The Role of Wnt Signaling in Adrenocortical Homeostasis

by

Elisabeth Marie Walczak

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Doctoral Committee:

Professor Gary D. Hammer, Chair
Associate Professor Scott E. Barolo
Professor Andrzej A. Dlugosz
Associate Professor Jorge A. Iñiguez-Lluhí
Associate Professor Jun Li
Associate Professor Deneen M. Wellik

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ABSTRACT

Wnt/beta-catenin (βcat) signaling is critical for adrenal homeostasis. To elucidate how Wnt/βcat signaling elicits homeostatic maintenance of the adrenal cortex, we characterized the identity of the adrenocortical Wnt-responsive population. We find that Wnt-responsive cells consist of both Sonic Hedgehog-producing (Shh) adrenocortical progenitors and differentiated, steroidogenic cells of the zona glomerulosa, but not the zona fasciculata. Additionally, Wnt-responsive cells are rarely actively proliferating. To determine potential direct inhibitory effects of βcat signaling on zona fasciculata-associated steroidogenesis, we utilized the mouse ATCL7 adrenocortical cell line that serves as a model system of glucocorticoid-producing fasciculata cells. Stimulation of βcat signaling caused decreased corticosterone release consistent with the observed reduced transcription of steroidogenic genes *Cyp11a1*, *Cyp11b1*, *Star* and *Mc2r.* Decreased steroidogenic gene expression was correlated with diminished Steroidogenic factor 1 (*Nr5a1*, Sf1) expression and decreased occupancy on steroidogenic promoters. Additionally, βcat signaling suppressed the ability of Sf1 to transactivate steroidogenic promoters independent of changes in Sf1 expression level. To investigate Sf1-independent effects of βcat on steroidogenesis, we utilized Affymetrix gene expression profiling of Wnt-responsive cells *in vivo* and *in vitro.* One candidate gene identified, *Ccdc80*, encodes a secreted protein with unknown signaling mechanisms. We report that *Ccdc80* is a novel βcat-regulated gene in adrenocortical cells. Treatment of adrenocortical cells with media containing secreted Ccdc80 partially phenocopies βcat-induced suppression of steroidogenesis, through an Sf1-independent

mechanism. The data presented in this thesis reveal multiple mechanisms of βcat-mediated suppression of steroidogenesis and suggests that Wnt/βcat signaling regulates adrenal homeostasis by inhibiting fasciculata differentiation in addition to promoting the undifferentiated state of progenitor cells.

CHAPTER I: INTRODUCTION

The adrenal cortex is an exceptionally dynamic endocrine organ that is homeostatically maintained throughout life. This chapter presents the current knowledge of how adrenal development and postnatal maintenance is regulated by a large array of signaling molecules, including combinatorial inputs from distinct paracrine signaling pathways and the endocrine system.

I. Adrenal Anatomy & Function

The adrenal gland is composed of two discrete endocrine organs with distinct embryological origins. The outer adrenal cortex, derived from the intermediate mesoderm, is the primary site of corticosteroid biosynthesis. The inner adrenal medulla, formed from the neural crest, produces catecholamines that serve as mediators of the 'fight-or-flight" response. The organization of the adrenal cortex was first described by Arnold in 1866, whose nomenclature remains standard today [1]. The adrenal cortex is subdivided into three separate zones histologically, each responsible for the production of steroid hormones that mediate different aspects of stress response and homeostasis. The outermost layer, the zona glomerulosa (zG), is composed of cellular rosettes that secrete mineralocorticoids, like aldosterone, under control of the renin-angiotensin-aldosterone system (RAAS) for maintenance of electrolyte balance. The middle zona fasciculata (zF) consists of radial columnar cells that produce glucocorticoids, notably cortisol in humans and corticosterone in mice, in response to the hypothalamic-pituitary-

adrenal (HPA) axis to mediate energy homeostasis and the mammalian stress response. The innermost zona reticularis (zR) contains a net-like arrangement of cells that synthesize sex-steroid precursors, such as androstenedione and dehydroepiandrosterone (DHEA). The developmental establishment of the adrenal cortex occurs similarly across mammals [2], yet zonal differences exist between species. While humans and primates have the three adrenocortical zones described above, rodents lack the zR (Figure 1.1).

II. Evidence for Adrenal Stem/Progenitor Cells

Somatic stem cells are responsible for post-developmental, homeostatic maintenance of precursor populations and have been described for many organs [3]. Stem cells are described as long-lived, slow cycling, clonogenic, possessing the ability to self-renew, and having multipotent differentiation potential. Whereas stem cells retain the capacity to proliferate indefinitely, the progenitor cells that they give rise to are more committed in lineage and thought to possess limited replicative potential. [4]. Many studies provide evidence for adrenocortical cells with stem and progenitor-like capacities.

