5).

There is a significant amount of tissue remodeling that occurs both before and after birth. During the third trimester, the definitive zone begins to differentiate into the zona glomerulosa and zona fasciculata, but the reticularis does not form until the third post-natal year. Approximately three months after birth, the fetal zone regresses, leaving a band of fibrous connective tissue surrounding the medulla. Adrenocortical remodeling is complete with the onset of adrenarche, which occurs during puberty and results in significantly increased production of sex steroid precursors from the reticularis (5, 9, 11). The mature adult cortex maintains organ homeostasis through constant replenishment by way of inward centripetal displacement of cells from the periphery of the gland (7, 12).

While there are definitely differences between the primate and murine adrenal, the developmental patterns share a great deal of similarity. The mouse adrenal, in a fashion similar to the primate adrenal, also develops from an adrenogonadal primordium that arises from the urogenital ridge. The process of invasion by neural crest cells, forming the medulla, and encapsulation by mesenchymal cells is also strikingly similar to human adrenal development, yet follows a different timetable (5, 9). A major advantage of mouse models is a greater ability to understand adrenal development through genetic loss of function studies. In both species, formation of the adrenogonadal primordium is dependent upon expression of the nuclear receptor, Steroidogenic Factor 1 (SF1), which

is expressed in the expanding coelomic epithelial cells (6, 13, 14). The contributions of SF1 to adrenal development will be discussed in further detail later in this chapter.

While the mouse and human adrenal have a great deal of genetic and developmental similarity, there are some differences between the species. The primary difference between the species is the lack of formation of a zona reticularis in mice, mainly because there is no expression of Cyp17, which is required for production of cortisol as well as sex steroids. As such, the mouse adrenal does not form a traditional fetal zone, but does have a fetal structure that expresses steroidogenic factor 1 under a fetal-specific enhancer (15). Additionally, the mouse forms a similar transient structure, called the X-zone, which appears at the second post-natal week and regresses at puberty in males and during the first pregnancy in females (16-18). While the relationship between the fetal zone and the X-zone has been debated, it seems that the X-zone is a remnant of the mouse fetal adrenal (15, 19). Interestingly, the orphan nuclear receptor, DAX1 (Dax1 in mice) seems to be required for regression of the human fetal zone and the murine fetal and X-zones (19-21). Dax1 will be discussed in greater detail later in this chapter.

The adult adrenal cortex is a dynamic organ that continuously repopulates. Injection with agents that label dividing cells, BrdU or tritiated thymidine, initially labels cells of the outer cortex, with the label progressively moving toward the medulla (12, 22, 23). Enucleation studies in rats, in which the inner contents of the adrenal are removed, demonstrated regeneration of the adrenal cortex into steroid-secreting zones within 30 days (24, 25). Additionally, transplantation of primary bovine adrenal cells that were derived from a single clone into adrenalectomized mice resulted in replacement of the

host animals' adrenal function (26, 27). The transplanted tissue even begins to form into architecture that resembles normal adrenal cortex. Finally, studies in transgenic mice, in which the beta-galactosidase reporter gene was expressed under the control of a steroid hydroxylase resulted in variegated expression and chord-like mosaic patches that extended throughout the entire cortex (28, 29). Taken together, all of these data suggest that the adrenal cortex is maintained by proliferation of cells at the periphery of the cortex followed by displacement of the cells inward toward the medulla.

Steroidogenic Factor 1

Steroidogenic Factor 1 (SF1) is a nuclear receptor (NR5A1) (30) that was first identified as a factor that bound an AGGTCA motif in the promoter regions of the genes encoding steroidogenic enzymes and is critically required for transcription of these genes in steroidogenic tissues, namely the adrenal cortex and gonad (31-33). Members of the nuclear receptor superfamily typically display conserved features such as a DNA binding domain (DBD), a ligand binding domain (LBD), and two activation domains that are either independent (the amino-terminal AF-1) or dependent (the carboxy-terminal AF-2) upon a ligand for activation (34). Nuclear receptors are DNA-dependent transcription factors that are often regulated by small lipophilic molecules, such as adrenal or gonadal steroids or other hormones, although a number of receptors, for which ligands do not exist or have not been identified, are termed, 'orphan receptors' (35). Many nuclear receptors translocate to the nucleus following ligand binding where they bind to DNA as homo- or heterodimers. However, SF1 is constitutively nuclear (36), contains a typical DBD, and binds DNA as a monomer (37). The amino-terminal region is very short and

lacks a typical AF-1 domain. However, SF1 contains a conserved AF-2 that interacts with coactivators and is required for transcriptional transactivation (36, 38-40). **(Figure 1.3)**

SF1 in Adrenal Development

As would be expected for the master regulator of steroidogenesis, SF1 is expressed in all major steroidogenic tissues, namely the three zones of the adrenal cortex, testicular Leydig and Sertoli cells, and ovarian, theca and granulosa cells (33, 41-43). SF1 is also expressed in the anterior pituitary and ventromedial hypothalamus (42-44). SF1 is first expressed in the urogenital ridge during the formation of the AGP (13, 14) and expression persists in the organs that are derived from this structure throughout adulthood. Following the initial cloning and isolation of SF1, it was thought that this nuclear receptor was only involved in the synthesis of steroid hormones in adrenals and gonads. Therefore, it was somewhat surprising that SF1 was also required for proper adrenal and gonadal development (45). Indeed, mice lacking expression of Sf1 do not develop adrenals or gonads, have male-to-female sex reversal with persistent Müllerian structures, and die shortly after birth due to adrenal insufficiency (46, 47). This requirement of SF1 suggests the reason complete deletions of the human SF1 gene have not been reported. However, human patients with insufficient SF1 expression, whether by mutations or deletions of one copy, demonstrate XY sex reversal and adrenal insufficiency (48, 49). Reconstitution of such mutations usually result in reduced transcriptional transactivation ability of the SF1 protein on relevant steroidogenic target gene promoters, indicating that SF1 is necessary for activation of some target genes during developmental processes (48).

The developmental role of SF1 is further strengthened by studies involving forced expression in SF1-negative cells. Expression of SF1 in embryonic stems cells resulted in induction of cholesterol side-chain cleavage enzyme and cAMP-induced progesterone synthesis (50). Using mouse and human bone marrow mesenchymal cells, SF1 expression resulted in transformation into cells that expressed most of the required enzymes for steroidogenesis and were able to synthesize progesterone, corticosterone, cortisol, dihydroepiandrosterone, testosterone, and estradiol in response to ACTH (51, 52). Finally, using adipose tissue-derived mesenchymal cells, a similar transformation occurred, but with a shift toward production of adrenal hormones, such as corticosterone, rather than gonadal hormones, such as testosterone, that was observed in the bone marrow-derived mesenchymal cells (53). Together, these studies show that SF1 is able to direct nascent cells toward competent steroid-producing cells.

Regulation of SF1 expression is quite complex and relies on tissue-specific cis-regulatory elements that control expression of SF1 in different tissues. Using transgenic mice, in which β-Galactosidase is expressed under the control of the contiguous *Sf1* gene, both pituitary and VMH enhancers have been mapped to intron 6 of the *Sf1* gene (54, 55). Using the same approach, the fetal adrenal enhancer (FAdE) has been mapped to intron 4 (15). FAdE expression is induced by a homeodomain complex, Hox-Pbx-Prep1, and maintained by that complex along with autoregulation by Sf1 itself. Interestingly, mice lacking the Pbx gene do not develop adrenals, yet do develop gonads in which Sf1 is expressed (56), suggesting that there is a different factor and/or enhancer site controlling expression of Sf1 in these tissues. FAdE expression does not persist through development of the adult adrenal (it disappears with the X-zone), suggesting that there is another adult

definitive adrenal enhancer (DAdE). However, lineage tracing has shown that FAdE-positive cells do ultimately become adult cortex but the enhancer that maintains Sf1 expression in the adult cortex has yet to be determined (19).

SF1-mediated Transcription

While many members of the nuclear hormone receptor family are activated by direct interaction with their cognate hormone ligand (34), transcriptional activation of SF1 by ACTH occurs through intermediate signaling cascades (57). ACTH binds to the transmembrane G protein-coupled receptor, MC2R, which results in activation of adenylyl cyclase, subsequent accumulation of cAMP, and activation of protein kinase A (PKA) which initiates the initial steps of conversion of cholesterol to pregnenolone in the steroidogenic pathway (58). Additionally, PKA activates enzymes within the adrenal cortex, namely phosphorylation of StAR, which transports cholesterol from the outer to the inner mitochondrial membrane and is the rate-limiting step of steroidogenesis (57, 59, 60). Even though gene expression of many of the enzymes involved in steroidogenesis is intimately linked to cAMP signaling and SF1 activity, it is not clear that PKA directly phosphorylates SF1 (61). Rather, it seems cAMP-dependent signaling dephosphorylates SF1 and affects recruitment of SF1 to active transcriptional foci as well as its dynamic interaction with regulatory factors (62-64).

ACTH has also been shown to signal through mitogen-activated protein kinases (MAPK) ERK1/2 in addition to PKA (63). SF1 is phosphorylated by ERK2 on Ser^{203} in the hinge region of the protein, which increases cofactor interaction, causes it to adopt an active conformation, irrespective of ligand binding, and increases transactivation of

reporter constructs (65, 66). However, in vivo studies have shown that Ser^{203} phosphorylation is coincident with cyclical interaction of SF1 with target chromatin DNA that is abrogated by pharmacological inhibition of either the MAPK or PKA pathway, indicating that the cyclical phosphorylation/dephosphorylation pattern is required for transcriptional activation (67). These pathways also coordinate SF1 transactivation of Glycoprotein Hormone alpha-Subunit in pituitary gonadotropes (68). It has recently been shown that other factors, such as CDK7 and SUMOylation, can also modify the extent of Ser^{203} phosphorylation and affect its basal transcriptional activity (69, 70). In short, several signaling pathways seem to converge to coordinate basal transcriptional activity of SF1 that sometime synergizes with cAMP induction.

Activity of nuclear receptors is also greatly affected by availability of and interaction with cofactors (71). SF1 has been shown to interact with a number of coactivators, such as CBP/p300, GCN5, and all three SRCs. SF1 also interacts with corepressors SMRT and RIP140. These cofactor interactions are facilitated by interaction of the conserved AF-2 domain of SF1 with an LXXLL binding motif found in many cofactors (72, 73). Interaction of SF1 with specific cofactors is often facilitated or modified by signaling cascades; cAMP and PKA promote interaction with SRC-1 and SRC-3, while inhibiting interaction with SRC-2 (74, 75). Indeed, mice deficient for Src-1 have altered HPA stress response, suggesting that certain cofactor interactions are preferred over others in certain cellular or genomic contexts (76).

SF1 also interacts with other transcription factors in a promoter-specific context. Not surprisingly, in light of its cooperative activation with cAMP, SF1 interacts with CREB to transactivate cAMP-responsive SF1 target genes (77). Notably, SF1 also

cooperates with β-Catenin to activate transcription of alpha-inhibin as well as the orphan nuclear receptor, Dax1 (78, 79). In addition to transactivating expression of Dax1, SF1 also interacts with the Dax1 protein, which binds via the same LXXLL motif that enables interactions with other coactivators (80). Due to this similar mode of interaction, Dax1 acts as a transcription repressor of SF1 by directly competing with coactivators such as SRC-1 in addition to its association with corepressors, such as NCoR and Alien (81, 82). It is very likely that SF1 interacts with additional transcription factors to cooperatively regulate transcription of certain promoters and future studies will be focused on identifying the entirety of the SF1 interactome.

Genominc Targets of SF1

Many of the genomic targets genes of SF1 have been identified by using transient transfection assays using a limited portion of the promoter or regulatory region of the target DNA. In adrenocortical cells, SF1 increases expression of all the enzymes required for steroid hormone biosynthesis, the ACTH receptor, and steroidogenic acute regulatory enzyme. Additionally, SF1 modulates the gene expression of various factors that are involved in detoxification of byproducts of steroidgenesis, such as aldose reductase-like protein, or cellular acquisition or synthesis of cholesterol, such as scavenger receptor B1 and HMG-CoA synthase (83-85). As would be expected, cells with a mutation that impaired SF1 function had dramatically reduced expression of many of the genes involved in steroid synthesis, such as MC2R, StAR, and CYP11A (86).

SF1 seems to be required to develop and maintain the male phenotype. Müllerian inhibitory substance (MIS), which suppresses formation of Müllerian structures into

female internal genitalia, is stimulated by SF1 (87, 88). SF1 also increases expression of insulin-like peptide 3, which is important for descent of testicles and for male germ cell survival (89, 90). Finally, SF1 plays a key role in sex determination by cooperating with SRY to activate the SOX9 gene and promote the differentiation of Sertoli cells (91). It is therefore not surprising that males heterozygous for SF1 mutations display various degrees of sex reversal.

Expression of SF1 in pituitary gonadotropes is responsible for regulating the reproductive axis, specifically by regulating the expression of LHβ and FSHβ, as well as GnRH receptor (44, 92, 93). Interestingly, SF1 expression in the VMH seems to play a role in appetite control and energy balance by driving expression of such factors as brain-derived neurotrophic factor (BDNF) and cannabinoid receptor (94, 95).

There have been several studies looking at global gene expression in adrenal cell lines that provide insight regarding genomic targets of SF1. In Y1 mouse adrenocortical carcinoma cells, ACTH treatment resulted in the expected increased expression of genes required for steroid synthesis and metabolism. Interestingly, though, there was a significant downregulation of transcripts involved in DNA replication, cell division and RNA processing (96). This study documented changes in well over 1200 transcripts, both up and down, of which only 56% were also regulated by cAMP and only 6% were regulated by PKC, suggesting that a significant portion of ACTH signaling occurs through other pathways, such as MAPK or Ca^{2+}. Another study compared the effect of ACTH stimulation on human adult and fetal primary adrenocortical cells (97). In these primary cells, there were many fewer transcripts activated than what was observed in Y1 cells, and hardly any were downregulated by ACTH. Surprisingly, no cell division or

DNA replication transcripts were downregulated, as was observed in the Y1 cells, suggesting that this phenomenon might be unique to the Y1 cells. Finally a third study performed global expression profiling after overexpressing SF1 in H295 human adrenocortical carcinoma cells, which resulted in upregulation of proliferation markers in addition to the expected steroidogenic transcripts (98). This finding was further verified by generation of a transgenic mouse that express multiple copies of the *Sf1* gene, which had many hyperplastic lesions and formed adrenal tumors. Interestingly, SF1 also seems to be overexpressed in some cases of childhood adrenocortical tumors (99). Together, these results suggest that SF1 has a much wider range of genomic targets than simply steroidogenic enzymes or components of the HPA or HPG axis, and includes factors that are involved in differentiation or proliferation in certain physiological contexts. Microarrays on ACTH-treated cells capture changes that are both SF1-independent and SF1-dependent. However, it is somewhat surprising that there are such dramatic differences in effect among the different cell types and they may simply be artifacts of the phenotype of the particular cells that were used in the studies, or a result of the different array platforms used.

Identification of an SF1 ligand

The putative ligand for SF1 has been the subject of debate for a number of years. Initially, it was thought that SF1 was an orphan receptor because of its constitutive activity when expressed in cells. Additionally, SF1 binds DNA as a monomer, whereas most ligand-activated receptors bind DNA as homo- or heterodimers, and the LBD appears to adopt a permanently stabilized conformation (34, 66). However, the AF2

within the LBD is conserved across species, suggesting its requirement for ligand binding (36, 39, 40). Initial reports that SF1 was activated by oxysterols were quickly dismissed after it was determined that this activation did not occur in relevant steroidogenic cells (100-102). When crystal structural analysis of bacterial-expressed SF1 was performed by several groups, it revealed large phospholipids, phosphatidyl glycerol (PG) and phosphatidyl ethanolamine (PE), within the LBD (103-105). While it was first dismissed as an artifact of crystal structure purification, the fact that mutations that inhibit phospholipid binding resulted in loss of the ability to recruit coactivators or transactivate target promoters was a compelling case for their authenticity.

Tandem mass spectrometry of immunoprecipitated SF1 purified from H295 cells revealed phosphatidic acid (PA) association with the LBD, with PA able to stimulate SF1-dependent transcription of the CYP17 promoter (106). Interestingly, it was also determined that SF1 interacts with diacylglycerol kinase theta (DGK-θ), which is involved in production of PA, and cAMP signaling results in production of both DGK-θ and PA. Additionally, sphingosine was observed to bind the LBD and inhibit SF1-dependent transcription, becoming the first natural SF1 antagonist (106). Structural analysis of SF1 bound to different phospholipids revealed that there is a conformational change in the LBD when phosphatidyl choline (PC) or phosphatidyl inositol di- and triphosphates (PIP2 and PIP3) are bound compared to bacterial phospholipids, suggesting that a dynamic exchange between different phospholipid molecules affects cofactor recruitment and, therefore, transcription (107).

Perhaps the strongest support for lipid ligands of SF1 stems from the finding that ACTH signaling activates sphingosine kinase, which converts sphingosine to sphingosine

1-phosphate (S1P). S1P is secreted from cells and also activates SF1-dependent transcription of the CYP17 gene (108). Interestingly, it was discovered that sphingosine 1-phosphate can also stimulate the production of cortisol and aldosterone from bovine fasciculata and glomerulosa adrenal cells, respectively (109, 110). These results strongly suggest interplay between ACTH signaling and lipid metabolism that is dependent upon SF1.