Undifferentiated adrenocortical cells with limited steroidogenic capacity have been described across mammalian species. In mice and humans, the outermost zG layer contains both differentiated, aldosterone-producing cells intermingled with clusters of undifferentiated cells [5, 6]. In rats, the zU represents a circumferential layer of undifferentiated cells between the zG and the zF [7]. In adrenals of the common seal (*Phoca vitulina vitulina*), groupings of large rounded cells described as ‘adrenocortical blastema’ are found adjacent to trabeculae of connective tissue extending from the capsule. Similar to rats, seals also possess a zona intermedia (zI) between the zG and zF that contains additional blastema [8].

The continued proliferative capacity of the adult adrenal gland to maintain adrenal volume and function throughout life is consistent with the presence of a stem-like population of cells. Tritiated thymidine (H3-T) and BrdU pulse-chase experiments in rodents have established that cells proliferate in the outermost layers of the adrenal cortex and capsule [9-12]. Labeled cells are centripetally displaced until they reach the cortical-medullary boundary where they undergo apoptosis. In addition to normal homeostatic proliferation, the adrenal cortex possesses the ability to regenerate and repair itself following injury. As early as the 1930s, experiments conducted with rats demonstrated that when most of the cortex and medulla were removed through enucleation (leaving behind just the capsule and underlying outermost cortical layer), the remaining adrenal components were able to restore the bulk of adrenocortical mass within 6 weeks of engraftment [13]. The regenerative capacity of adrenal cortex is also evident from reconstitution experiments where primary cultures of bovine adrenals are able to reconstitute functional, vascularized, steroid-secreting adrenal tissue when transplanted into adrenalectomized, immunocompromised mice [14, 15].

Finally, the aforementioned pulse-chase and regeneration experiments also support the clonal lineage of differentiated adrenocortical cells from adrenal stem cells. More recent genetic experiments (discussed below) have uncovered a clonal relationship between inner adrenocortical cells and overlying capsular and subcapsular cells (Figure 1.2). This was originally observed over 70 years ago with pulse-chase experiments utilizing trypan blue injections, where labeled capsular cells became evident within the adrenal cortex following a 2-30 day chase period [16]. The clonal lineage of adrenocortical cells is further supported with the use of chimeric transgenic reporter animals that exhibit variegated transgene expression in radial columns throughout the adrenal cortex [17-19].

III. Establishment and Maintenance of the Adrenocortical Stem Cell Niche

A. Overview of adrenal organogenesis (Establishment)

Cells destined to become adrenocortical cells originate from the coelomic epithelium and condense to form a combined adrenogonadal primordium (AGP) around three to four weeks post conception (wpc) in humans and embryonic day 9.0 (e9.0) in mice [20, 21]. This initial step in adrenal development is critically dependent on the expression of *Nr5a1*, which encodes the nuclear receptor Steroidogenic factor 1 (Sf1). Genetic loss of *Nr5a1*, or transcriptional regulators that activate *Nr5a1* expression, such as Pbx1 [22], Wt1 [23, 24], and Cited2 [25, 26], results in complete adrenal agenesis [21]. Establishment of the AGP is followed by its separation into a respective adrenal and gonadal primordium, with subsequent mesenchymal cell encapsulation of the fetal adrenal by 9 wpc or e12.5 in mice. Concomitantly, sympathoadrenal (SA) precursors destined to become the adrenal medulla begin migrating into the fetal adrenal from the neural crest [2, 27]. Following encapsulation, the developing adrenal grows due to the emergence of definitive or 'adult' adrenocortical cells between the fetal adrenal and the capsule, while fetal adrenal cells begin to regress (Figure 1.2). By birth, definitive adrenocortical cells constitute the majority of the cortex, while only a small ring of the fetal adrenal zone remains [2]. This remaining layer of fetal adrenal cells regresses shortly after birth in humans but in mice disappears by puberty in males and the first pregnancy in females.

B. Postnatal Growth and Homeostasis (Maintenance)

Definitive adrenocortical cells emerge during late embryogenesis, with zonation of the adrenal cortex into distinct steroidogenic layers completed in the perinatal period with the onset of zG-specific expression of *Cyp11b2* [28]. In humans and select nonhuman primates, the zR

emerges during adrenarche [29]. Homeostasis of the postnatal adrenal gland is maintained by the balance between proliferation at the outer cortex, centripetal displacement of differentiating cells, and apoptosis of cells at the cortical-medullary boundary. As discussed above, historic studies observed that adult adrenals contain a subset of peripheral cells with continual proliferative potential and the capacity to regenerate adrenal tissue. Revisiting the paradigm of BrdU pulse-chase experiments, recent data from the Morley laboratory established that nascently BrdU-labeled, proliferating cells in the outer adrenal cortex do not colocalize with steroidogenic markers, but become differentiated as they are centripetally displaced [12]. Additionally, a small fraction of label-retaining cells, consistent with a slowly cycling or relatively quiescent stem cell, remains in the capsule and subcapsular cortex 23 weeks post BrdU pulse [12]. These data provide further support that homeostatic adrenal growth is maintained by long-term retained, undifferentiated cells existing in both the capsule and outer adrenal cortex.