DAX1

DAX1 (Dosage-sensitive sex reversal, Adrenal Hypoplasia Congenita critical region on the X chromosome gene 1) was first identified as the gene altered in X-linked Adrenal Hypoplasia Congenita (AHC) (20, 111, 112). The DAX1 gene encodes an atypical nuclear hormone receptor (NR0B1), containing a ligand binding domain in its carboxy-terminal region, and in place of a traditional DNA binding domain, a 70 amino acid glycine-rich repeat motif in its amino-terminal region (112-114). **(Figure 1.3)**

DAX1 Inhibition of Nuclear Receptors

Soon after DAX1 was cloned, it was determined that it acts as a transcriptional repressor and is able to inhibit steroidogenesis, specifically by binding to hairpin loop structures in the StAR promoter (115). DAX1 contains a transcriptional repressor domain within it carboxy-terminus that is often absent in patients with AHC (113, 114). The repeat motif within the amino-terminal region contains an LXXLL-related motif, similar to many coactivators, that enables DAX1 to compete for binding to other nuclear receptors (80). Most notably, this motif directs DAX1 to interact with SF1 or LRH1

(NR5A2), but it is also important for interaction of DAX1 with activated estrogen receptors (ERα and ERβ), androgen receptor (AR), and glucocorticoid receptor (GR) (116-118). Unlike coactivators, DAX1 associates with factors that repress transcription, such NCoR and Alien (81, 82). In vivo evidence for repression ability of DAX1 is provided by the compound $Sf1^{+/-}$, $Dax1^{-Y}$ mouse that rescues the adrenal haploinsufficiency observed in the $Sf1^{+/-}$ mice. Specifically, adrenal steroidogenesis is returned to wild-type levels in the compound knockout (119). The Dax1-deficient mouse will be discussed in greater detail below.

DAX1 in Embryogenesis and Cancer

In light of its role in inhibiting transcription, mainly in steroidogenic tissues, it was surprising to find that Dax1 is highly expressed in mouse embryonic stem (ES) cells (120). While initially it was thought to be an artifact, the importance of Dax1 in mouse ES cells was strengthened by the knock-down experiments demonstrating differentiation induced by loss of Dax1 (121). Additionally, Dax1 has been placed within the protein interaction network of transcription factors required to maintain pluripotency in ES cells (122, 123). Similar to its role in steroidogenic cells, Dax1 acts as a transcriptional repressor in ES cells as myriad differentiation and proliferation genes are expressed early after Dax1 expression is knocked down (124). However, it is not entirely clear if DAX1 plays a role in human embryogenesis given that its expression is very low in human ES cells (125).

There is an inverse correlation between DAX1 expression and steroid production in adrenocortical adenomas, while its expression in adrenocortical carcinomas is highly

variable (126, 127). Given that SF1 expression seems to induce proliferation of adrenocortical cells and is overexpressed in childhood adrenocortical tumors, it is not unexpected that DAX1 would be expressed at low levels in these same tumors (98, 99). Studies have demonstrated that DAX1 expression is correlated with various aspects of tumorigenesis in ovarian and breast cancer, yet DAX1 expression is inversely correlated with endometrial and prostate cancer progression (128-131). However, DAX1 plays a significant role in non-endocrine cancers as well. DAX1 is enriched in the tumorigenic side-population of the A549 lung cancer cell line (132). Interestingly, when DAX1 expression was knocked down in these cells, there was decreased invasion ability in Matrigel, colony formation, and xenograft growth (133).

Dax1-deficient Mouse Model

A mouse model was generated in which exon 2 of the Dax1 gene was floxed (21). This portion of the gene was then deleted by crossing to a line that expressed cre recombinase under the control of a cytolomegalovirus promoter, resulting in germline transmission of the deleted allele. These mice appear to have a mild adrenal phenotype, in which the x-zone persists. However, they display a much more dramatic testicular dysgenesis, in which there is progressive degeneration of the germinal epithelium and failure of the spermatic chords to develop properly (21, 134). They do not show signs of hypogonadotropism, yet are infertile because of Leydig cell hyperplasia and decreased spermatogenesis due, in part, to overexpression of Cyp19 (aromatase), which converts testosterone to estradiol and is normally inhibited by Dax1 and results in elevated levels

of estradiol. Interestingly, treatment with the selective estrogen receptor modulator, tamoxifen, restores partial fertility (135).

The phenotype of Dax1-deficient mice becomes more apparent when viewed in the context of Sf1 haploinsufficiency, which results in a blunted response to different types of stress (136). Consistent with its role as a repressor of Sf1, Dax1-deficiency was able to increase the acute stress response of $Sf1^{+/-}$ mice to levels similar to wild-type mice (119). This seems to indicate that the primary role of Dax1 in the adrenal is repression of Sf1 specifically. Interestingly, this study also demonstrated that $Dax^{-/Y}$ adrenals are hypersteroidogenic and have significantly elevated ACTH sensitivity compared to wild-type mice (119). In testes, however, Dax1 seems to work cooperatively with Sf1 to promote development of a testicular phenotype (137). Indeed, on some genetic backgrounds, $Dax^{-/Y}$ display gonadal sex reversal due to insufficient expression of Sox9 (138, 139).

Adrenal Hypoplasia Congenita

Patients diagnosed with AHC present with adrenocortical failure, secondary to a hypoplastic/aplastic gland manifesting with symptoms of cortisol and aldosterone deficiency, such as hyperpigmentation, salt-wasting crisis with hyponatremia, hyperkalemia and hypoglycemia (140, 141). An appropriate regimen of steroid hormone supplements is prescribed, after the initial adrenal crisis is brought under control, for such patients and their hormone levels are faithfully monitored. There are two forms of AHC: miniature adult and cytomegalic. The miniature adult form of AHC is characterized by adrenals having a small amount of cortical tissue, comprised of normal adult cortex with

normal structure and organization, with little or no fetal cortex. This form of AHC is sporadic or autosomal recessive and most often associated with defects in the central nervous system or pituitary (142). The cytomegalic form of AHC is characterized by adrenals with an absent, or nearly absent, adult cortex. However, the remaining cells are large, cytoplasmic, and eosinophilic with disorganized structure (143-145). **(Figure 1.4)** This X-linked, recessive form of AHC has been linked to mutations or deletions of the DAX1 gene.

X-linked AHC has a bimodal age of onset. In approximately 60% of individuals, X-linked AHC manifests within the first few weeks of life with the remaining 40% of the patients diagnosed in childhood (typically between ages 1 and 10) (140). There is considerable variability in the DAX1 mutations found in AHC patients with no single event being predominant. Nonsense and frameshift mutation occur very evenly along the entire gene, while missense mutations cluster at the carboxy-terminus in the LBD (140, 146, 147). This mutation analysis suggests that the repressor function of DAX1, which is located at the carboxy-terminus, is missing or nonfunctional in individuals with AHC. Functional analysis of DAX1 mutations reveals that the capability to transcriptionally repress SF1 is greatly reduced (140). Interestingly, there does not appear to be a genotype/phenotype correlation, as siblings with the same genetic mutation can manifest at ages different by many years (148, 149). Individuals with X-linked AHC often have hypogonadotropic hypogonadism, which becomes evident upon delayed or abnormal puberty (150).

There are case reports that demonstrate rare instances of variant presentations of X-linked AHC associated with DAX1 changes. Several reports have noted cases of early

puberty in young children, evidenced by penile and/or testicular enlargement, which may be dependent upon excess circulating ACTH (151, 152). There have been several cases where DAX1 mutations manifest in a phenotype in females. In the first case study of a kindred of affected individuals, all of the carrier females had delayed puberty, but had normally functioning adrenals (153). Another case reported AHC in a female infant due to skewed X inactivation (154). Finally, a third case reported a non-symptomatic male having a common DAX1 mutation with his daughter who had mild AHC (155). In this case the mild phenotype was attributed to the mutation causing only a slight defect in the ability of DAX1 to repress SF1 transactivation.

A variable phenotype of X-linked AHC that is somewhat more common, but still quite rare, is late onset AHC. In such cases, adult men are diagnosed with adrenal insufficiency and, subsequently found to have mutations in DAX1 that result in a mild defect (156-158). Often, these patients initially present with hypogonadotropic hypogonadism and suffer an adrenal crisis during times of extreme crisis or physical illness.

Variability of AHC phenotypes provides an interesting avenue of pursuit toward elucidating a possible role of DAX1 in progression to adrenal failure. One interesting observation stems from presymptomatic diagnosis of AHC. In these cases, usually brothers of affected individuals, some adrenal hormones are elevated above normal levels prior to progression to adrenal insufficiency (149, 159). The siblings have the same genetic mutation, yet their age of diagnosis is different by several years. In one particular instance, the sibling of an infant patient had cortisol elevated above the normal levels, then manifested with AHC when he was eight years old, with corresponding cortisol

levels below the normal range (149). Additionally, some children demonstrate early puberty, meaning that they have elevated levels of sex steroids (151, 152). Taken together, these phenotypes suggest that, as would be expected, loss of DAX1 results in a hyperfunctional adrenal cortex, but only for a brief period.

Summary

SF1 and DAX1 are nuclear receptors that play an integral role in development and function of the adrenal cortex. SF1 is a transcription factor that strongly activates expression of the genes encoding several of the cholesterol transporters and P450 enzymes required for the conversion of cholesterol into the steroid hormones of the adrenal cortex. Additionally, SF1 regulates the basal expression of several genes, including genes involved in steroidogenesis, endocrine signaling, and growth and development. The tissue and temporal specificity of several of these genes demonstrates the subtlety with which SF1-dependent transcription is regulated. It is likely that several different signaling pathways regulate SF1-dependent transcription by altering cofactor availability or by modulating signaling molecules that impinge on SF1 transactivation. These studies describe the regulation by SF1 of several factors that are involved in SF1-dependent transcription. In chapter 2, I describe the results of a chromatin immunoprecipitation-chip microarray, in which genomic fragments of SF1 immunoprecipitate were analyzed on a promoter microarray. Promoter regions of a large number of genes were enriched. Of particular interest were those involved in sphingolipid signaling and metabolism, which would constitute a novel genomic target of SF1-dependent transcription. I also describe an association of SF1 on the Adcy4

promoter. While in most cases SF1 acts as an activator, on the Adcy4 promoter, SF1 acts to repress transcription of Adcy4. Finally, I describe an interaction between SF1 and GR to activate expression of the gene encoding Dax1. In all three cases, the genomic target of SF1 encodes a factor that can modulate SF1-dependent transcription. This suggests the existence of several mechanisms by which SF1-dependent transcription is uniquely modulated. In chapter 3, I describe evidence that Dax1-deficient mice develop adrenal failure. While mutations of DAX1 result in Adrenal Hypoplasia Congenita in humans, the Dax1-deficient mouse has a mild adrenal phenotype. These results potentially reconcile the disparity of the phenotypes between the two species. This dissertation contributes to the understanding of both SF1-dependent gene regulation and the significance of DAX1 in homeostasis of the adult adrenal cortex.

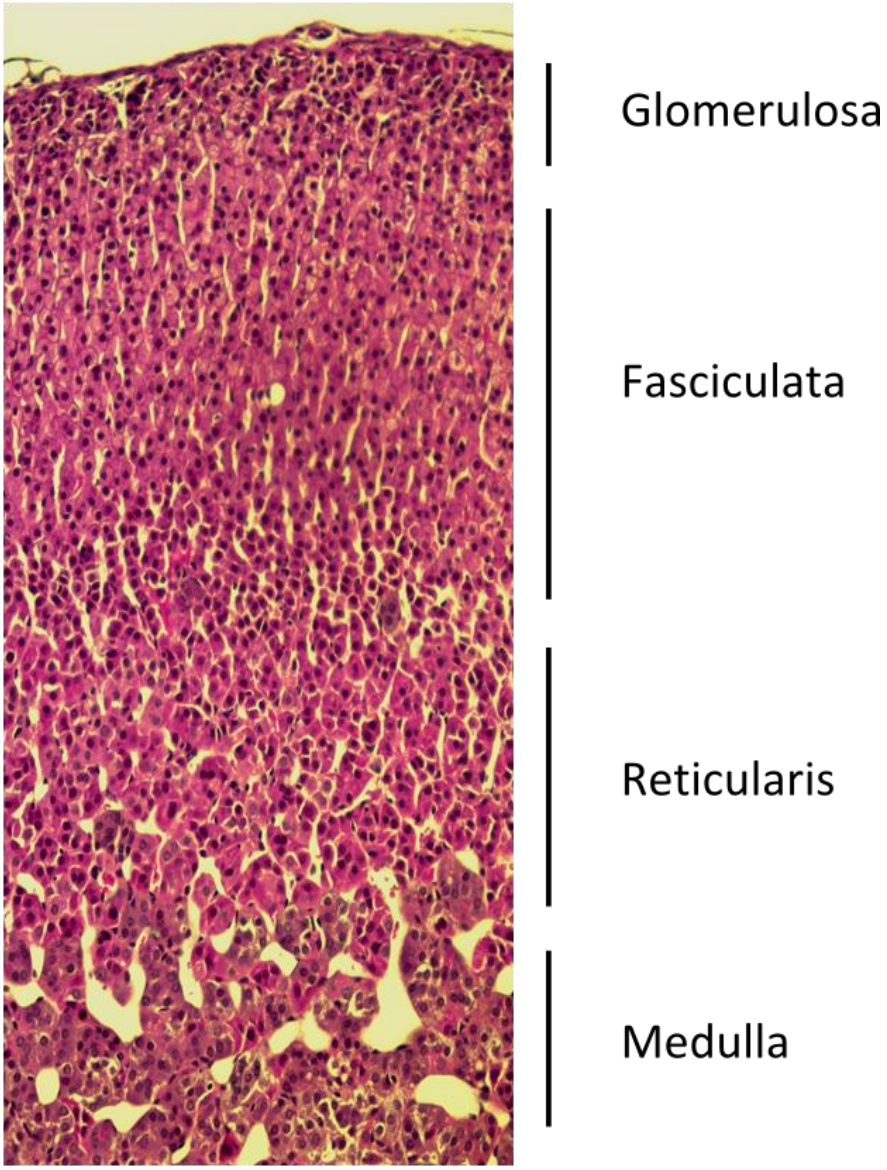

Figure 1.1 Gross histology of the human adrenal.

Section of human adult adrenal tissue stained with hematoxylin and eosin.

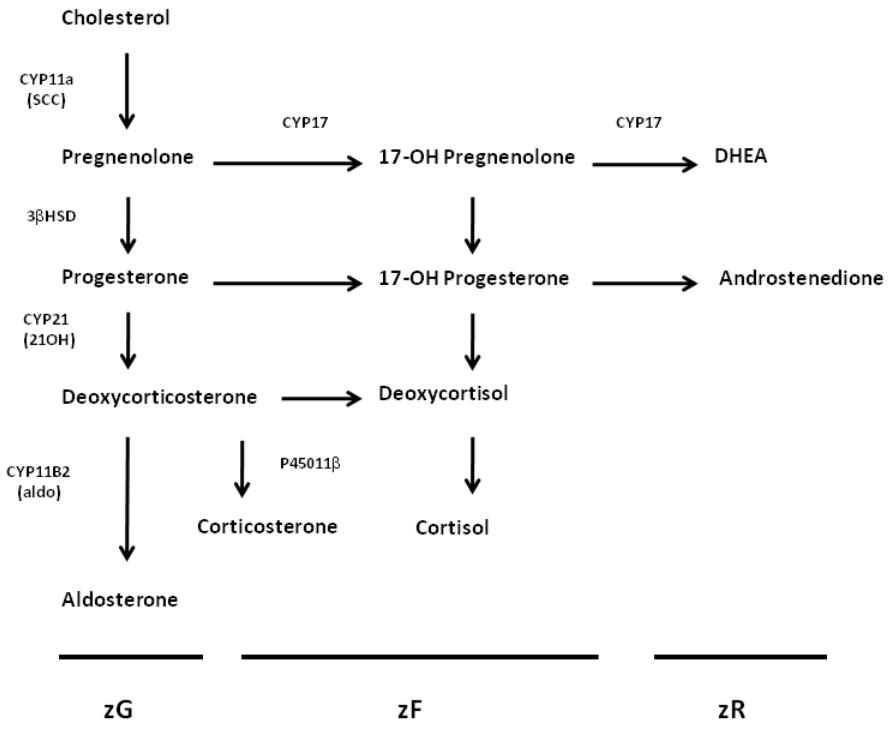

Figure 1.2 Adrenal steroidogenesis pathway.

The process by which cholesterol is converted into adrenal steroid hormones is specific for each zone of the cortex. All of the enzymes represented on this diagram are regulated by SF1. Portions of this pathway are commonly shared between humans and mice. However mice do not express CYP17, which means that only corticosterone is produced in the zF and they do not have a zR. DHEA and Androstenedione are converted to Testosterone and Estradiol in the gonads.

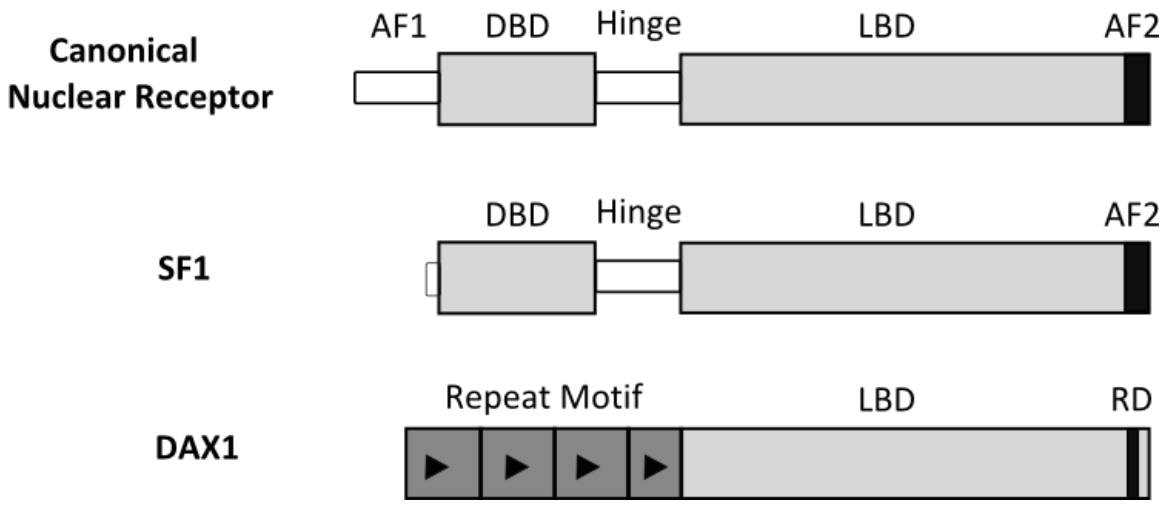

Figure 1.3 Diagram of nuclear receptors SF1 and DAX1.

SF1 shares many of the features of a canonical nuclear receptor, except for an AF1. DAX1 has a repeat motif at the amino-terminus that also contains LXXLL motifs that allow for interaction with other nuclear receptors. DAX1 also has a repression domain (RD) at the carboxy-terminus. The RD is mutated or absent in many AHC patients.

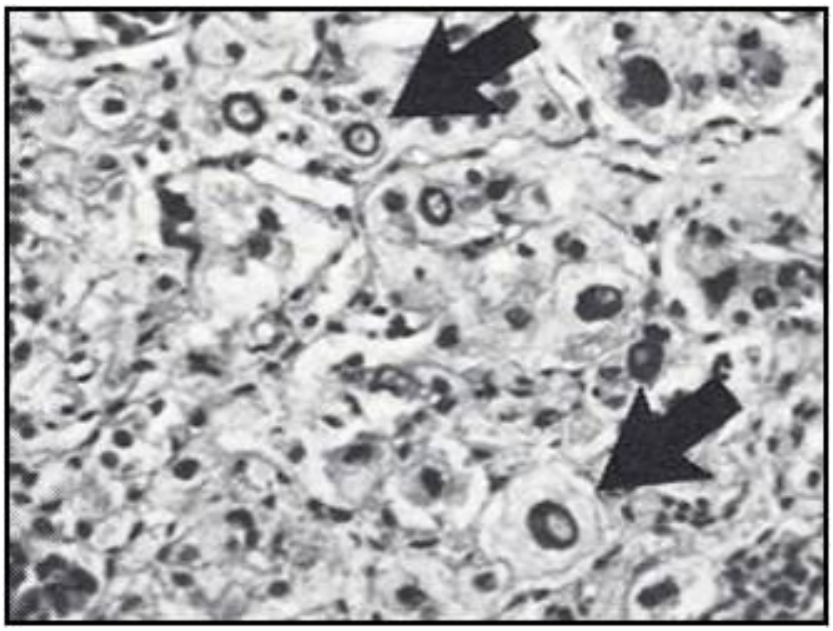

Figure 1.4 Cytomegalic cells in an adrenal from an AHC patient.

Patients with X-linked AHC often have large cytomegalic cells that are eosinophilic. Very few normal cortical cells remain. Image adapted from Lack, EE Pathology of the Adrenal Glands, 1990 (160).

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CHAPTER 2: IDENTIFICATION OF NOVEL SF1 TARGETS

This chapter comprises work on several projects with the common theme of identification or characterization of SF1 target genes. Figure 2.4 comprises a portion of a manuscript that was published in collaboration with Dr. Bernard Schimmer (*Endocrinology* 149(7):3668-78). Figures 2.5 – 2.7 comprise a manuscript published in *Molecular Endocrinology* 20(11):2711-23. Permission was obtained from The Endocrine Society to reproduce copyrighted images.

Introduction

Steroidogenic Factor 1 is a DNA-dependent transcription factor that activates the gene promoters of steroidogenic enzymes and is required for steroidogenesis (1-3). SF1 binds to a DNA response element and recruits a variety of cofactors, which remodel DNA or recruit additional factors, resulting in transcriptional activation of these target genes (4). However, the context by which SF1 activates specific promoters and not others is not completely understood. SF1 regulates the basal expression of several genes, including genes involved in steroidogenesis, endocrine signaling, and growth and development. Additionally, SF1 activates transcription of genes encoding factors involved in steroid hormone synthesis in response to endocrine signals. Specifically, in response to ACTH, SF1 activates MC2R or other zF-specific gene promoters to facilitate

the production of cortisol. However, the fact that steroidogenesis is not required for survival until after birth and SF1 is required for development of the adrenals and gonads, suggests that SF1 has an additional, developmental role in steroidogenic tissues. Therefore, SF1 is likely responsible for activation of a different subset of developmental genes in these tissues (5).

Previous studies have compared gene expression changes in Y1 mouse adrenocortical carcinoma cells when SF1 is stimulated by ACTH or cAMP (6). As expected, many steroid hydroxylases are upregulated in response to these signaling cascades. However, there are large numbers of additional transcripts that are modulated as well. From this study, it appears that ACTH induces transcription of genes essential for differentiation, while repressing genes that are associated with proliferation, such as DNA replication, cell division, and RNA processing (6). Similarly, steroidogenic enzyme transcripts were highly upregulated in both human fetal and adult primary adrenocortical cells after ACTH stimulation (7). However, proliferation markers were not downregulated in these cells. Gene expression analysis on human H295 adrenocortical carcinoma cells, in which SF1 was overexpressed, suggests that SF1 plays a role in proliferation of adrenocortical tumors (8). Additionally, mice overexpressing Sf1 develop hyperplastic lesions and adrenal tumors. While these studies may provide useful information regarding the SF1 transcriptome, they do not necessarily provide evidence of direct activation or repression by SF1, which would require a more direct approach.

Microarray experiments performed using SF1 overexpressing cells or cells in which Sf1 was knocked down, have demonstrated its myriad effects on global gene

expression. Interestingly, all previous studies have shown that SF1 activates transcription of target genes. Yet, gene expression changes in both directions following manipulation of SF1 expression, suggesting that it can also behave as a repressor of gene expression in some contexts (6). SF1 does, in fact, repress CYP11B2, which converts corticosterone into aldosterone, in the zF (9). This restricts CYP11B2 expression to the zG, which ensures that aldosterone is only secreted from this zone. However, SF1 has not been shown to suppress transcription of any additional genomic targets to date. But, in light of the microarray data, it is feasible that SF1 suppresses additional targets as well.

Recent data has shown that SF1interacts with phosphatidic acid (PA) and sphingosine, with PA stimulating and sphingosine inhibiting SF1-dependent transcription of the CYP17 promoter (10). PKA signaling results in increased production of PA. Structural analysis of SF1 bound to different phospholipids revealed conformational changes in the LBD dependent upon the phospholipid species, suggesting that a dynamic exchange between different phospholipid molecules affects cofactor recruitment and, therefore, transcription (11). ACTH signaling activates sphingosine kinase, which converts sphingosine to sphingosine 1-phosphate (S1P). S1P is secreted from cells and also activates SF1-dependent transcription of the CYP17 gene (12). SIP can also stimulate the production of steroid hormones from bovine fasciculata and glomerulosa adrenal cells (13, 14). These results strongly suggest interplay between ACTH signaling and lipid metabolism that is dependent upon SF1. However no specific link has been shown, to date.

Adenylyl cyclase is an enzyme that produces cAMP from ATP in response to various stimuli and is an important effector of ACTH action (15, 16). There are nine

isoforms that vary in their tissue distribution and by the signaling that mediates their action (17). However, the factors that regulate Adcy expression in the adrenal cortex are not well understood. Adcy4, which is activated by β/γ G protein subunits is expressed in the adrenal and is deficient in a mutant clone of the Y1 adrenal cell line (18, 19). These Y1 clones have a defect in SF1 that impairs coactivator association, but maintains DNA binding, and have impaired Adcy4 promoter activity, suggesting a regulatory role of SF1 in Adcy4 expression (20, 21).

Cholesterol is converted into bile acids or steroid hormones. P450 enzymes, specific to the liver or adrenal cortex, metabolize this reaction (22). The nuclear receptor Liver Receptor Homolog-1 (LRH-1, NR5A2), activates transcription of these enzymes as well as the orphan receptor Short Heterodimer Partner (SHP, NR0B2)(23, 24). SHP is synergistically activated by LRH-1 and Farnesoid X Receptor (FXR), the receptor to which bile acids bind, and then inhibits transcription of the enzymes required for bile acid synthesis (25). In addition to the parallels between the two methods by which cholesterol is metabolized, there are also striking parallels between the nuclear receptors that are involved. LRH-1 and SF1, as well as SHP and DAX1, share significant homology and are closely related within the nuclear receptor superfamily (26, 27). These similarities suggest that a similar feedback loop exists for regulation of glucocorticoid synthesis within the adrenal cortex.

In this chapter, unique, non-classical interactions involving SF1 are explored. I describe the results of a chromatin immunoprecipitation-chip microarray (ChIP-chip), in which genomic fragments of SF1 immunoprecipitate were analyzed on a promoter microarray. Promoter regions of a large number of genes were enriched. Of particular

interest were those involved in sphingolipid signaling and metabolism, which constitutes a novel genomic target of SF1-dependent transcription. I further show that Edg5 may be a unique target for SF1 and may interplay with more traditionally known SF1 signaling effectors. I also describe an association of SF1 on the Adcy4 promoter. While in most cases SF1 acts as an activator, on the Adcy4 promoter, SF1 acts to repress transcription of Adcy4. Finally, I describe an interaction between SF1 and GR to activate expression of the gene encoding Dax1. In all three cases, the genomic target of SF1 encodes a factor that can modulate SF1-dependent transcription. This suggests the existence of several mechanisms by which SF1-dependent transcription is uniquely affected.

Materials and Methods

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was performed as described previously (4). Mouse Y1 adrenocortical carcinoma cells were grown to approximately 90% confluence in DMEM supplemented with 7.5% horse serum, 2.5% bovine serum, and antibiotics. Cells were then placed in serum-free DMEM containing 0.05% BSA for 48 h and then treated with 2.5 μM α-Amanatin (Sigma) for 2 h. Cells were washed twice with PBS and then incubated in serum free DMEM containing 0.1 μM ACTH or vehicle control. At various time points following treatment, DNA/protein complexes were cross-linked by incubating the cells in formalin (1% formaldehyde) for 10 min. The cross-linking reaction was terminated by incubating with 0.125 μM glycine for 5 min. Cells were washed twice with ice-cold PBS and then collected by scraping into PBS containing protease inhibitor cocktail (Sigma). Crude nuclei were prepared by hypotonic lysis (28).

Nuclei were then resuspended in 150 µl of lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1x protease inhibitor cocktail] per 2 x 10^6 cells and sonicated four times for 10 sec each at 30% power (Fisher Sonic Dismembrator, model 300; Fisher Scientific, Hampton, NH) followed by centrifugation at 4°C for 15 min. Supernatants were collected and diluted in buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, 1x protease inhibitor cocktail] followed by immunoclearing with 2 µg sheared salmon sperm DNA (Invitrogen), 5 µl preimmune serum, and 50 µl protein A-Agarose [50% slurry in 10 mM Tris-HCl (pH 8.1), 1 mM EDTA, 0.5 mg/ml BSA, 0.05% sodium azide, 200 µg/ml sheared salmon sperm DNA) for 2 h at 4°C. Immunoprecipitation was performed overnight with specific antibodies. Immune complexes were recovered by addition of 50 µl protein A-Agarose and incubation at 4°C for an additional hour. Precipitates were washed sequentially for 5 min each in buffer TSE I [0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], buffer TSE II [0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl], buffer III [0.25 M LiCl,1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], followed by two washes in Tris-EDTA. Immunoprecipitates were extracted two times by agitation in fresh 1% SDS, 0.1 M $NaHCO_3$. Eluates were combined and cross-links were reversed by heating at 65°C for 4h. DNA fragments were isolated using a DNA isolation kit (Qiagen). Isolated DNA was used as template in subsequent PCR or qPCR.

Serial Chromatin Immunoprecipitation

ChIP assays were performed as described above through the Tris EDTA (pH 8.0) washes. Complexes were eluted from primary immunoprecipitates by incubation in 10 mM DTT for 30 min at 37°C. Elutes were diluted 1:50 in re-ChIP buffer [1% Triton X-100; 2 mM EDTA; 150 mM NaCl; and 20 mM Tris-HCl (pH 8.1)] followed by immunoprecipitation with second antibodies (see above) at 4°C overnight. Immunocomplexes were recovered, washed, eluted, and analyzed as described above.

Chromatin Immunoprecipitation on chip microarray (ChIP-chip)

For ChIP-chip, chromatin immunoprecipitation was performed as described above with an antibody directed against Sf1 using sheared chromatin from Y1 adrenocortical cells with and without ACTH treatment. After final elution, bound DNA fragments were amplified using a Whole Genome Amplification Kit (Sigma) (29). Amplified DNA fragments were then labeled, hybridized to a NimbleGen MM8 RefSeq Promoter ChIP-chip Microarray, which contains over 17,000 2 Kb promoter regions of well-characterized genes. This process was performed by NimbleGen. Signal intensity data were obtained from the scanned files using NimbleScan software to generate log2-ratio files. Ratio and peak data were visualized using NibleGen SignalMap software using $p \leq 0.05$ for the confidence level cutoff. Enriched promoter regions were analyzed using the online Transcription Element Search System (TESS, www.cbil.upenn.edu/tess) to identify relevant transcription factor binding sites (30). Functional annotation of genes associated with enriched promoter regions was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (31).

Quantitative PCR

Isolated ChIP DNA was amplified with the appropriate primers spanning response elements within the promoter region of target genes using SYBR Green PCR Master Mix (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta C(T)}$ method (32).

Quantitative RT-PCR

Total RNA was isolated from tissues using Trizol Reagent (Invitrogen) and 1 µg was reverse transcribed using iScript system (BioRad). The resulting cDNA was diluted 1:5 and 2 µl was amplified with the appropriate primers using SYBR Green PCR Master Mix (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta C(T)}$ method (32).

Cell Culture and Primary Adrenal Cell Culture

Y1 adrenocortical cells were maintained in DMEM supplemented with 7.5% horse serum, 2.5% fetal bovine serum, and antibiotics at 37°C under humidified atmosphere of 5% CO_2. Cells were grown on Primaria-coated tissue culture plates. Primary cells were derived from B6/SJL F2 mice. Adrenals were harvested, cleaned, and minced by hand. Single cell suspensions were generated by incubating minced adrenal glands in serum-free medium containing 2 mg/ml collagenase, 0.05 mg/ml deoxyribonuclease I, and 5 mg/ml BSA at 37°C for 45 min with mechanical disruption (pipetting up and down) every 15 min. Primary adrenal cells were plated on to fibronectin-coated plates in Knockout DMEM containing 15% Knockout serum replacement (Invitrogen) at a density of 16 adrenals per 6-cm culture plate. Primary cells were allowed to adhere to plates 48 h before treatment or use.

Statistics

Statistical significance was determined by using an unpaired student's t-test to compare data from $Dax1^{-/Y}$ mice and wild type mice at each age. Results were considered significant if $p < 0.05$ and are denoted with an asterisk.

Results

Sf1 immunoprecipitation enriches a variety of gene promoters

Previous studies have shown wide-ranging effects of ACTH signaling in Y1 cells (6). The results of these studies suggest that in addition to activating expression of steroidogenic enzymes, ACTH may also induce differentiation of adrenal cells. In order to discover possibly novel Sf1 target genes, ChIP-chip was performed. Y1 cells were treated with 100 nM ACTH or vehicle control and then harvested after 90 minutes as described in the methods section. Nuclear extracts were then immunoprecipitated using an antibody directed against Sf1. Immunoprecipitated DNA was then labeled and hybridized to a ChIP-chip promoter array. Interestingly, there were a variety of gene targets enriched, both in the vehicle-treated group and the ACTH-treated group. Analysis of peak data revealed that under basal condition, Sf1 is enriched, or putatively bound, at 95 different gene promoters. However, after ACTH stimulation, Sf1 is associated wih 189 different gene promoters. **(Figure 2.1, A)** Interestingly, only 10 gene promoters were common to both lists. Analysis of the promoter regions using TESS, revealed that many of the enriched promoter regions contain canonical SF1 response elements.