C. Cell lineages

The search for adrenocortical stem/progenitor cells has revealed very complex cell lineage relationships in which the precursors for adult adrenocortical cells are capsular cells and fetal adrenal cells that go through a capsular intermediate (Figure 1.2). The data supporting our current understanding of cell lineages during adrenal development and maintenance are discussed below.

1) Fetal adrenal cells are precursors to definitive adrenocortical cells

In addition to the adrenals and gonads, Sf1 expression is found in non-steroidogenic tissues such as the ventromedial hypothalamus [30], pituitary gonadotropes [31] and the spleen [32]. Expression of Sf1 in these organs results from distinct tissue-specific enhancers of the

Nr5a1 gene. Analysis of the transcriptional regulation of *Nr5a1* identified a fetal adrenal enhancer (*FAdE*), whose activity is solely restricted to fetal adrenal cells. *FAdE* expression is initiated by complexes of the homeobox proteins Pbx1, Pknox1, and Hox transcription factors, and later maintained by Sf1 itself [33]. Lineage-tracing experiments utilizing *FAdE*-driven Cre recombinase (*FAdE-Ad4bp-Cre*) revealed that *FAdE*-expressing cells were precursors to most, if not all, Sf1+ cells in the adult adrenal cortex [34]. Interestingly, a temporal window existed in which fetal adrenal cells could serve as precursors for definitive adrenal cells. Using a *FAdE-CreERT2* inducible allele, only *FAdE*-expressing cells marked prior to fetal adrenal encapsulation could serve as precursors for adult adrenal cells. When induced on or after e14.5, *FAdE*-derived adult adrenocortical cells were no longer detected [34]. These data suggest that an initial population of fetal adrenal cells contributes to lineage of adult adrenocortical cells only early in adrenal development.

2) Sf1-negative capsular cells give rise to Sf1-positive definitive adrenocortical cells

Trypan blue lineage tracing experiments discussed above suggested that adrenocortical cells are derived from the overlying Sf1-negative (-) mesenchymal capsule [16]. More recently, data from three independent laboratories studying the Hedgehog (Hh) pathway in the adrenal gland also provided evidence for this phenomenon. In mammals, a family of secreted Hh molecules, Sonic (Shh), Desert (Dhh) and Indian (Ihh), bind to the cell surface receptor Patched1 (Ptch1). In the absence of Hh ligands, Ptch1 inhibits the positive signal transducer Smoothened (Smo), resulting in proteolytic processing of Gli transcription factors to their co-repressor form. Hh binding to Ptch1 relieves inhibition of Smo resulting in downstream signaling that inhibits proteolysis of Gli proteins. Hh target genes, including *Gli1* and *Ptch1*, are induced by active Gli transcription factors and are important for feedback regulation. In the adrenal gland, Shh is

expressed in peripheral Sf1-positive (+) adrenocortical cells from e12.5 onward [35-37] (Figure 1.3). These cells rarely colocalize with steroidogenic markers during development or postnatal life and therefore are relatively undifferentiated [35]. The Shh-receiving cells are contained within an inner layer of Sf1- capsular cells, which express *Smo*, *Ptch1* and *Gli1* [35-37]. Cell lineage analysis with *ROSA26-YFP* reporter mice crossed with mice harboring a Cre recombinase replacing the endogenous *Shh* locus (Shh^{gfpcre}*:R26-YFP*) revealed that postnatally, all cells of the cortex (but not capsule or medulla) were derived from *Shh*-expressing fetal cells [35]. Further experiments utilizing an inducible *Shh*-Cre allele crossed to GFP reporter mice (*Shh CreT2:R26 mR/mG*) showed that shortly after labeling, *Shh*-expressing cells remain restricted to the peripheral cortex [35]. Over time, these cells and their descendents form clonal, radial stripes that extend deep into the cortex and colocalize with steroidogenic cells of both the zG and zF. In addition to *Shh*-expressing cells, the lineage of Gli1+ (Shh-receiving) cells was also investigated. Employing an inducible *Gli1-CreERT2* allele, investigators demonstrated that during adrenal development, Sf1-;Gli1+ capsular cells gave rise to Sf1+;Gli1- undifferentiated Shh+ cells, as well as Sf1+; Gli1- steroidogenic cells [35]. Postnatal lineage tracing experiments unveiled similar results. Collectively, these data suggest that Sf1-;Gli1+ capsular cells serve as precursor/stem cells to Sf1+, Shh+ undifferentiated progenitors and differentiated steroidogenic cells during adrenal organogenesis and postnatal maintenance.