Functional annotation of the enriched gene lists using Database for Annotation, Visualization, and Integrated Discovery (DAVID) demonstrated that there is a dramatic

difference in the functional categories for the gene promoters to which Sf1 is bound under the two conditions. Under basal conditions, Sf1 is bound to promoters of genes encoding cytoskeleton, proliferation, and signaling proteins. After treatment with ACTH, Sf1 is bound to promoters of genes encoding transmembrane, DNA binding and differentiation proteins. Multiple transcriptional regulators are represented on both lists. However, because DNA binding in this assay does not infer activation or repression, this result is not unexpected. **(Figure 2.1, B)**

Known ACTH-responsive Sf1 target genes encoding steroidogenic enzymes (such as CyB5r4 (33)), were enriched in the ACTH-treated group, albeit not representing the most highly enriched sequences. This may indicate that in the moderately steroidogenic Y1 adrenocortical carcinoma cell line, Sf1 participates in functions beyond transcriptional regulation of steroidogenic enzymes. However, promoters of steroidogenic enzymes tend to be underrepresented on this particular array. Consistent with recent reports of sphingolipids and phospholipids binding to SF1, there was enrichment of genes involved in sphingolipid biology in both the basal and ACTH-treated group. Interestingly, there seemed to be a shift following ACTH treatment, as the genes in this category were unique to each group. While the phosphodiesterase Smpd2 is enriched under basal conditions, both the sphingosine receptor Edg5 and the lyase Sgpl1 are enriched following ACTH treatment. Notably, two genes specifically enriched in the absence of ACTH are Notch2 and Tcf23, which both play major roles in the maintenance of progenitor multipotency and inhibition of differentiation. Interestingly, a number of the highly enriched genes in the ACTH-treated group, including Edg5, retinoic acid binding protein Crabp1, and the entire reproductive homeobox 4 gene cluster Rhox4 are

well-characterized regulators of differentiation of multiple cell lineages (34-36). **(Figure 2.2)**

Edg5 is a target of Sf1

Using the TESS database, it was confirmed that Edg5 does, indeed, have an SF1 response element. Additionally it corresponded to the area of the promoter that had probe enrichment. To verify that Edg5 is indeed a SF1 target gene, a traditional ChIP assay was performed using the same anti-SF1 antibody that was used for the ChIP-chip. Immunoprecipitated DNA was amplified using primer sequences designed to flank the response element within the promoter region of Edg5. PCR was also performed using primers that correspond to well-know SF1 targets, StAR and Mc2r. Amplification using the proximal Edg5 showed a 4-fold increase in SF1 promoter occupancy compared to IgG control. **(Figure 2.3, A)** StAR and Mc2r both confirm adequate immunoprecipitation by SF1 and they both have between four and 14 fold higher SF1 occupancy. Interestingly SF1binding at the Edg5 promoter did not change significantly in response to ACTH. This is at odds with the ChIP-chip data. The argument that the ACTH has lost its potency is dismissed by the fact that occupancy was induced at the StAR and Mc2r promoters in response to ACTH stimulation.

Because Edg5 was enriched in the ACTH-treated and not the basal ChIP-chip array, it would be expected that ACTH stimulation would be accompanied by changes in gene expression. To determine if ACTH can effect change in Edg5 expression, qRT-PCR was performed. ACTH induces a modest, but significant, 1.5 fold increase in Edg5 mRNA expression. **(Figure 2.3, B)** StAR expression was used as a positive control and

its expression was also increased, but only by two-fold, which is somewhat less than what was expected.

Sf1 represses activity of the Adenylyl cyclase 4 promoter

Recently, it was discovered that Adcy4, which is activated by β/γ G protein subunits, is expressed in the adrenal and is deficient in a mutant clone of the Y1 adrenal cell line (18, 19). These Y1 clones have a defect in SF1 that impairs coactivator association, but maintains DNA binding, and have impaired Adcy4 promoter activity, suggesting a regulatory role of SF1 in Adcy4 expression (20, 21). In collaboration with Dr. Bernard Schimmer at the University of Toronto, the observation was made that both SF1 and Sp1 regulatory sequences are present in the 5′ flanking region of the Adcy4 gene. **(Figure 2.4, A)** After electromobility shift and deletion analysis indicated that SF1 and Sp1 are able to bind the Adcy4 promoter region and SF1 along with Sp1B represses its activity, a ChIP assay was performed to determine if SF1 interacts with the Adcy4 promoter in vivo. The proximal Adcy4 promoter was amplified from immunoprecipitates of anti-SF1 ChIP, but a distal region was not. **(Figure 2.4, B)**

To determine the effect of modulating SF1 expression on the activity of Adcy4, a luciferase assay was performed in Y1 cells transfected with an Adcy4 luciferase reporter plasmid and a SF1 expression vector. Overexpression of SF1 resulted in 35% decrease in Adcy4 promoter activity, whereas SF1 antisense had no effect. **(Figure 2.4, C)** Finally SF1 expression was knocked down in Y1 cells, which resulted in a nearly 3-fold increase in Adcy4 mRNA expression, while SF1 and Mc2r expression were both reduced by over

90%. **(Figure 2.4, D)** Together, these data indicate that SF1 acts to repress activity of Adcy4 expression in adrenocortical cells.

SF1 and GR form a complex on the murine Dax1 promoter

In the liver, SHP is synergistically activated by LRH-1 and Farnesoid X Receptor (FXR), the receptor to which bile acids bind, and then inhibits transcription of the enzymes required for bile acid synthesis (25). Because of the parallels between these nuclear receptors and the adrenal nuclear receptor that mediate cholesterol methabolism into steroid hormones, the promoter of Dax1 was explored. It was determined that Dax1 is synergistically activated by GR and SF1 in response to the glucocorticoid, dexamethasone. **(Figure 2.5, A)** Additionally, GR and SF1 interact with each other and form a protein complex on the Dax1 promoter. This complex is formed in the presence of dexamethasone and specifically disrupted by ACTH **(Figure 2.5, B)**

ACTH treatment results in SF1 clearance from the Dax1 promoter and recruitment to the StAR and Mc2r promoters

Previous studies have shown that ACTH induces cyclical Sf1 recruitment to promoters of steroidogenic genes (4). Therefore, it was somewhat interesting to find that ACTH resulted in clearance of SF1 from the Dax1 promoter. To confirm that this was a specific effect of ACTH, parallel ChIP assays were performed analyzing SF1 immunoprecipitates on the Dax1, StAR , and Mc2r promoters. While ACTH treatment resulted in clearance from the Dax1promoter, there was 5.5-fold and 8.9-fold increase of SF1 occupancy on the Mc2r and StAR promoters, respectively. **(Figure 2.6)**

Dax1 and StAR are conversely regulated by ACTH and dexamethasone

To determine if binding of SF1 and GR to the Dax1 promoter had functional consequences, gene expression was measured following dexamethasone and ACTH treatment in primary adrenal cells. Primary adrenocortical cell were treated with vehicle, dexamethasone, or dexamethasone plus ACTH for 18 hr. Quantitative RT-PCR was performed on RNA using primers specific to Dax1 and StAR. Dexamethasone treatment alone resulted in 4.5-fold increase in Dax1 expression and a 4.7-fold decrease in StAR expression. Concomitant treatment with dexamethasone and ACTH resulted in attenuated Dax1 expression (2.8-fold increase) and a dramatic 10.9-fold increase in StAR expression. **(Figure 2.7)**

Discussion

In this chapter, unique, non-classical interactions involving SF1 were explored. I described the results of a chromatin immunoprecipitation-chip microarray (ChIP-chip), in which genomic fragments of SF1 immunoprecipitate were analyzed on a promoter microarray. Promoter regions of a large number of genes were enriched. Of particular interest were those involved in sphingolipid signaling and metabolism, which constitutes a novel genomic target of SF1-dependent transcription. I further showed that Edg5 may be a unique target for SF1 and may interplay with more traditionally-known SF1 signaling effectors. I also described an association of SF1 on the Adcy4 promoter. While in most cases SF1 acts as an activator, on the Adcy4 promoter, SF1 acts to repress transcription of Adcy4. Finally, I described an interaction between SF1 and GR to activate expression of the gene encoding Dax1. In all three cases, the genomic target of

SF1 encodes a factor that can modulate SF1-dependent transcription. This suggests the existence of several mechanisms by which SF1-dependent transcription is uniquely affected by short intracellular feedback loops.

Using an unbiased approach for discovery of novel target genes is very useful. Previous studies that determined SF1 target sites mainly consisted of analysis of known members of the steroidogenesis pathway or factors known to play a role in the HPA or HPG axes. Approaches such as ChIP-chip or the newer technique, ChIPseq (37), offer an unbiased method for discovering novel targets of SF1. Previous gene expression analyses suggest that SF1 plays a role in ACTH-mediated differentiation, and that SF1 can contribute to tumorigenesis in some contexts (6, 8). The scope would need to be expanded outside of the limited cell types in which previous studies were performed. There are a limited number of adrenocortical cell lines and it cannot be assured that they behave as normal adrenocortical cells would. Ideally, future ChIP-chip experiments would be done on primary adrenocortical cells, similar to the approach used by Xing et al (7).

Sphingosine signaling interacting with SF1 has become an emerging area of research. Most often, however, sphingosine is upstream of SF1 transactivation, yet I have shown that it sphingosine signaling may also be downstream. Further work must be done to fully characterize sphingosine metabolism as a bona fide target of SF1. It is interesting to speculate that there may be a feedback mechanism by which SF1 can affect intracellular concentrations of sphingosine and sphingosine 1-phosphate to regulate cell- and promoter-specific activation or repression. Future studies would require the use of SF1 mutants deficient in their ability to bind different lipid moieties. Would these

mutants transactivate traditional steroidogenic targets? It is entirely possible that interaction with sphingolipids/phospholipids is required for steroidogenesis. However, it is also possible that interaction with sphingolipids may direct SF1 toward a distinct subset of genomic targets. Future studies will focus on revealing this distinction.

In most cases, SF1 has been shown to activate transcription. However, there is one exception: the repression of CYP11B2 in the zona fasciculata (9). The identification of adenylyl cyclase 4 as a target of SF1 is just the second target that SF1 actively represses. Interestingly, the response element to which SF1 binds to repress Adcy4 is nearly identical to the response element it binds to activate traditional steroidogenic target genes. Therefore, the information to activate or repress transcription is not delivered by the response element, but must come from surrounding transcription factors acting on the same promoter region. Future studies will focus on identifying genomic sequences surrounding the SF1 response element that result in either activation or repression of the genomic target.

Repression of Adcy4 by SF1 might also have an effect on SF1-depenent transcriptional activation. Adcy4 is regulated by β/γ G-protein subunits, so repression of this factor would drive cAMP signaling to be influenced more predominantly by other signaling pathways.

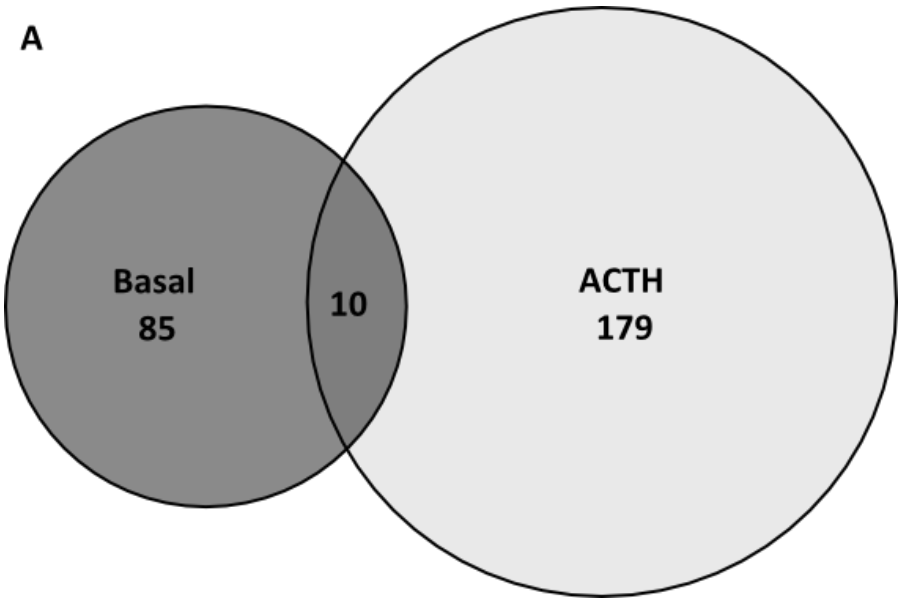
DAX1 is yet another SF1 target that feeds back on SF1-dependent transcription. In fact this feedback mechanism and the consequences of such appear fairly well defined compared to the other two targets reported here. DAX1 and SF1 share quite an unusual relationship. While they are coincidently expressed in many tissues, they seem to play

quite unique roles in adrenal physiology. The mechanism provided here is sufficient for explaining the role of DAX1 in mediating glucocorticoid production and suggests an intra-adrenal feedback loop that maintains a balance between d differentiated steroidogenically competent cells and undifferentiated progenitor cells. However, this mechanism does little to account for the developmental role that DAX1 has in the adrenal cortex. In fact, the fact that mutations in both SF1 and DAX1 result in adrenal hypofunction cannot be explained by a simple repression of SF1-dependent steroidogenesis by DAX1. As DAX1 is a repressor of transcription, there must be other critical factors in the developing adrenal that DAX1 represses. In chapter three, I will go into more detail concerning DAX1 in the adrenal cortex.

Acknowledgements

Mapping of the Adcy4 promoter and the experiments in Figures 2.4 C and 2.4 D were performed by Dr. Bernard Schimmer. Mapping of the Dax1 promoter and experiments in figure 2.5 were performed by Brian Gummow. All other experiments were performed by Joshua Scheys under the mentorship of Gary Hammer.

A



B

Basal		ACTH	
Functional Category	**# of Genes**	**Functional Category**	**# of Genes**
Cytoskeleton	8	Transmemebrane	25
Transcription	11	Transcription	21
Proliferation	12	Differentiation	9
Sphingolipid Metabolism	1	Sphingolipid Signaling	2

Figure 2.1 Analysis of DNA enrichment following αSF1 immunoprecipitation.

αSF1-immunoprecipitated DNA was hybridized to a NimbleGen MM8 RefSeq promoter and results were analyzed using NimbleScan software. A) Venn diagram showing total number of enriched DNA regions in presence or absence of ACTH. B) Table listing top functional categories of genes near enriched DNA regions. Functional categories were determined using DAVID (31).

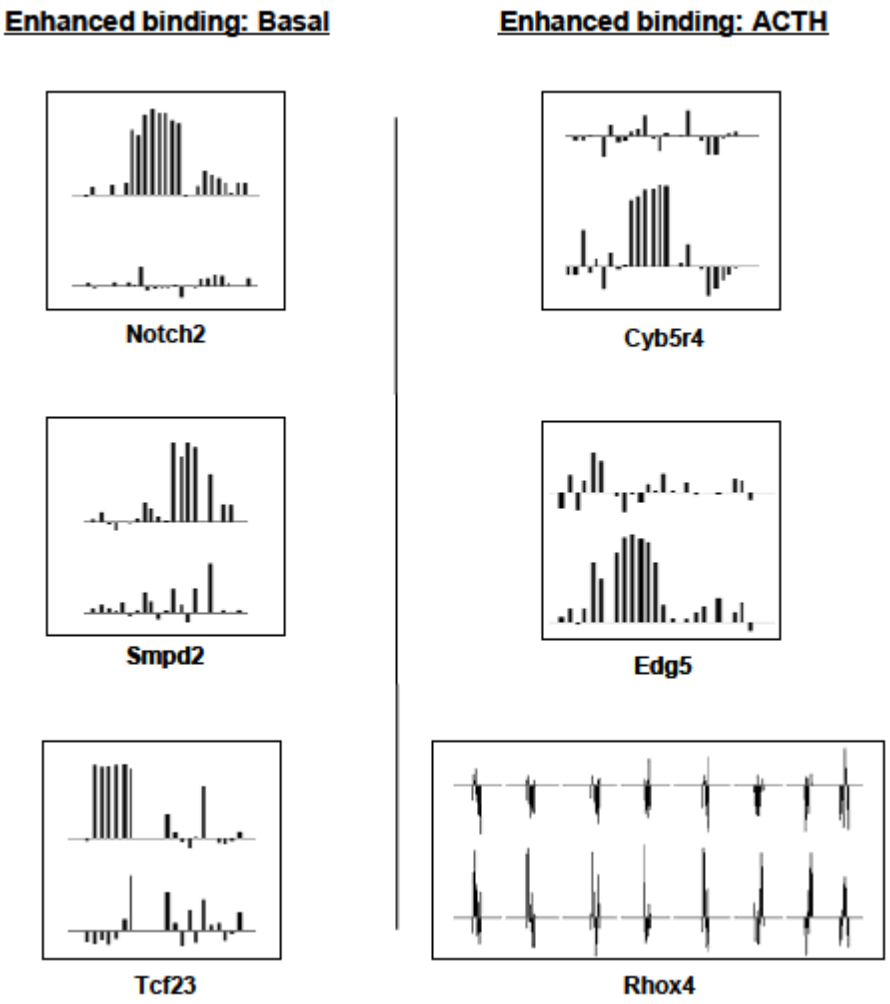


Figure 2.2 Visualization of peak data for select enriched DNA regions.

αSF1-immunoprecipitated DNA was hybridized to a NimbleGen MM8 RefSeq promoter and peak data was visualized using NimbleScan software. Each boxed area represents the tiled promoter region for a given gene: upper histogram – SF1 binding under basal conditions; lower histogram – SF1 binding following ACTH stimulation. All histograms are represented on the same scale.