Pathological stress can engage different pools of stem/precursor cells to participate in tissue regeneration in a variety of organ systems [38]. During normal maintenance of the adult cortex, Wt1+ mesenchymal capsular cells can contribute to the steroidogenic lineage, yet only infrequently [39]. However, following gonadectomy, engagement of the Wt1+ capsular population is more robust. Utilizing *Wt1:CreERT2,* $ROSA^{mG/mT}$ mice, cords of GFP+ Wt1

descendents give rise to Wt1-; Sf1+ cells that express the gonadal genes *Cyp17* and luteinizing hormone receptor (*Lhr*) [39]. These data indicate that the capsule contains several populations of multipotent adrenocortical stem/progenitor cells that have the capacity to differentiate into adrenal or gonadal cells in response to differing endocrine stressors.

3) Sf1+ fetal adrenal cells give rise to Sf1-, Gli1+ capsular cells

Data discussed above indicates that fetal adrenal cells expressing *Nr5a1* from *FAdE* and Sf1- capsular cells both serve as precursors to definitive adrenocortical cells. A unifying model integrating these observations would predict that a subpopulation of fetal adrenal cells could contribute to the forming capsule and switch off *Nr5a1* expression [40]. These cells or their descendants would express *Gli1* and give rise to adult adrenocortical cells expressing *Nr5a1* from the definitive enhancer as demonstrated previously [35]. Experimental evidence for this model recently emerged from our laboratory. To investigate whether *FAdE*-expressing fetal adrenal cells gave rise to capsular cells, *FAdE-Ad4bp-Cre* mice (described above) were crossed with *ROSA26-tdTomato/eGFP* reporter mice (*FAdE-Ad4bp-Cre:R26R*$^{mT/mG}$) and transgenic adrenals were evaluated at e18.5 and adulthood. In *FAdE-Ad4bp-Cre:R26R*$^{mT/mG}$ adrenals, GFP+ descendents from FAdE-expressing cells colocalize with steroidogenic cells of the adult adrenal cortex as observed previously [34]. Additionally, a subset of GFP+ cells reside within the Sf1- capsule [41]. When *FAdE-Ad4bp-Cre:R26R*$^{mT/mG}$ mice were bred with *Gli1-LacZ* reporter mice, *FAdE-Ad4bp-Cre:R26R*$^{mT/mG}$*:Gli1-LacZ* adrenals exhibited capsular cells that co-expressed GFP and LacZ [41]. These data collectively demonstrate that descendants from fetal adrenal cells extinguish Sf1 expression and contribute to the population of Sf1-; Gli1+ capsular cells (Figure 1.2). These data unify the seemingly disparate cell lineage relationships of adult adrenocortical precursors.

4) Zonation and lineage conversion of differentiated adrenocortical cells

While definitive adrenocortical cells arise during embryogenesis, zonation of the adrenal cortex into distinct steroidogenic layers occurs perinatally and is relatively static under normal physiological conditions. Several models explain the derivation of differentiated, steroidogenic cells of each layer. Gottschau first proposed the centripetal migration model in which progenitor cells in the peripheral cortex first differentiate into mineralocorticoid-producing cells in the zG and upon centripetal displacement transform into glucocorticoid-producing cells of the zF [42]. Based on the disparate effects of hypophysectomy on the zG and zF, along with the distinct functions of the steroids produced from these zones, a separate model hypothesized that each steroidogenic layer contains its own progenitor pool [43]. H3-T, BrdU and trypan blue pulse-chase experiments support the centripetal migration model, however, none of these studies directly tested lineage conversion of a zG to zF cell. Recently, a series of zG cell lineage tracing experiments provided genetic evidence for the conversion of zG cells into zF cells. In mice that contain Cre recombinase inserted into the *Cyp11b2* locus (AS^{Cre}), Cre is expressed in terminally differentiated cells of the zG [44]. When crossed with $R26R^{mT/mG}$ reporter mice (described above), GFP+ cells appear in the zG the outset of *Cyp11b2* expression and eventually label the entire zG [44]. *Cyp11b1*-expressing cells in the zF (that do not express *Cyp11b2*) become GFP+ over time, indicating these cells underwent lineage conversion from a zG cell. By twelve weeks of age, nearly the entire cortex is GFP+ [44]. This phenomenon also occurs under forced homeostatic maintenance of the cortex, namely regeneration following dexamethasone (dex) induced zF atrophy. Eight weeks following dex withdrawal, $AS^{+/Cre}::R26R^{+/mTmG}$ exhibited GFP+ zF cells [44], indicating lineage conversion of zG to zF still occurs under this homeostatic paradigm.

IV. Factors involved in regulation of adrenal stem cells

A. Transcription factors

1) Sf1.

As introduced above, Sf1 is an essential regulator of adrenal development as well as steroidogenic function. Both the Parker and Morohashi laboratories originally identified Sf1 (also known as Ad4-binding protein, Ad4bp) as the nuclear receptor that activates transcription of the steroidogenic enzymes responsible for catalyzing steroid biosynthesis [45, 46]. Shortly thereafter, the importance of Sf1 in adrenal specification and growth became evident utilizing genetic loss-of-function studies *in vivo*. Sf1 expression initiates at e9.0 in the urogenital ridge, specifying precursors of the steroidogenic lineage and forming the AGP [47]. Mice globally deficient for Sf1 exhibit degeneration of the AGP due to apoptosis between e11.5 and e12.0, resulting in agenesis of the adrenals and gonads [21]. Unlike Sf1 knockout animals, mice heterozygous for Sf1 survive to adulthood yet possess smaller, hypertrophic adrenals that show reduced capacity for corticosterone production when stressed [48]. Similarly, humans heterozygous for Sf1 mutations exhibit primary adrenal failure and gonadal dysgenesis (see [49] for review). In a genetic model where Sf1 overexpression is mediated by the fetal adrenal enhancer of *Nr5a1*, mice exhibit ectopic formation of adrenal tissue throughout the thoracic cavity [50]. Similarly, forced expression of Sf1 in embryonic [51] and mesenchymal stem cells [52] is sufficient to promote steroidogenic cell differentiation *in vitro*. These data collectively demonstrate the importance of Sf1 in specification and differentiation of steroidogenic precursors. Additionally, Sf1 heterozygotes are unable to mount a compensatory growth response following unilateral adrenalectomy due to lack of peripheral adrenal cell proliferation [53]. These data, coupled with studies demonstrating increased subcapsular proliferation in mice

with transgenic overexpression of Sf1 [54], highlight a critical role for Sf1 in the regulation of adrenocortical cell proliferation in addition to differentiation. How does Sf1 mediate the seemingly opposing tasks of proliferation vs. differentiation? Regulation of the transcriptional activity of Sf1 by post-translational modifications and/or stimulatory and inhibitory ligands are posited to dictate what genetic programs are enacted by Sf1. Post translational modification of Sf1 has been extensively studied and reviewed elsewhere [55, 56]. The role of these modifications in homeostatic maintenance is an area of active investigation.

2) Dax1.

Nr0b1 encodes Dax1 (Dosage-sensitive sex reversal, Adrenal Hypoplasia Congenita (AHC) critical region on the X chromosome gene 1), an atypical nuclear receptor (NR) found mutated or deleted in patients afflicted with X-linked AHC. Dax1 lacks a classical NR DNA-binding domain, instead containing three and a half repeated stretches of glycine and alanine rich regions [57]. Initial reports demonstrated that Dax1 functioned as a negative regulator of Sf1-mediated transcription [58] and subsequently these findings were extended to other NRs as well [59]. Dax1 recruits the corepressors NCoR and Alien for transcriptional inhibition [60, 61], however data suggests Dax1 has a dose-dependent coactivator capacity [62]. Dax1 inhibits Sf1-mediated steroidogenesis *in vitro* and *in vivo* [63], but also directly represses *Star* by binding to hairpin structures present in the promoter region [64]. These early studies were performed in cell culture systems and the role of Dax1 *in vivo* remained elusive for some time. It was unclear whether Dax1 exerted its inhibitory actions on steroidogenesis in a differentiated cell or in a progenitor cell where steroidogenesis was uniquely downregulated. Indeed, a number of studies have shown that Dax1 is essential to maintain ES cell pluripotency [65-68]. In the adult adrenal cortex, Dax1 is critical for maintenance of progenitor cells as Dax1 deficient mice display

worsening adrenal dysplasia with age [69]. When compared to wildtype littermates, Dax1 knockout mice exhibit enhanced steroidogenesis and proliferation at early ages, with progressive loss of proliferating cells over time [69]. These data suggest that without Dax1, adrenal progenitors precociously differentiate leading to their continued depletion, ultimately manifesting in adrenal failure in these animals.

B. Paracrine signaling pathways

1) Wnt Pathway.

The mammalian Wingless-Type MMTV Integration Site (Wnt) pathway is a paracrine signaling pathway critically involved in development and stem cell maintenance in multiple organ systems [70]. Secreted Wnt molecules bind to a variety of receptors (including Frizzleds (Fzd), RORs and RYKs) to elicit distinct downstream signaling through the canonical/beta catenin (βcat) pathway and/or the noncanonical/planar cell polarity and calcium signaling pathways [71]. For activation of the canonical pathway, Wnts bind to Fzd receptors resulting in the inactivation of multi-protein complex that promotes the destruction of the transcription factor βcat. Once freed from the destruction complex, βcat enters the nucleus and transactivates Wnt-responsive genes through interaction with DNA-bound TCF/LEF transcriptional regulators. The developing adrenal exhibits βcat expression as early as e12.5 [72]. Employing transgenic reporter mice, active canonical Wnt/βcat signaling is evident in a few scattered cells in the periphery of the gland at this time. By e18.5, Wnt/βcat signaling is more broadly restricted to the outer subcapsular region of the cortex [72]. To ablate βcat expression in adrenocortical cells, Kim et al. crossed *Ctnnb1*^{tm2kem} mice that have floxed βcat alleles with *Sf1/Cre*^{high} mice, which contain five copies of the Sf1/Cre transgene resulting in high Cre expression in Sf1+ cells [72].