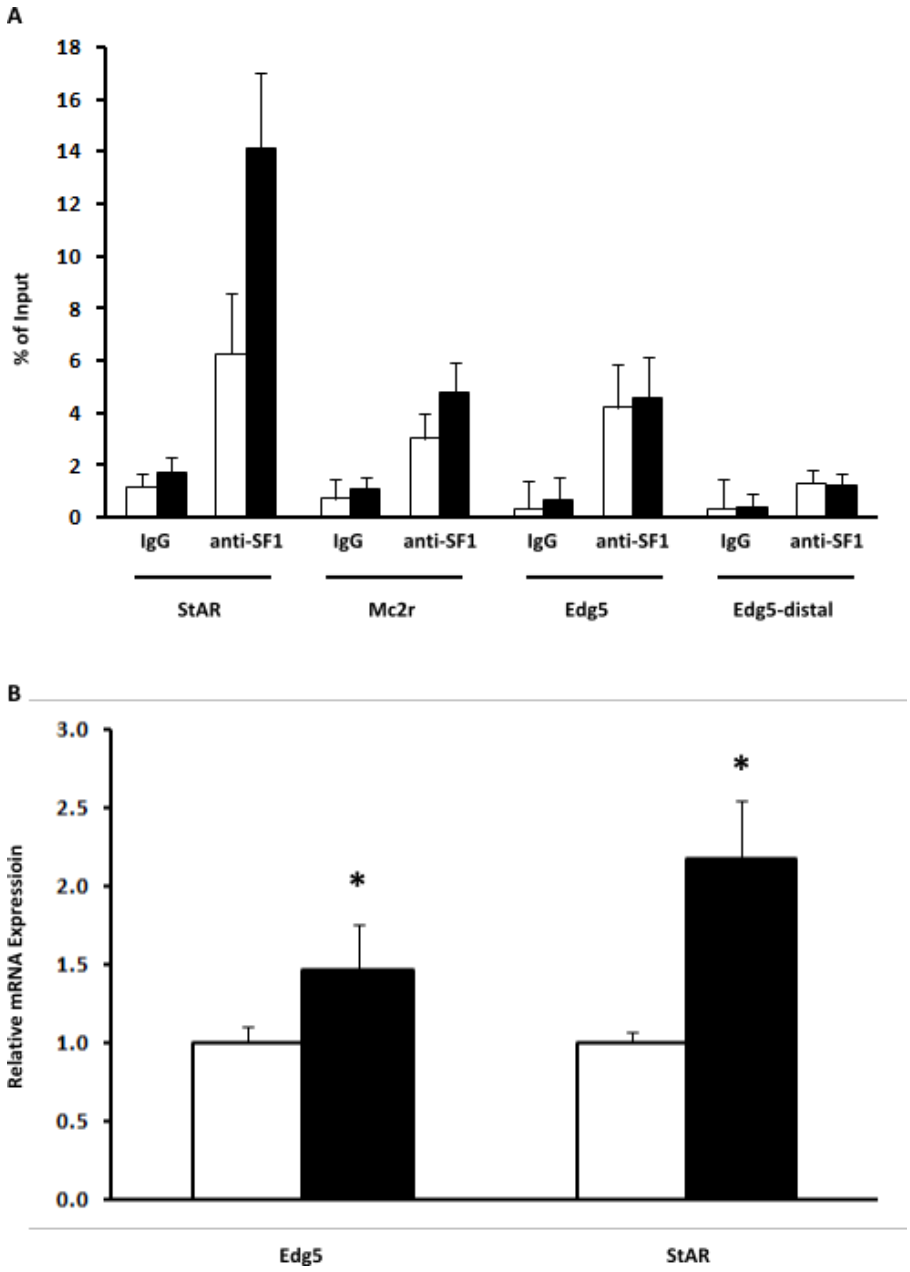


Figure 2.3 Confirmation of Edg5 as SF1 target by ChIP and qRT-PCR.

A) ChIP assays were performed on Y1 cells using an anti-SF1 antibody after 80 min 10 nM ACTH stimulation. Immunoprecipitates were analyzed by qPCR using primers spanning SF1 RE in the proximal promoters of StAR, Mc2r, and Edg5 genes, as well as the distal promoter of Edg5. Results are plotted as a percentage of the input DNA. B) Quantitative RT-PCR of Edg5 and StAR transcripts following 24 hr 10 nM ACTH stimulation. Results are plotted relative to vehicle-treated, which was set to one. For both figures, white bars are vehicle, and black bars are ACTH-treated. (*, $P \leq 0.05$)

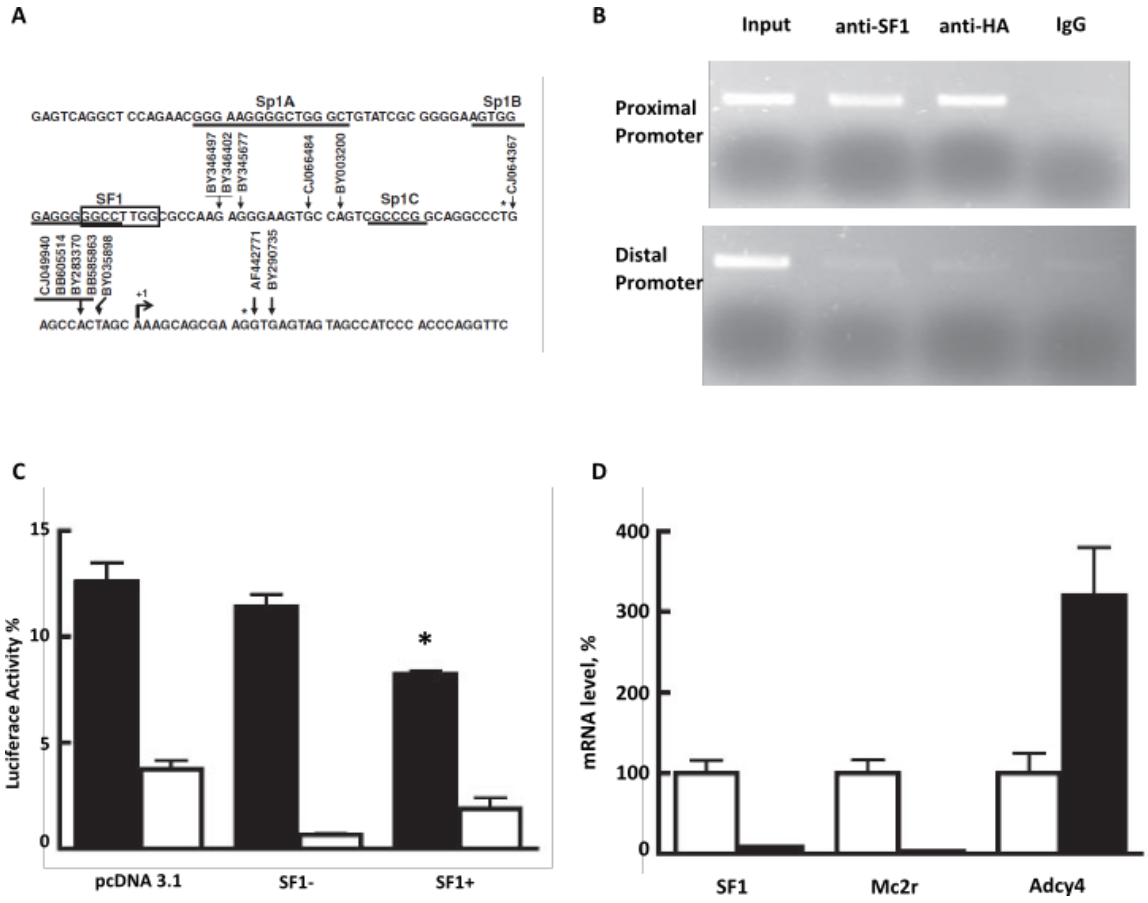


Figure 2.4 SF1 interacts with Adcy4 promoter and affects its transcription.

A) Promoter region of Adcy4 contains Sp1 binding sites and SF1 response element. B) ChIP assays were performed on Y1 cells stably expressing myc-HA-SF1 using anti-SF1 and anti HA antibodies or control IgG. Immunoprecipitated DNA was amplified using primers spanning the SF1 RE (upper gel), or a distal region (lower gel), +2744 downstream of the transcription start site. C) Luciferase assays were performed on Y1 cells transfected with reporter plasmid containing 970 bp (black bars) or 18 bp (white bars) of the Adcy4 promoter along with a HIS-tagged SF1 expression vector (SF1+), an antisense control (SF1-), or empty vector, pcDNA 3.1. D) Quantitative RT-PCR of SF1, Mc2r, and Adcy4 in Y1 cells following transfection with siRNA targeting SF1. Results are expressed as a percentage of SF1 siRNA (black bars) to control siRNA (white bars). (*, $P \leq 0.05$) *Copyright 2008, The Endocrine Society*

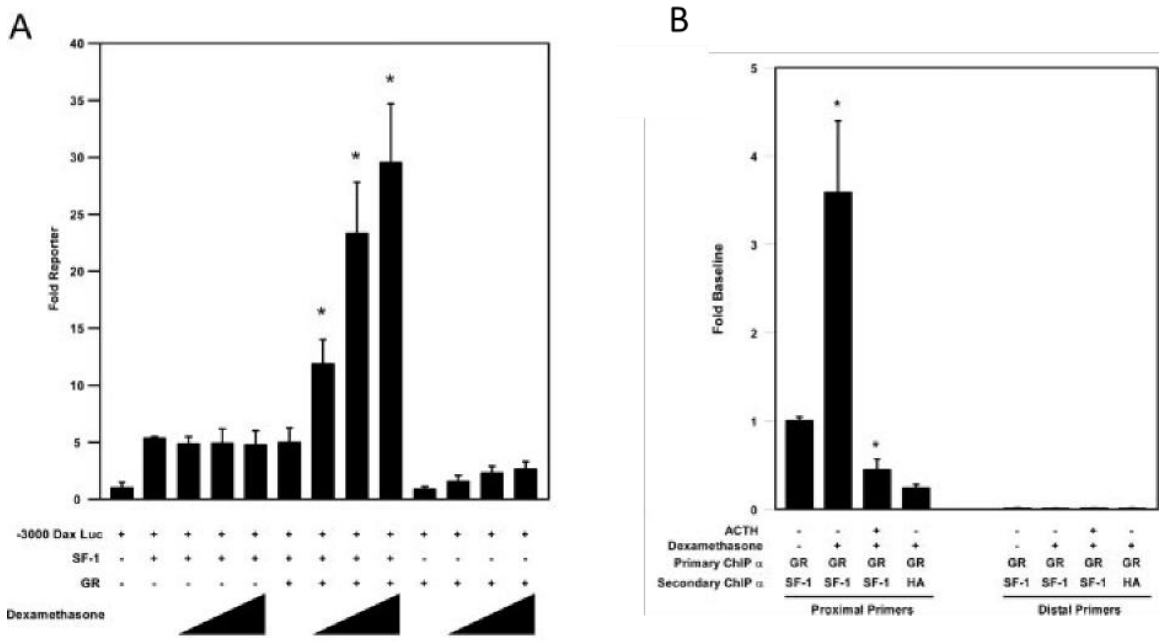


Figure 2.5 SF1 and GR synergistically activate Dax1 and form a complex on the Dax1 promoter.

A) Luciferase assays were performed after transfecting JEG3 cells with a Dax1 luciferase construct, along with SF1 expression vector, GR expression vector, or both. Cells were treated with increasing amounts of dexamethasone for 12 h. Results were plotted as fold over baseline reporter activity. B) Serial ChIP assays were performed on primary adrenal cells using anti-GR antibodies followed by anti-SF1 antibodies following 40 min treatment with 10 μM dexamethasone or 100 nM ACTH, or both. Immunoprecipitates were analyzed by qPCR using primers designed against the proximal and distal Dax1 promoter. Results were plotted as fold over baseline. (*, $P \leq 0.05$) *Copyright 2006, The Endocrine Society*

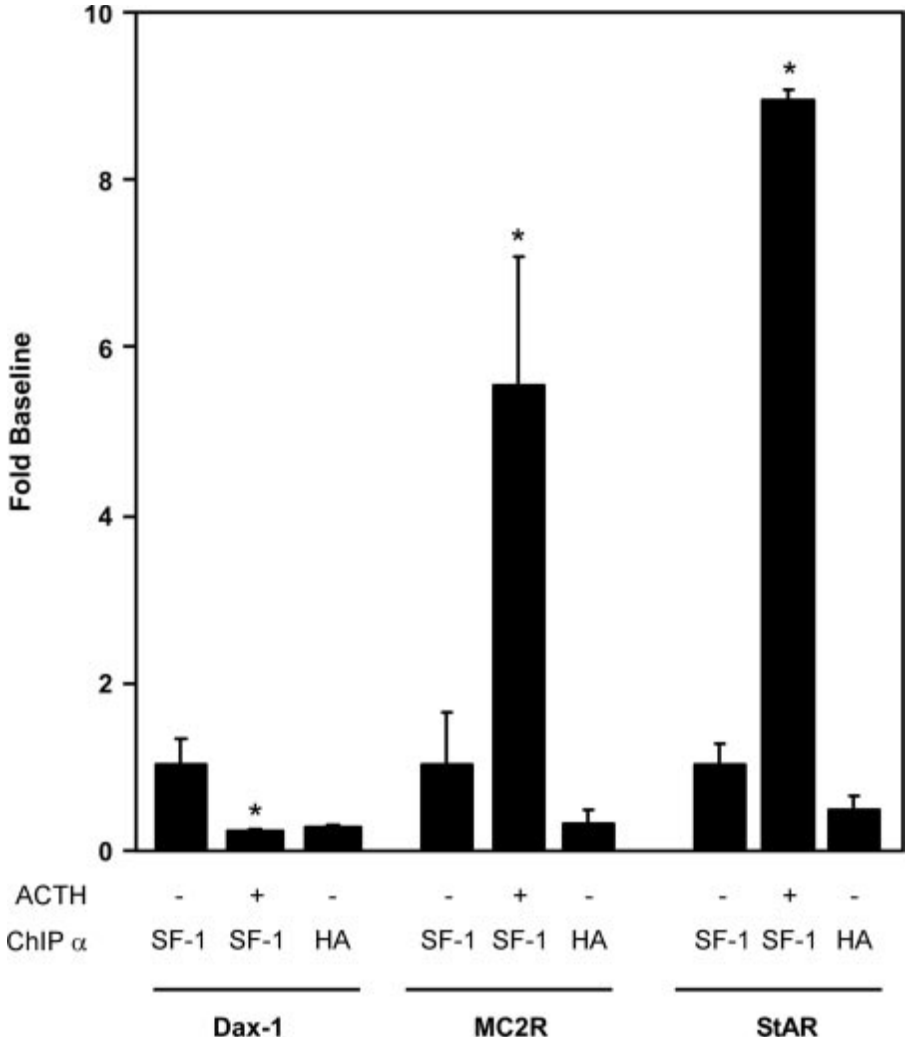


Figure 2.6 ACTH shifts SF1 from Dax1 promoter to MC2R and StAR promoters.

ChIP assays were performed in primary adrenal cells using anti-SF1 and anti-HA antibodies after stimulation with 10 nM ACTH for 40 min. Immunoprecipitates were analyzed by qPCR using primers that spanned the SF1 response elements within Dax1, Mc2r, and Star proximal promoters. Results were plotted as fold change over baseline (SF1 IP with no ACTH). (*, $P \leq 0.05$) *Copyright 2006, The Endocrine Society*

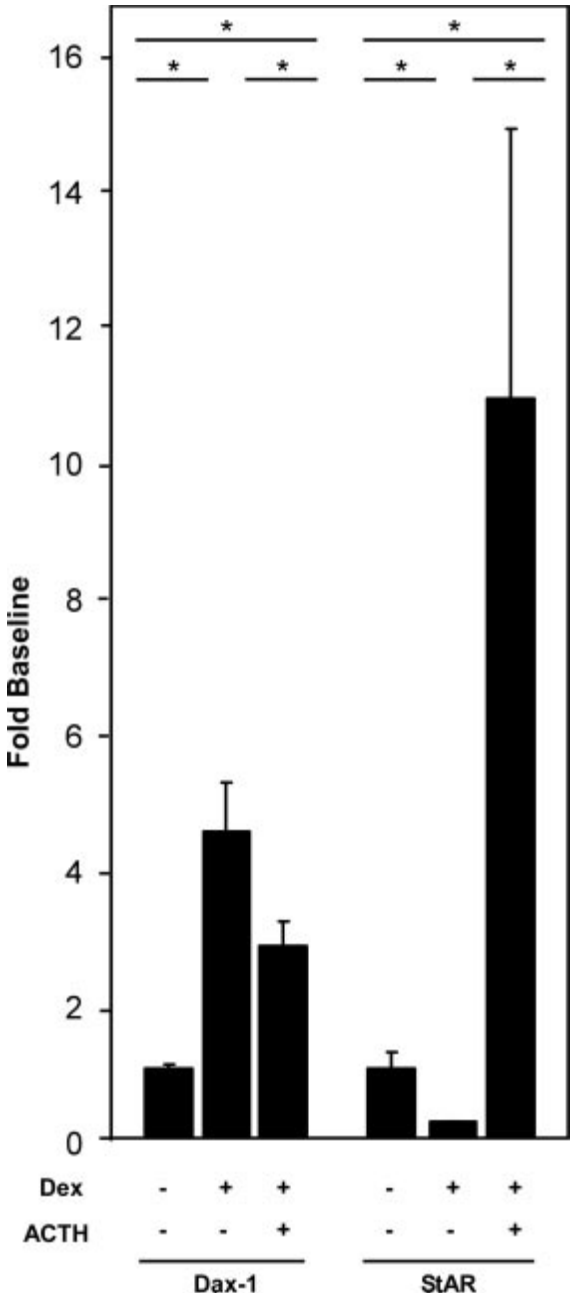


Figure 2.7 Dexamethasone and ACTH have reciprocal effects on expression of Dax1 and StAR.

Primary mouse adrenocortical cells were treated with 10 uM dexamethasone alone or in combination with 100 nM ACTH for 18 hr. qRT-PCR was performed on isolated mRNA using primers specific to Dax1 and StAR. Results were plotted as fold change over basal expression. (*, $P \leq 0.05$) *Copyright 2006, The Endocrine Society*

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CHAPTER 3: DAX1-DEFICIENT MICE DEVELOP ADRENAL FAILURE

All work presented in this chapter was performed independently under the mentorship of Dr. Gary Hammer. The data obtained from this work resulted in a first-author manuscript submitted to *Endocrinology*.