Adrenal development initiates in the absence of βcat, however, adrenocortical cells exhibit a remarkable loss of proliferation between e12.5-e14.5 with disappearance of the entire gland by e18.5 [72]. *Sf1/Cre^{low}* mice only contain one copy of the Sf1/Cre transgene and exhibit inefficient recombination. In *Sf1/Cre^{low}:Ctnnb1^{tm2kem}* mice, a subset of cells escapes recombination leading to an overall 50% decrease in βcat-mediated Wnt signaling [72]. *Sf1/Cre^{low}:Ctnnb1^{tm2kem}* adrenals develop relatively normally until 15 weeks of age, where after progressive thinning of the adrenal cortex, marked by loss of Sf1+ cells, results in adrenal failure by 45 weeks of age [72]. It is interesting to note that *Nr0b1*/Dax1 (discussed above) is a βcat target gene and Dax1 deficient adrenals in part phenocopy *Sf1/Cre^{low}:Ctnnb1^{tm2kem}* adrenals [69]. Data emerging from the Val laboratory suggests βcat contributes to differentiation of zG cells through the indirect regulation of aldosterone production [73]. Collectively, these data support a role for Wnt/βcat signaling in maintenance and commitment of the adrenal progenitor pool.

The Wnt4 ligand is expressed in the developing adrenal as early as e11.5, with restricted expression in the periphery of the cortex observable by e14.5 [5]. It remains unclear whether Wnt4 expression is a cause or consequence of the activated canonical Wnt pathway, as it has been shown to be genetically downstream of βcat in other tissues [74, 75]. In humans, *Wnt4* mutations result in SERKAL syndrome, an autosomal-recessive disorder that includes female sex reversal and dysgenesis of the kidneys, lungs and adrenal glands [76]. SERKAL-afflicted fetuses exhibit reduced adrenal size but relatively normal histology [76]. In contrast, mice globally deficient for *Wnt4* have relatively similar adrenal size and morphology during development, but exhibit reduced *Cyp11b2* expression at birth resulting in less aldosterone production [5]. *Wnt4* knockout mice also contain ectopic adrenocortical cells at the anterior tips of the gonads, the

region closest in proximity to the forming adrenal glands. Given the shared lineage between the adrenal and gonad, these data suggest that Wnt4 regulates adrenal cell homing or specification during separation of the AGP in early adrenal development [5, 77].

2) Shh Pathway.

Shh signaling is implicated in developmental patterning and stem cell biology in many tissues, from flies to humans [78]. Evidence for the importance of Shh signaling in adrenal development arose from the discovery that frameshift mutations that result in truncation of Gli3 into a constitutive repressor are the underlying cause of Pallister Hall syndrome (PHS). PHS is characterized by a constellation of congenital anomalies including polydactyly, hypothalamic hamartoma, kidney abnormalities and adrenal insufficiency. A genetic mouse model of PHS utilizing a truncated Gli3Δ699 allele has lead to discrepant findings; one study reported these mice displayed adrenal aplasia [79], while Laufer et al. found no adrenal malformations [80]. Nonetheless, recent reports have highlighted a critical role for the Shh in adrenocortical progenitor biology (see above). Mice with global *Shh* ablation, as well as conditional ablation of *Shh* in steroidogenic cells, exhibit hypoplastic adrenals embryonically due to reduced cortical and capsular cell proliferation [36, 37]. Adult *Sf1/Cre:Shh*$^{fl/-}$ exhibit hypertrophic cells yet retain the capacity for steroid secretion [37]. Despite smaller adrenal volume in *Shh* deficient mice, adrenal histology and organization is comparable to wildtype counterparts, indicating adrenocortical encapsulation and zonation, as well as migration of medullary cells, does not rely on Shh signaling. However, *Sf1/Cre:Shh*$^{fl/-}$ embryonic and adult adrenals possess markedly thinner capsules compared to wildtype [35, 37]. Coupled with the observation that *Shh* deficient mice have reduced capsular proliferation, these data indicate Shh is may be a regulator of mitogenic signaling for capsular stem/progenitor cells.

3) FGF Signaling.