Introduction

DAX1 (Dosage-sensitive sex reversal, Adrenal Hypoplasia Congenita critical region on the X chromosome gene 1) was first identified as the gene altered in X-linked Adrenal Hypoplasia Congenita (AHC) (1-3). Patients diagnosed with AHC present with adrenocortical failure, secondary to a hypoplastic/aplastic gland manifesting with symptoms of cortisol and aldosterone deficiency, such as hyperpigmentation, salt-wasting crisis with hyponatremia, hyperkalemia and hypoglycemia (4, 5). An appropriate regimen of steroid hormone supplements is prescribed for such patients. While the disease typically manifests within the first few weeks of life, there is wide variation in age of onset, even when the underlying DAX1 mutation is identical; siblings with the same genetic mutation can manifest at ages different by many years (6). Though X-linked AHC has been definitively linked to alterations in DAX1, the mechanism by which loss of functional DAX1 results in adrenal failure has yet to be delineated.

X-linked AHC has a bimodal age of onset. In approximately 60% of individuals, X-linked AHC manifests within the first few weeks of life with the remaining 40% of the patients diagnosed in childhood (typically between ages 1 and 10) (4). There is considerable variability in the DAX1 mutations found in AHC patients with no single event being predominant. Nonsense and frameshift mutation occur very evenly along the entire gene, while missense mutations cluster at the carboxy-terminus in the LBD (4, 7, 8). This mutation analysis suggests that the repressor function of DAX1, which is located at the carboxy-terminus, is missing or nonfunctional in individuals with AHC. Functional analysis of DAX1 mutations reveals that the capability to transcriptionally repress SF1 is greatly reduced (4). Interestingly, there does not appear to be a genotype/phenotype correlation, as siblings with the same genetic mutation can manifest at ages different by many years (6, 9). Individuals with X-linked AHC often have hypogonadotropic hypogonadism, which becomes evident upon delayed or abnormal puberty (10).

There are case reports that demonstrate rare instances of variant presentations of X-linked AHC associated with DAX1 changes. Several reports have noted cases of early puberty in young children, evidenced by penile and/or testicular enlargement, which may be dependent upon excess circulating ACTH (11, 12). There have been several cases where DAX1mutations manifest in a phenotype in females. In the first case study of a kindred of affected individuals, all of the carrier females had delayed puberty, but had normally functioning adrenals (13). Another case reported AHC in a female infant due to skewed X inactivation (14). Finally, a third case reported a non-symptomatic male having a common DAX1 mutation with his daughter who had mild AHC (15). In this

case the mild phenotype was attributed to the mutation causing only a slight defect in the ability of DAX1 to repress SF1 transactivation. A phenotype of X-linked AHC that is somewhat more common, but still quite rare, is late onset AHC. In such cases, adult men are diagnosed with adrenal insufficiency and, subsequently found to have mutations in DAX1 that result in a mild defect (16-18). Often, these patients initially present with hypogonadotropic hypogonadism and suffer an adrenal crisis during times of extreme crisis or physical illness.

Variability of AHC phenotypes provides an interesting avenue of pursuit toward elucidating a possible role of DAX1 in progression to adrenal failure. One interesting observation stems from presymptomatic diagnosis of AHC. In these cases, usually brothers of affected individuals, some adrenal hormones are elevated above normal levels prior to progression to adrenal insufficiency (9, 19). The siblings have the same genetic mutation, yet their age of diagnosis, is different by several years. In one particular instance, the sibling of an infant patient had cortisol elevated above the normal levels, then manifested with AHC when he was three years old, with corresponding cortisol levels below the normal range (9). Additionally, some children demonstrate early puberty, meaning that they have elevated levels of sex steroids (11, 12). Taken together, these phenotypes suggest that, as would be expected, loss of DAX1 results in a hyperfunctional adrenal cortex, but only for a brief period.

The DAX1 gene encodes an atypical nuclear hormone receptor (NR0B1), containing a ligand binding domain in its carboxy-terminal region, and in place of a traditional DNA binding domain, a 70 amino acid repeat motif in its amino-terminal region (2, 20, 21). Murine expression of Dax1 is restricted to tissues involved in

steroidogenesis and reproduction, both during development and in the adult (22). The confirmed primary function of Dax1 is as a transcriptional inhibitor of Steroidogenic factor 1 (Sf1, Nr5a1), the master regulator of development and function of the HPA and HPG axes (23). Because of its association with corepressors such as NCoR (24) and Alien (25), as well as the presence of LXXLL binding motifs (26), Dax1 is believed to directly compete at cofactor binding sites of Sf1 (20, 24). Dax1 has also been shown to have additional, less well characterized properties, such as binding to a stem-loop structure in the Star promoter (27), acting as a shuttling RNA binding protein (27), and inhibiting RNA splicing (28). None of these ascribed functions of Dax1 adequately explain the mechanism by which X-linked AHC develops from loss of DAX1. However, recent proteomics studies have identified Dax1 as a binding partner of ES cell factors Oct4 and Nanog, which implicate Dax1 as an important factor within the transcriptional network that maintains stem cell pluripotency (29-31). Indeed, knock down of Dax1 in ES cells results in spontaneous differentiation (32, 33). Additionally, Dax1 also plays a role in the etiology of some cancers – its expression correlates with advanced stage in ovarian carcinoma and with the stem cell population of lung cancer cells (34, 35). Taken together, these data suggest an important general role for Dax1 in maintaining or promoting an undifferentiated state, which suggests a similar function in cells within the adult adrenal cortex.

Consistent with its role as a steroidogenesis inhibitor, Dax1 knockout ($Dax1^{-/Y}$) mice, in which exon 2 of the Dax1 gene locus was deleted (36), have enhanced adrenal steroidogenesis that rescues adrenal haploinsufficiency of Sf1-deficient mice (37). These mice also have elevated gonadal steroidogenesis, which renders them infertile partly due

to elevated expression of p450 aromatase (Cyp19) (38). However, all of the studies performed with Dax1$^{-/Y}$ mice used relatively young animals (less than 10 weeks). To date, steroidogenic analysis of Dax1$^{-/Y}$ mice over the lifespan of the mouse has not been performed.

A current model of Dax1 action implicates it in maintenance of adrenocortical progenitor cells, primarily because expression within the adrenal cortex is enriched in the subcapsular zone , which is the location of putative adrenocortical progenitor cells (39). Indeed, earlier studies have demonstrated that the adrenal cortex is populated from the subcapsular zone by inward centripetal displacement (40, 41). Dax1 expression in the adrenal is activated by both Wnt signaling and glucocorticoids, yet inhibited by ACTH, placing it within an intra-adrenal feedback loop that regulates steroidogenesis in adrenal cells in response to developmental or endocrine signals and plays a critical role in differentiation of adrenal progenitors in response to limiting corticosterone levels (42-44). In this model, Dax1 deficiency would result in premature progenitor cell differentiation (with elevated steroidogenesis) followed by eventual exhaustion. There is clinical support for this model in a handful of case studies in which siblings of X-linked AHC patients were monitored before manifesting with clinical adrenal failure (9, 19). In these reports, several patients with DAX1 mutations had elevated adrenal steroid hormone production before ultimately failing, suggesting similar consequences for loss of Dax1 in mice and humans.

In an effort to reconcile the disparate phenotypes of mice and humans deficient in Dax1, I decided to observe adrenals of Dax1-deficinet mice over an extensive time period. In this study I demonstrate that Dax1-deficient mice have elevated adrenal

steroidogenesis – marked by both increased capacity and increased sensitivity – which diminishes with age. Additionally, Dax1-deficient mice have progressively worsening adrenocortical dysplasia , which is absent in wild type mice. Finally, both young and old $Dax1^{-/Y}$ mice have markedly different adrenal proliferation rates than their wild type counterparts. This is the first study to demonstrate evidence of adrenal failure in $Dax1^{-/Y}$ mice, which, taken together, suggests they may be an appropriate model for human AHC.

Materials and Methods

Experimental Animals

All experiments involving animals were performed according to institutionally approved and current animal care guidelines. Dax1 deficient mice were obtained previously (37) and maintained on a 129S1/SvImJ background. To obtain $Dax1^{-/y}$ mice, wild-type males were mated with heterozygous females. Experimental animals were housed with 2-4 animals per cage for up to 100 weeks (n=5 per genotype, per group). 24 hours before sacrifice, mice were injected with 5-bromo-2-deoxyuridine (BrdU, Roche, Basel Germany). Mice were transferred to single cages and housed in stress-free conditions until sacrifice by rapid decapitation at 0800 h. Trunk blood was collected in EDTA-coated collection tubes (Sarstedt). After decapitation, both adrenal glands, pituitaries, and a portion of small intestine were removed and placed in ice cold phosphate-buffered saline.

ACTH Stimulation Test

ACTH stimulation test was performed as described previously (45). Briefly, the hypothalamic-pituitary axis was suppressed by injecting 5 μg/g body weight dexamethasone (Sigma, St. Louis, MO) i.p. at 1800 h one day prior and at 0800 h on the day of the assay. At 1000 h, 1 μg/g body weight ACTH 1-24 (Bachem, Torrance, CA) was injected i.p. and blood was collected from venous tail puncture at 0, 15, 30, and 60 minutes post ACTH injection.

Plasma Hormone Measurements

Serum ACTH and corticosterone levels were determined by radioimmunoassay using a ^{125}I RIA kit (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. Samples were run in duplicate and quantified using a Gamma Counter.

Immunohistochemistry

Tissues were fixed for 2-3 hours in formalin (4% formaldehyde in PBS) and then dehydrated in graded ethanol solutions before embedding in paraffin. Embedded tissues were sectioned in 7 μm sections and mounted on microscope slides. Sections were then deparaffinized in xylenes (2 X 5 minutes) and rehydrated in graded ethanol. Antigen retrieval was achieved by boiling in Citrate Buffer (10 mM sodium citrate, 0.05% Tween-20, pH 2.0) for 15 minutes. Tissue sections were incubated in antibody diluent for 1 hour at room temperature and then in primary antibody (custom rabbit anti-Sf1 – 1:2000; rat anti-BrdU – 1:500, AdB Serotec; mouse anti-PCNA – 1:1000, Santa Cruz) overnight at 4°C. Sections were then incubated with fluorescent dye-conjugated secondary antibody for 1 hour (FITC anti-rat – 1:1000; Alexa-fluor anti-rabbit/mouse – 1:1000).

Quantitative RT-PCR

Total RNA was isolated from tissues using Trizol Reagent (Invitrogen) and 1 μg was reverse transcribed using iScript system (BioRad). The resulting cDNA was diluted 1:5 and 2 μl was amplified with the appropriate primers using SYBR Green PCR Master Mix (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta C(T)}$ method (46). Primer sequences for each amplified gene are: *Gapdh*: Fwd-5′ aatgtgtccgtcgtggatct, Rev-5′ cccagctctccccatcacta; *Cyp11b2*: Fwd-5′ gcaccaggtggagagtatgc, Rev-5′ gccattctggcccatttag; *Cyp21a1*: Fwd-5′ ctgcttcaccaccctgaga, Rev-5′ agctgcattcggttcctgt, *Star*: Fwd-5′ gctgctcagtattgacctgaag, Rev-5′ gcgataggacctggttgatg; *p450scc*: For-5′ aagtatggccccatttacagg, Rev-5′ tggggtccacgatgtaaact.

Immunoblotting

Total cell lysates were prepared in lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, and protease inhibitor cocktail]. Lysates were allowed to rotate at 4 C for 30 min, and protein contents of the high-speed supernatant were measured using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equivalent quantities of protein (20–45 μg) were resolved on polyacrylamide-SDS gels, transferred to nitrocellulose membrane (Bio-Rad), and immunoblotted with specific antibodies. Results were visualized using the Supersignal West Dura Extended Duration Substrate kit (Pierce Chemical Co., Rockford, IL.).

Statistics

Statistical significance was determined by using an unpaired student's t-test to compare data from Dax1$^{-/Y}$ mice and wild type mice at each age. Results were considered significant if $p < 0.05$ and are denoted with an asterisk.

Results

Dax1$^{-/Y}$ mice have altered acute HPA axis responsiveness and baseline HPA axis profile

To determine if Dax1-deficient mice have an altered steroidogenic profile past 6 weeks (37), we performed ACTH stimulation tests on 15, 30 and 60 week wild type and Dax1$^{-/Y}$ mice. ACTH stimulation following dexamethasone suppression is a good readout of maximal adrenal steroidogenic capacity or responsiveness (45). 15 week Dax1$^{-/Y}$ mice have a significantly higher corticosterone output in response to ACTH, particularly at the 30 and 60 minute intervals (534 ± 56 *vs*. 267 ± 81 ng/mL, $p < 0.05$; 551 ± 50 *vs*. 322 ± 27 ng/mL, $p < 0.05$; respectively). **(Figure 3.1 A)** However, 30 week Dax1$^{-/Y}$ mice have a similar corticosterone output to wild type mice. **(Figure 3.1 B)** Notably, 60 week Dax1$^{-/Y}$ mice have significantly reduced ACTH-induced corticosterone output at the 60 minute interval (261 ± 63 vs. 540 ± 31 ng/mL, $p < 0.05$. **(Figure 3.1 C)** Based on this initial observation, we next decided to investigate the basal hormonal activity of the hypothalamic pituitary axis in Dax1-deficient mice more extensively. We measured plasma hormone levels of wild type and Dax1$^{-/Y}$ mice between 15 and 100 weeks old and plotted ACTH to corticosterone ratio. ACTH/corticosterone ratio is an ideal method for illustrating actual adrenal steroidogenic responsiveness as a more sensitive adrenal will produce a given amount of corticosterone in response to a lower amount of ACTH (37). In agreement with our initial observation, 15 week Dax1$^{-/Y}$ mice

have a lower ACTH to corticosterone ratio (3.16 ± 1.02 vs. 8.31 ± 2.05; $p < 0.05$), indicating increased adrenal ACTH sensitivity. **(Figure 3.2)** Mice in the intermediate age groups did not have a significant difference in ACTH/corticosterone ratio. However, 100 week $Dax1^{-/Y}$ mice had a greater ACTH/corticosterone ratio (10.44 ± 1.61 vs. 4.47 ± 0.76; $p < 0.05$; Figure 2), indicating loss of both adrenal steroidogenic sensitivity and capacity with age.

Steroidogenic enzyme gene expression in $Dax1^{-/Y}$ adrenals declines from 15 to 100 weeks

Differentiation of the adrenal cortex can be analyzed by gene expression of relevant enzymes required for steroidogenesis. To further delineate the changes in steroidogenic output of the Dax1-deficient mice, we evaluated gene expression of several steroidogenic enzymes at each age by quantitative PCR. As expected, 15 week $Dax1^{-/Y}$ mice have elevated adrenal expression of Steroidogenic acute regulatory enzyme (Star) mRNA (2.88 ± 0.31 *vs*. 1.00 ± 0.11 fold, p , 0.05), Side-chain cleavage enzyme (Scc) mRNA (6.61 ± 0.88 *vs*. 1.00 ± 0.09 fold, $p < 0.05$), and P45011b1 mRNA (3.59 ± 0.41 *vs*. 1.00 ± 0.19 fold, $p < 0.05$) compared to wild-type mice. **(Figure 3.3 A)** In intermediate ages, expression of steroidogenic enzyme mRNA is not significantly different between $Dax1^{-/Y}$ and wild-type mice. However, 100 week $Dax1^{-/Y}$ mice have lower adrenal expression of Star mRNA (0.39 ± 0.02 *vs*. 1.00 ± 0.16 fold, $p < 0.05$), Scc mRNA (0.36 ± 0.11 *vs*. 1.00 ± 0.28 fold, $p< 0.05$), and 11b1 mRNA (0.36 ± 0.02 *vs*. 1.00 ± 0.24 fold, $p < 0.05$) compared to wild type mice.

In order to further confirm the results obtained by qRT-PCR, adrenal protein lysates were analyzed by Western blot. While there was no appreciable change in levels

of P450Scc, it is quite striking that StAR protein levels are elevated in the 15- and 30-week Dax1$^{-/Y}$ adrenals. However, at 80 weeks, StAR protein levels are dramatically lower than its wild-type counterpart. **(Figure 3.3 B)**

Adrenal glands from Dax1-deficient mice display progressively worsening dysplastic adrenal morphology

Because failing adrenals often display abnormal architecture, we compared Dax1 and wild-type adrenals histologically. Histological analysis revealed that Dax1$^{-/Y}$ adrenals have severe morphological abnormalities when compared to wild type adrenals. 15 weeks Dax1$^{-/Y}$ mice have a somewhat enlarged adrenal cortex, yet maintained normal adrenal morphological structure. **(Figure 3.4)** By 55 weeks, adrenals of Dax1$^{-/Y}$ mice began to display abnormal architecture. 100 week Dax1$^{-/Y}$ adrenals were severely deformed and dysplastic while wild type adrenals maintained normal architecture. Additionally, Dax1$^{-/Y}$ adrenals contained large eosinophilic cytomegalic cells.

Proliferation of Dax1$^{-/Y}$ adrenals declines from 15 to 100 weeks

To determine if Dax1-deficient mice had altered adrenocortical proliferation, we measured BrdU-positive foci by immunohistochemical staining of adrenals with an antibody directed against BrdU, and quantified the number of BrdU foci in each adrenal and averaged three sections per adrenal. Adrenals of 15 week Dax1$^{-/Y}$ mice had significantly higher levels of BrdU-positive foci than their wild-type counterparts (13.0 ± 2.5 vs 2.7 ± 0.2; $p < 0.05$). 30 week Dax1$^{-/Y}$ adrenals had more BrdU-positive foci than wild type (6.0 ± 1.0 vs. 2.2 ± 0.2; $p < 0.05$). At intermediate ages, the number of BrdU-

positive foci was not significantly different between the genotypes. However, 100 week Dax1$^{-/Y}$ mice had fewer BrdU-positive foci than their wild-type counterparts (1.9 ± 0.8 vs 4.1 ± 0.4; $p < 0.05$). **(Figure 3.5 A)** These results were confirmed by performing immunostaining on 15-, 30-, 70-, and 100-week adrenal sections using an antibody directed against proliferating cell nuclear antigen (PCNA). A greater number of cells in each adrenal section stained positive for PCNA, but there was an appreciable difference between the genotypes. Adrenals of 15 week Dax1$^{-/Y}$ mice had significantly higher levels of PCNA-positive foci than their wild-type counterparts (110.4 ± 27.4 vs 42. ± 3.6; $p < 0.05$). The number of PCNA-positive foci was not significantly different in the 30- and 60-week adrenals. However, 100 week Dax1$^{-/Y}$ mice had fewer PCNA-positive foci than their wild-type counterparts (9.0 ± 3.8 vs 24.8 ± 3.8; $p < 0.05$). **(Figure 3.5 B)**

PCNA staining was visualized along with anti-SF1 staining to appreciate the differences in young and old mice. 15 week Dax1$^{-/Y}$ adrenals have considerably more anti-PCNA staining than wild type, specifically in the subcapsular zone. Conversely, 100 week Dax1$^{-/Y}$ adrenals have less anti-PCNA staining than wild-type adrenals, confirming that proliferation dramatically decreases in old Dax1$^{-/Y}$ adrenals. **(Figure 3.6)** Additionally, PCNA protein levels were analyzed by Western blot. There is appreciably more PCNA in 15- and 30-week Dax1 adrenals than in wild-type adrenals. However, at 80 weeks, PCNA protein is appreciably lower than in its wild-type counterpart. **(Figure 3.3 B)**

Discussion

The primary goal of this study was to reconcile the interspecies variation observed in mice and humans deficient in DAX1. In this study, a similarity in the phenotype of Dax1-deficient mice to that of human patients with X-linked AHC is observed. As expected based on earlier studies, young $Dax1^{-/Y}$ mice have enhanced adrenal steroidogenesis, which is consistent with the well known repressor function of Dax1in steroidogenesis. However, hormone monitoring and gene expression has demonstrated that $Dax1^{-/Y}$ mice have progressively diminishing adrenal steroidogenesis as they age. Although young $Dax1^{-/Y}$ mice display enhanced subcapsular proliferation, a progressively worsening dysplasia, which is accompanied by a marked decrease in proliferation, is also observed with age.

Symptoms of adrenal failure serve as prompt for sequencing the DAX1 allele, leading to diagnosis of X-linked AHC when mutations or deletions are discovered. Because hormone monitoring is not standard practice in asymptomatic children, little data has been collected on hormone levels of patients with DAX1 mutations prior to manifestation of adrenal insufficiency, explaining the paucity of data detailing steroidogenic profiles prior to adrenal failure. However, recent case reports indicate that some individuals with DAX1 mutations have elevated cortisol or other steroid hormones prior to diagnosis of AHC, which is reversed upon development of the syndrome (9, 19). This would reconcile the human data with the mouse data and support a model in which loss of Dax1 results in premature differentiation of the adrenal cortex.

It is noteworthy that there has been speculation that Dax1$^{-/Y}$ mice might express a hypomorphic Dax1 allele, which could explain interspecies phenotype differences (47). This speculation is largely due to the method used to generate the knockout – deletion of the relatively small exon 2, leaving the larger exon 1 intact. An alternatively spliced isoform, DAX1α/DAX1A has been identified in human tissues, consisting of all of exon 1 and a very short intronic sequence, which would resemble a protein that might be produced by the knockout mouse (48, 49). However, expression of this isoform is extremely low compared to the main isoform, raising questions regarding the physiological role of DAX1a in steroidogenic tissue development and function (50). Additionally, neither a mouse orthologue to DAX1a nor a specific hypomorphic Dax1 in the Dax1$^{-/Y}$ mouse has been identified. Using an antibody that would recognize a truncated protein, our lab showed no evidence of truncated protein expression in the Dax1$^{-/Y}$ adrenals (37). Lastly, X-linked AHC still develops in patients with mutations at the C-terminus of DAX1 (51). While the gonadal phenotypes between X-linked AHC patients and Dax1$^{-/Y}$ mice are strikingly similar, there are still some noticeable differences – mainly that Dax1$^{-/Y}$ mice do not have hypogonadotropic gonadism (36). In all, this may simply reflect heterogeneity and variation along the spectrum of phenotypes for DAX1 mutations rather than indicate a hypomorphic allele in the mouse model.

Recently Dax1 was shown to act as a coactivator when associated with steroid receptor RNA activator (SRA) (52). Interestingly, this interaction occurs at the amino-terminal region of Dax1, suggesting that it could be preserved in cases where only the carboxy-terminus of Dax1 is deleted. Perhaps Dax1 interaction with SRA provides the mechanism by which Dax1 is able to activate genes required for maintenance of

adrenocortical progenitor cells. Interestingly, in light of the fact that his study was performed in mouse cells, it is entirely plausible that this interaction is a species-specific phenomenon. Further studies must be performed looking at the SRA in the context of Dax1-deficiency as well as the role of SRA in the human adrenal cortex.

This study is consistent with a role for Dax1 in maintenance of subcapsular adrenocortical progenitor cells. However, Dax1 is clearly not the sole protein that functions in such a capacity. Previous studies have demonstrated that both Wnt-signaling and glucocorticoids transcriptionally activate Dax1 (42, 43) and loss of beta-catenin results in similar adrenocortical failure and cellular depletion (44, 53). These data, combined with the similar restricted expression of beta-catenin and Dax1 in the subcapsular region, lend credence to a physiologic interaction between these pathways in maintenance of undifferentiated progenitor cells within the adrenal cortex. It is, therefore, easy to speculate that loss of Dax1 results in premature differentiation of adrenocortical progenitor cells, which ultimately leads to adrenal failure. Loss of a factor leading to exhaustion of organ-specific progenitor cells may be fairly common within endocrine tissues: human PROP1 mutations result in dramatic variability of pituitary morphology and longitudinal imaging revealed pituitary enlargement followed by hypoplasia (54, 55). Data from Prop1-deficient mice, in which a hypoplastic pituitary follows expantion of trapped undifferentiated cells, suggests that this may be due to the role of Prop1 in migration of progenitor cells into the anterior pituitary (56).

Cytomegaly is a hallmark phenotype of an adrenal cortex from a patient diagnosed with X-linked AHC, though the reason remains unknown (5, 57). Cytomegaly is also a phenotype of other genetic syndromes in which adrenal failure occurs, such as

IMAGe Syndrome (58). Additionally, several mouse models display a similar phenotype upon adrenal failure such as the *acd* mouse that is defective in telomere maintenance (59) as well as the adrenal specific beta-catenin knockout mouse (44). In the current study, adrenal sections of $Dax1^{-/Y}$ mice also display dysmorphic cytomegaly, that increases in intensity with age. It is provocative to speculate that AHC with cytomegaly may represent a compensatory morphological endpoint induced by progenitor cell exhaustion. Indeed, perhaps the most intriguing phenotype observed in the current study is the proliferation difference between $Dax1^{-/Y}$ and wild type mice, particularly at either end of the age spectrum. Young $Dax1^{-/Y}$ mice have a significantly higher proliferation rate (by BrdU-positive foci and PCNA staining), while it is dramatically lower in old mice. This is in stark contrast to the relatively constant rate of proliferation seen in the wild type mice and to previous assumptions that Dax-deficient adrenals would sacrifice proliferation for differentiation (60). Whether the data reflect loss of direct inhibition by Dax1 on subcapsular proliferation or activation or a secondary compensatory mechanism due to premature differentiation of cortical cells will require further analysis.

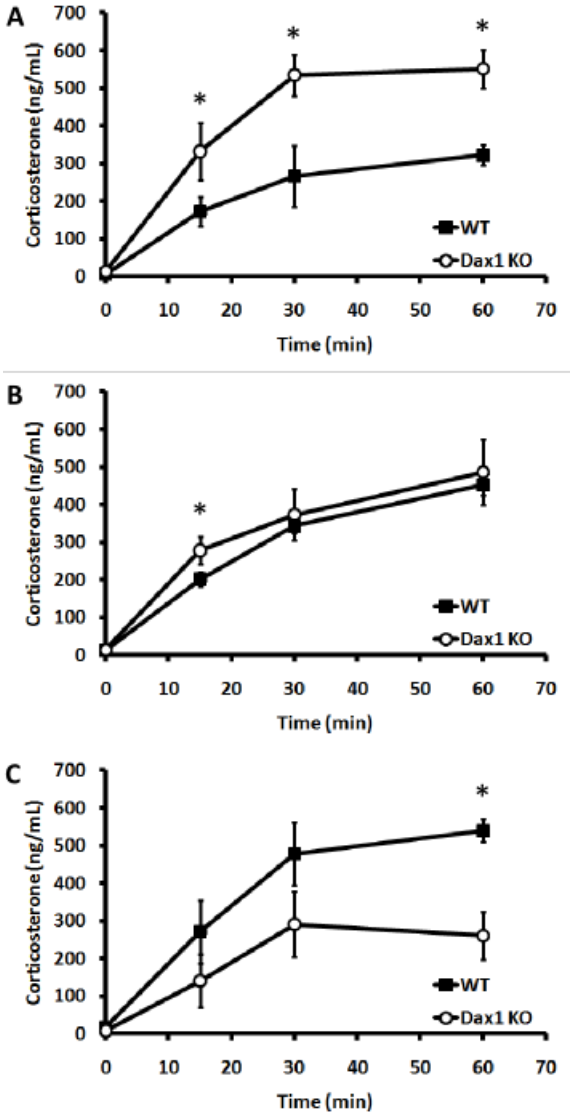


Figure 3.1 ACTH stimulation test in 15-, 30-, and 60-week mice.

ACTH stimulation test was performed by dexamethasone suppression overnight followed by injection of ACTH with blood samples collected at 15, 30, and 60 min post-injection. Corticosterone was measured by RIA and values were plotted for 15- (A), 30- (B), and 60-week mice (C). Wild-type are represented by black sqaures, $Dax1^{-Y}$ are represented by white circles. (*, $P \leq 0.05$)

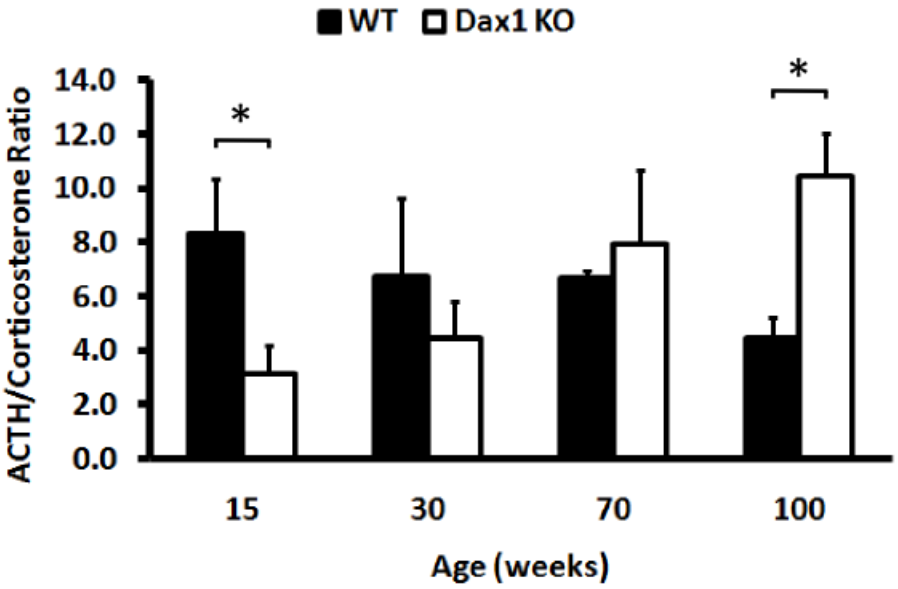


Figure 3.2 ACTH/Corticosterone ratio at various ages.

Trunk blood was collected and basal hormone levels were measured using RIA. The level of ACTH was plotted as a ratio to the level of corticosterone for each age. Wild-type are represented by black bars, Dax1^{-Y} are represented by white bars. (*, $P \leq 0.05$)

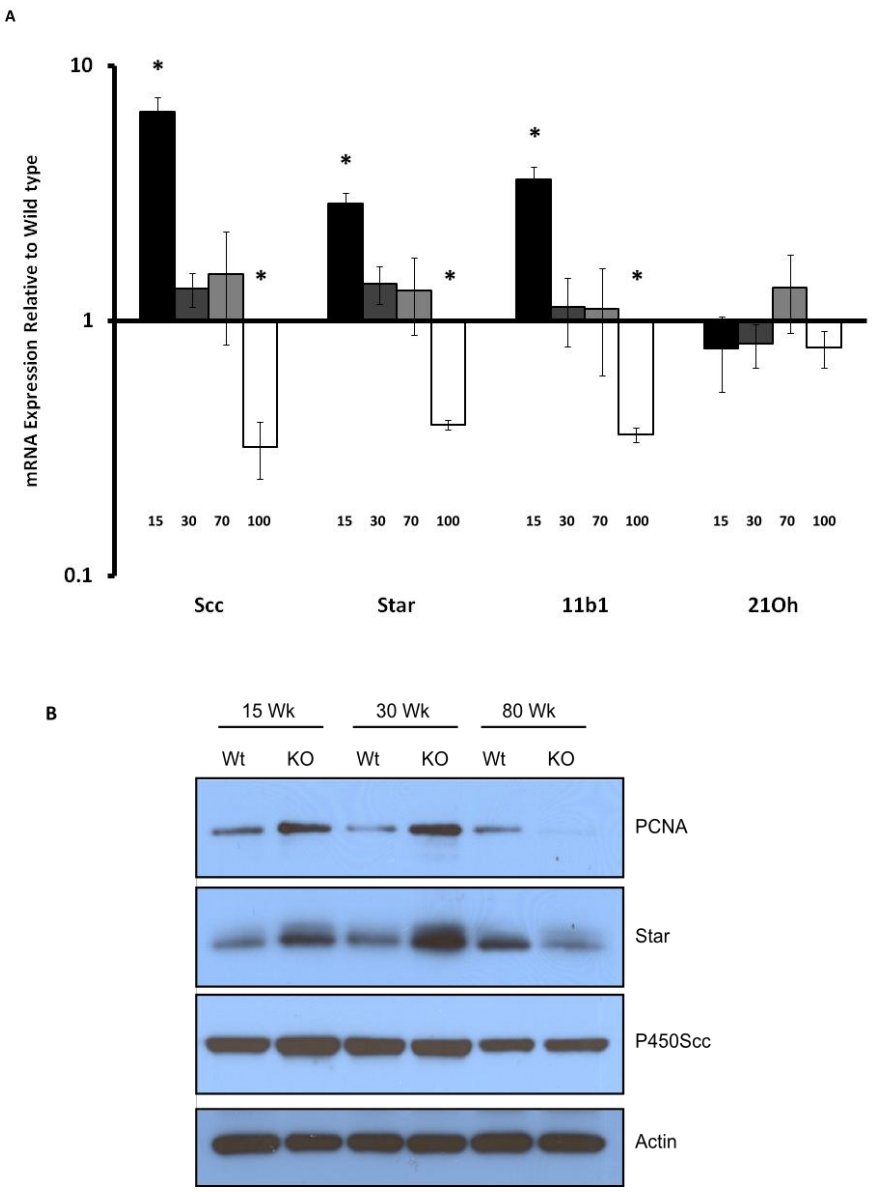


Figure 3.3 Gene and protein expression levels at various ages.

A) mRNA was harvested from fresh adrenals and analyzed by qRT-PCR using primers directed against side-chain cleavage enzyme, steroidogenic acute regulatory protein, Cyp11b1, and 21-hydroxylase. Results were plotted relative to wild-type levels, which were set to one. (*, $P \leq 0.05$) B) Protein was isolated from fresh adrenals, run on a denaturing gel and then immunoblotted using specific antibodies.