The complexity of the fibroblast growth factor (FGF) signaling pathway is due to the diversity of ligands, receptors and their isoforms, as well as the intracellular signaling cascades that participate in FGF signal transduction. Binding of FGFs to FGF receptor tyrosine kinases (FGFRs) is facilitated by transmembrane heparan sulfate proteoglycans (HSPG). Ligand binding promotes FGFR dimerization and autophosphorylation, which can elicit multiple signaling pathways downstream, including JAK/STAT, PLCγ, PI3K and Erk pathways [81]. Both the adrenal capsule and cortex express components of the FGF pathway (Figure 1.3). FGF2 and FGF9 expressed in the capsule signal to FGFR1 IIIc and FGFR2 IIIc expressed in both the cortex and capsule [82, 83]. FGF1 secreted from the cortex is thought to elicit signaling from FGFR1 IIIc, FGFR2 IIIb and IIIc expressed in both compartments [82, 83]. Ablation of FGFR2 in the steroidogenic cell lineage results in adrenal hypoplasia observable by e15.5 [84]. The effects of FGFR2 deletion are predicted to be solely due to loss of the IIIb isoform as global deletion of FGFR2 IIIb results in embryonic adrenal hypoplasia [82, 85], whereas FGFR2 IIIc knockout mice have no discernable adrenal phenotype at postnatal day 2 [86]. Adrenal hypoplasia evident in FGFR IIIb deficient mice results from reduced cortical proliferation during embryogenesis [82], demonstrating a requirement for mitogenic FGF signaling for proper adrenal development. FGFs also exert mitogenic effects during postnatal adrenal maintenance. FGF2 elicits proliferation in both primary adrenocortical cultures and tumor cell lines [87, 88]. Adrenal re-growth under the paradigms of reconstitution by primary culture or compensatory growth following unilateral adrenalectomy is critically dependent on the pro-proliferative and angiogenic properties of FGFs [15, 89, 90].

4) TGFβ signaling and Inhibin.

The transforming growth factor beta (TGFβ) signaling pathway elicits a wide variety of cellular processes and is involved in maintenance and differentiation of stem cells [91]. The TGFβ superfamily consists of a diverse array of ligands, including (but not limited to) bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and inhibins. Ligand binding to TGFβ type II receptors promotes phosphorylation of type I receptors and results in phosphorylation and nuclear translocation of Smad proteins that modulate transcription of target genes. Activins and inhibins are present throughout the fetal and adult adrenal cortex [92] and profoundly influence adrenocortical differentiation. Activins augment Adrenocorticotropic hormone (ACTH)-induced steroidogenesis in fetal adrenal cells and stimulate Angiotensin II (AII) and ACTH-induced aldosterone production in adult adrenal cells [92, 93]. Unlike activins, it is unclear if inhibins directly affect adrenal steroidogenesis [94]; however, Inhibin-α (Inhα) has been demonstrated to greatly impact adrenal progenitor biology. Following gonadectomy, *Inhα* deficient mice develop adrenocortical tumors [95] dependent on high levels of circulating luteinizing hormone (LH) [96]. Loss of *Inhα* results in constitutive Smad3 activation with subsequent formation of neoplastic cells in the adrenal that express ovarian theca and granulosa cell markers, LH receptor (Lhr) and anti-Mullerian hormone (Amh), respectively [97]. Ectopic ovarian differentiation of adrenal cells results from the LH-induced switch from adrenal-specific Gata6 expression to gonadal-restricted Gata4 expression only in the context of *Inhα* deficiency [97]. Altogether, these data establish a role for Inhibin-α in the maintenance the adrenal fate of adrenocortical progenitor cells.

5) IGF Signaling.

The insulin-like growth factor (IGF) signaling pathway has been implicated in both the growth and differentiation of the adrenal cortex. The IGF family consists of two ligands structurally similar to proinsulin, IGF1 and IGF2. These secreted proteins interact with IGF1-receptor (IGF1R), a receptor tyrosine kinase structurally similar to the insulin receptor, to elicit pro-growth and survival signaling through MAPK and/or PI3K/Akt pathways [98]. IGF2R is distinct from IGF1R; it does not contain a tyrosine kinase domain and acts as a molecular sink to restrict the bioavailability of IGF2 [98]. Six IGF-binding proteins (IGFBPs) bind to and exert stimulatory or inhibitory effects on IGFs, however their actions in the adrenal are poorly understood. All components of the IGF pathway are expressed throughout the adrenal across mammalian species [2] (Figure 1.3). IGFs exert strong mitogenic effects on fetal and adult adrenal cells [99, 100]. IGF2 is highly expressed in the fetal adrenal however in the adult adrenal IGF2 expression is decreased and equivalent with IGF1 levels [98]. IGFRs are expressed throughout all zones of the human adrenal cortex, with enrichment of IGF1R in the subcapsular region [101]. Mice deficient for both the insulin receptor (Insr) and Igf1r exhibit gonadal dysgenesis, male-to-female sex reversal and adrenal aplasia [102]. Adrenal agenesis is evident by e16.5 and is likely due to global loss of cell proliferation throughout the genital ridge and AGP as early as e10.5 [102]. Insr, Igf1r double knockout mice also contain almost half the number of Sf1+ cells in the AGP at e.11.5, as well as reduced *Nr5a1* expression. These data collectively indicate that IGF signaling is a potent adrenal mitogen and is critical for adrenal specification early in development. Additionally, IGFs augment basal and ACTH-induced steroidogenesis in both fetal and adult adrenal cells *in vitro* [103], suggesting a role for IGF signaling in adrenocortical differentiation in addition to adrenal proliferation.