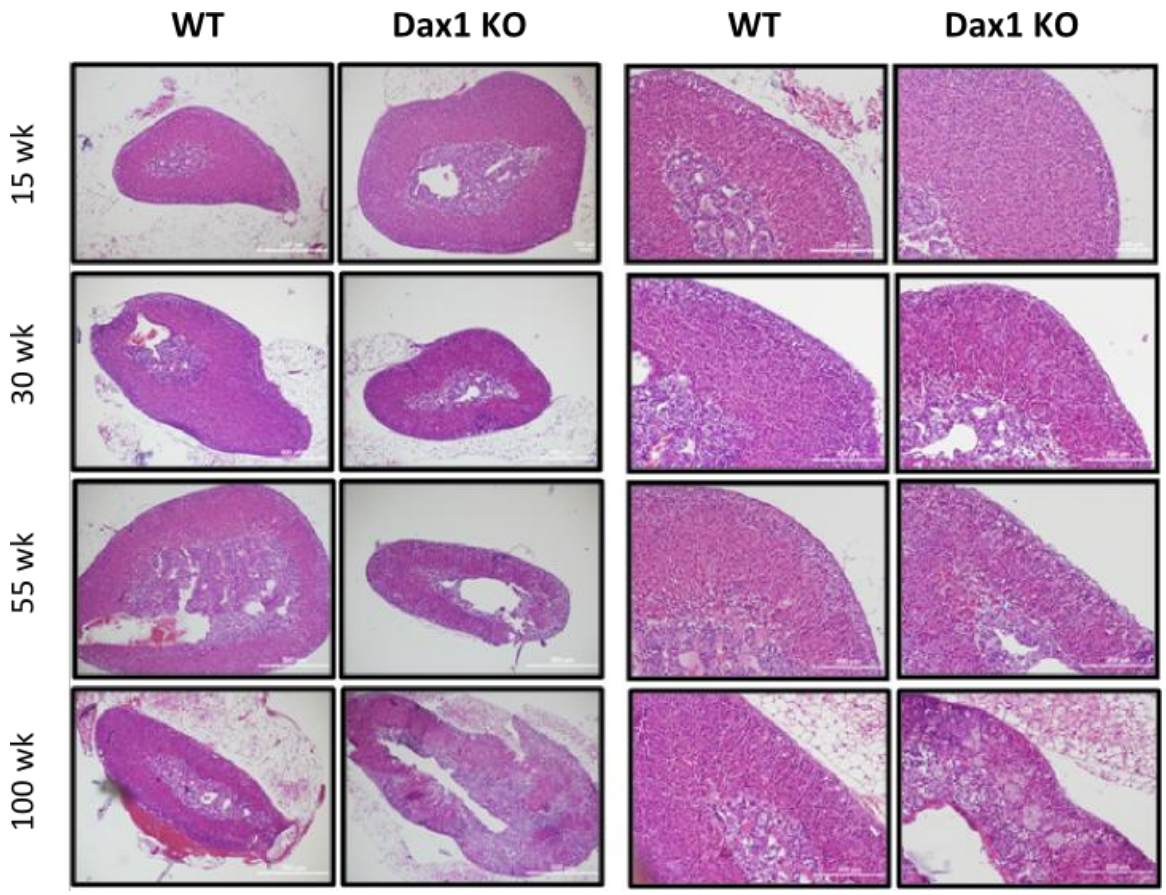
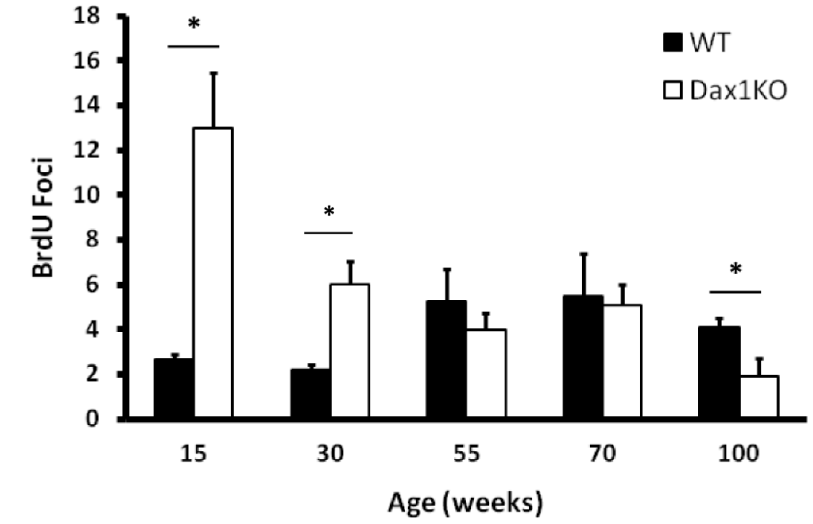


Figure 3.4 Adrenal histology at various ages.

Adrenal sections were deparaffinized and stained with Hemtoxylin and Eosin to visualize gross changes in histology or morphology. The two left panels are low magnification and the two right panes are higher magnification of the same adrenal section.

A



B

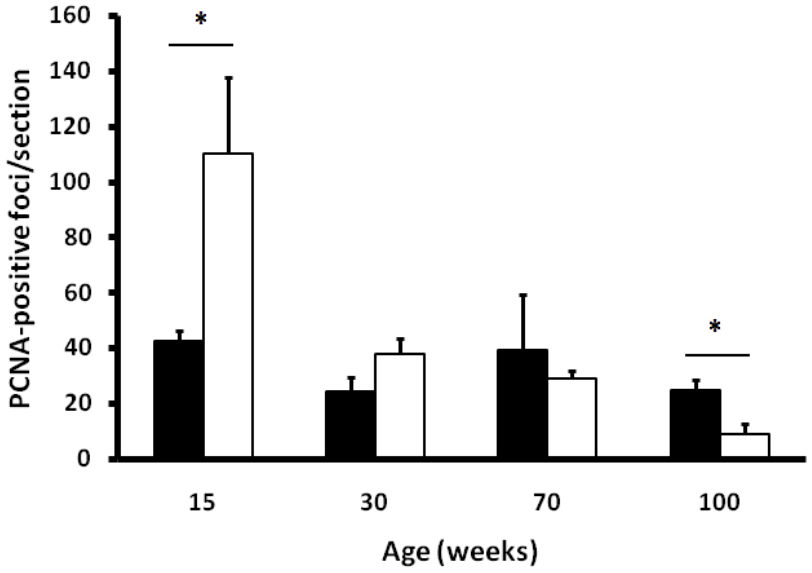


Figure 3.5 Proliferation markers at various ages.

Quantitation of BrdU (A)and PCNA-positive (B) foci per adrenal section. Foci were counted in three adrenal sections for each animal. Results for each age were averaged. Wild-type are represented by black bars, $Dax1^{-Y}$ are represented by white bars. (*, $P \leq 0.05$)

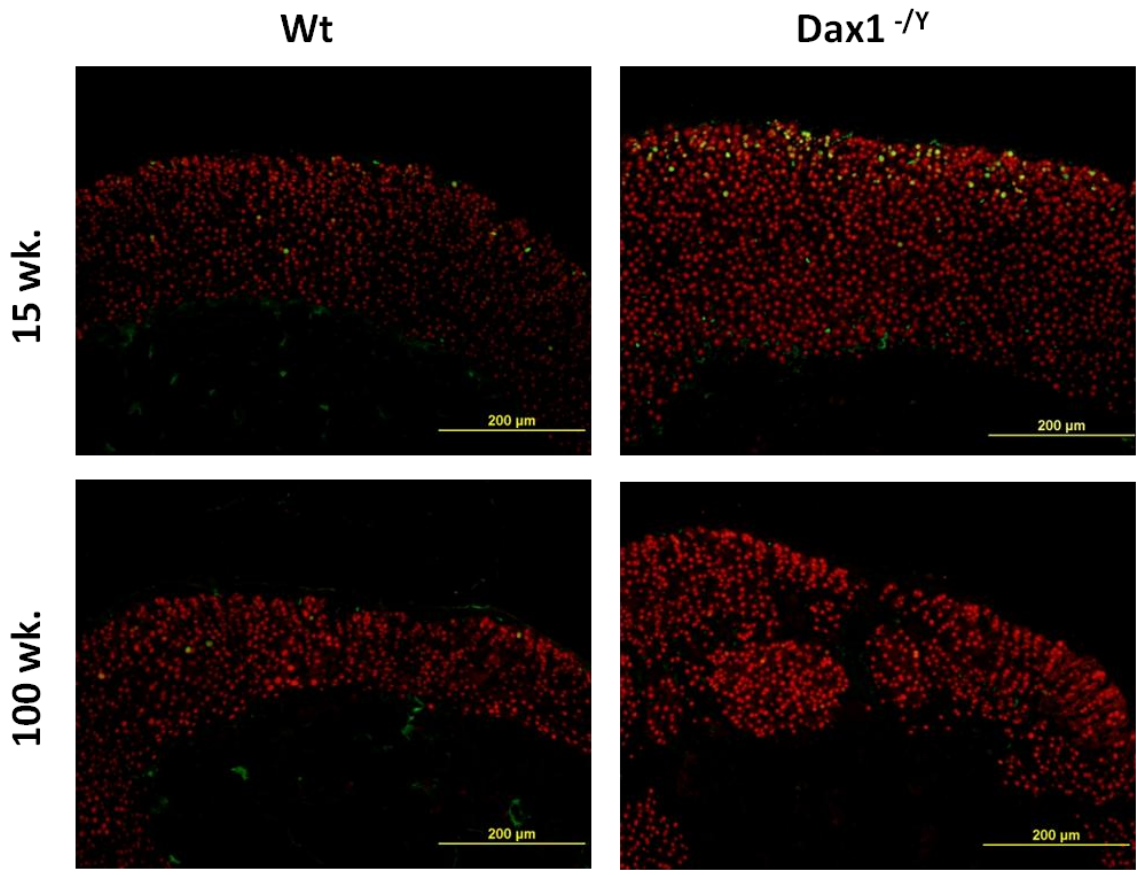


Figure 3.6 PCNA immunofluorescence in 15- and 100-week adrenal sections.

Adrenal sections were probed with antibodies directed against PCNA (green) and SF1 (red). Images were merged.

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SUMMARY AND FUTURE DIRECTIONS

Summary

SF1 and DAX1 are nuclear receptors that play an integral role in development and function of the adrenal cortex. SF1 is a transcription factor that strongly activates expression of the genes encoding several of the cholesterol transporters and P450 enzymes required for the conversion of cholesterol into the steroid hormones of the adrenal cortex. Additionally, SF1 regulates the basal expression of several genes, including genes involved in steroidogenesis, endocrine signaling, and growth and development. The tissue and temporal specificity of several of these genes demonstrates the subtlety with which SF1-dependent transcription is regulated. It is likely that several different signaling pathways regulate SF1-dependent transcription by altering cofactor availability or by modulating signaling molecules that impinge on SF1 transactivation. These studies describe the regulation by SF1 of several factors that are involved in SF1-dependent transcription.

In chapter two, I described the results of a chromatin immunoprecipitation-chip microarray (ChIP-chip), in which genomic fragments of SF1 immunoprecipitate were analyzed on a promoter microarray. Promoter regions of a large number of genes were enriched. Surprisingly, there were not a large number of know steroidogenic target

genes. However, of particular interest were enriched targets involved in sphingolipid signaling and metabolism, specifically Edg5, which constitutes a novel genomic target of SF1-dependent transcription. Edg5 is the sphingosine 1-phosphate receptor and S1P has been shown to stimulate release of steroid hormones from adrenocortical cells. ACTH signaling also increases sphingosine kinase activity, resulting in increased levels of S1P. It is interesting to speculate that SF1 increases production of Edg5 to compensate for higher levels of intra- and intercellular S1P. I also described an association of SF1 on the Adcy4 promoter. While in most cases SF1 acts as an activator, on the Adcy4 promoter, SF1 acts to repress transcription of Adcy4. This could possibly be a mechanism by which SF1 alters the cellular environment of upstream effectors that result in its transcriptional activation. Finally, I described an interaction between SF1 and GR to activate expression of the gene encoding Dax1. Once again, Dax1 is a factor that alters the transcriptional activity of SF1, by inhibiting its transactivation. In this case, Dax1 is fine-tuning the production of glucocorticoids from the adrenal cortex, but this work increases the knowledge of what role Dax1 plays in the adrenal cortex. In all three cases, the genomic target of SF1 encodes a factor that can modulate SF1-dependent transcription. This suggests the existence of several mechanisms by which SF1-dependent transcription is uniquely modulated.

In chapter 3, I described evidence that Dax1-deficient mice develop adrenal failure. While mutations of DAX1 result in Adrenal Hypoplasia Congenita in humans, the Dax1-deficient mouse has a mild adrenal phenotype. These results potentially reconcile the disparity of the phenotypes between the two species. While the known function of DAX1 is to inhibit SF1-dependent transcription, it was, and still remains,

puzzling why loss of DAX1 results in adrenal failure. The Dax1-deficient mouse has adrenal hyperfunction, which would be expected based on the ability of Dax1 to inhibit SF1. However, I have shown that Dax1-deficient mice develop adrenal hypofunction late in life, suggesting that Dax1 may be maintaining a population of cells in an undifferentiated state and that loss of Dax1 results in depletion of this population. This hypothesis is supported by cases of DAX1-deficient patients with elevated hormone levels before their adrenals ultimately fail. In sum, this dissertation contributes to the understanding of both SF1-dependent gene regulation and the significance of DAX1 in homeostasis of the adult adrenal cortex.

Future Directions

Identifying all of its genomic targets will result in tremendous advancement in the understanding of SF1-mediated transcription. Future studies stemming from the work presented here would include fully characterizing the enriched targets of the SF1 ChIP-chip. Starting with Edg5, all of the promoters of genes that belong to the sphingolipid signaling and metabolism cluster will require further characterization. This includes cloning the full promoter regions and performing luciferase assays to determine the context in which these genes are activated. This can be done under various conditions of different hormone treatments or activation of specific signaling pathways. Finally, the SF1 response elements within the promoters would be mutated to determine the requirement of SF1 in their transcriptional activation. Various activators or inhibitors of signaling pathways could be used to tease apart the interaction between sphingosine 1-phosphate signaling and ACTH signaling in adrenocortical cells.

Confirmation of the ChIP-chip data presented in this thesis could be performed using newer, more powerful techniques, such as ChIPseq. Because this method sequences all DNA regions that are enriched following chromatin immunoprecipitation, it is not limited in scope by genomic region, whereas ChIP-chip will only identify regions that are on the particular chip that is being used. The possibility of identifying considerably more targets of SF1 makes this technique an ideal choice for future studies.

The fact that SF1 can sometimes act as a repressor of transcription is quite intriguing in light of its usual mechanism of action; as an activator of transcription. Even more compelling is the fact the SF1 response element is not different from an element that results in activation of target genes. How then is the protein able to differentiate between a gene that is supposed to be activated and one that is to be repressed? Because the sequences are identical, the answer must lie in the sequence surrounding the SF1 response element. In the case of Adenylyl cyclase, it seems that specific Sp1 sites result in repression of this gene by SF1. Perhaps this is a unique configuration that always results in repression. Future studies could focus on fully chararterizing sequences surrounding SF1 response element in genes that are repressed by SF1. This would provide information regarding the specific transcription factors that, when they interact with SF1, result in transcriptional repression. The ChIP-chip data would prove useful for this type of analysis. Promoters in which ACTH specifically recruits or expels SF1 can be aligned and compared for commonalities. This would provide information regarding specific genomic sequences that regulate this type of interaction.

The Dax1-deficient mice provide a potentially very useful tool for elucidating the function and molecular mechanism of DAX1 in the adrenal cortex. A personal

communication with a collaborator revealed an unpublished case report of a patient with late onset AHC that suffered adrenal crisis after a period of extreme physical exertion. Perhaps adrenal crisis could be forced in $Dax1^{-/Y}$ mice by prolonged exposure to ACTH. A simple experiment would be to activate the HPA axis in the $Dax1^{-/Y}$ mice to observe signs of premature failure compared to the wild-type mice. This could be accomplished by giving daily ACTH injections for a period of time or by fasting, which induces the HPA axis. However, if longer periods of HPA axis activation are needed, a pellet could be implanted under the skin. If Dax1 mice demonstrated adrenal failure earlier than wild-type mice, it would be consistent with Dax1 maintaining the undifferentiated state of the adrenal cortex. This will provide greater insight regarding the function of DAX1 in maintaining the adult adrenal cortex and would further reconcile human and mouse DAX1 deficiencies.

The contribution of Dax1 to the adult adrenal cortex would probably best be modeled using system in which Dax1 is genetically inactivated after the adrenal has already formed. By crossing floxed Dax1 mice with mice that express a tetracycline-inducible *cre* recombinase, Dax1 could be inactivated at any age. Many of the experiment performed in chapter 3 of this thesis could be performed in this genetic context as well. Ideally, lineage tracing would be employed so that cells in which Dax1 was deleted could be traced throughout the adrenal. I would predict that this scenario would reveal cells continuously populating the adrenal cortex after the deletion of Dax1. The strength of this type of assay is the ability to observe Dax1-ablated adrenals at any point after inducing the *cre* recombinase. Presumably, cells in which Dax1 had been

recently deleted would be found close to the capsule, while cells in which Dax1 had been deleted for a longer period of time would be observed within the adrenal cortex.

Finally, the Dax1-deficient mouse could be used in developmental studies. Because it was previously shown the Dax1 specifically inhibits expression of FAdE, breeding the $Dax1^{+/-}$ mouse with the FAdE reporter mouse could provide compelling results. In this cross FAdE could be traced in the context of Dax1 deficiency. Because, Dax1 normally represses FAdE in the developing adrenal cortex, it might be predicted that FAdE expression would persist into the adult adrenal. It would be interesting to determine if the adult adrenal forms properly, or if the fetal adrenal persists, but then eventually fails. All together the Dax1-deficient mice will provide a useful tool for understanding the complexities of the development and maintenance of the adrenal cortex.

To fully understand the molecular mechanism of Dax1 action in the adrenal cortex, the full complement of genomic targets must be elucidated. While it is know that Dax1 inhibits SF1-dependent transcription, this mechanism is insufficient to explain how Dax1 maintains undifferentiated adrenocortical cells. It is highly likely that Dax1 is inhibiting other transcription factors as well. Future studies could make use of genomic profiling techniques, such as ChIP-chip and ChIPseq to determine the genomic targets of Dax1 in the subcapsular cells of the adrenal cortex. Additionally, techniques such as tandem affinity purification could be used to define the proteins with which Dax1 interacts. I believe that these types of studies will be used to further define the role of Dax1 in the adrenal cortex and put it in its rightful place at the forefront of adrenal biology.