C. Endocrine Signaling

The peptide hormones AII and ACTH mediate different aspects of endocrine homeostasis through regulation of adrenocortical steroidogenesis and differentiation (Figure 1.3). Under the control of RAAS, AII promotes aldosterone production through activation of phospholipase C (PLC), calcium signaling and protein kinase C (PKC) (see [104] for in-depth review). In contrast, proopiomelanocortin (POMC)-derived ACTH, under regulation by the HPA axis, induces glucocorticoid synthesis by stimulating cyclic-AMP/Protein kinase A (PKA) and MAPK/Erk signal transduction pathways (for in depth review, see [105]). Mice deficient for AII type 1 receptors (*Agtr1a*-/-; *Agtr1b*-/-), the ACTH receptor (*Mc2r)* or *Pomc* exhibit normal adrenal histology at birth [106-108], indicating AII and ACTH are dispensable for normal fetal adrenal development. However, these endocrine signals profoundly influence adrenocortical remodeling and maintenance during adult life. Activation of the RAAS and the HPA axis affect adrenal architecture similarly; initial differentiation and hypertrophy of steroidogenic cells occurs acutely, with chronic stimulation resulting in increased proliferation through mechanisms that are not well established [109]. Rats subjected to a low salt diet exhibited hypertrophic cells that had increased *Cyp11b2* expression in the zG over the initial 3 days, followed by hyperplasia peaking 3-5 days after commencement of diet [110]. It is predicted the increase in Cyp11b2+ cells prior to proliferation is the direct result of differentiation of *Shh*-expressing cells into zG cells (see above), followed by proliferation for progenitor replacement. In accord with this notion, rats subsisting on a low salt diet for 3 days gained *Cyp11b2*-expressing cells without induction of proliferation, commensurate with a ~50% reduction in *Shh* expression [111]. Additionally, the bulk of replicating cells under chronic RAAS or ACTH stimulation initially reside within the undifferentiated zU before extending to the other zones [110, 112]. Conflicting data exist on whether ACTH is mitogenic in itself [113]. Rather, it has been suggested that ACTH stimulates expression of growth factors, such as IGF2 [114, 115].

V. Summary

While a number of signaling pathways have been shown to participate in the growth and homeostatic maintenance of the adrenal cortex, it is unclear how paracrine and endocrine signals are spatially and temporally integrated. In this thesis, the role of the Wnt/βcat pathway in regulation of adrenal progenitor biology is explored. In chapter two, characterization the Wnt-responsive population *in vivo* finds that Wnt-responsive cells are heterogeneous, supporting the notion that Wnt signaling plays diverse roles in the adrenal cortex. Utilizing an adrenocortical cell model, I identified a novel aspect of the relationship between βcat and Sf1, wherein βcat inhibits Sf1-mediated steroidogenesis. Gene expression profiling *in vitro* and *in vivo* uncovered novel genes regulated by the Wnt/βcat pathway in the adrenal cortex. One such gene, *Ccdc80*, encodes the secreted factor that also participates in suppression of zF cell steroidogenesis. These data implicate Wnt/βcat signaling in the promotion of unidirectional differentiation of adrenocortical progenitors into zG cells, and not zF cells. Collectively, this dissertation provides mechanistic insight into how Wnt signaling contributes to adrenal homeostasis.

FIGURES

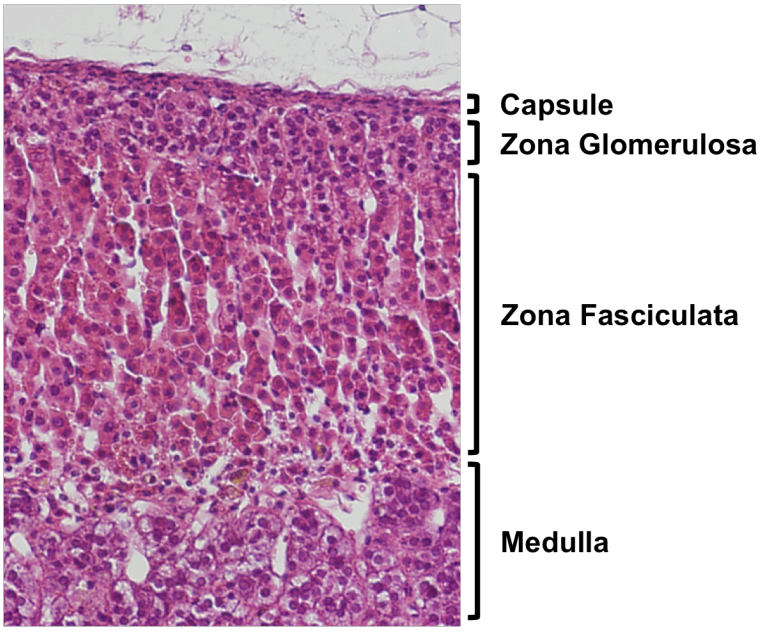

Figure 1.1: Histology of the mouse adrenal.

Transverse section of a wildtype adult mouse adrenal stained with hematoxylin and eosin.

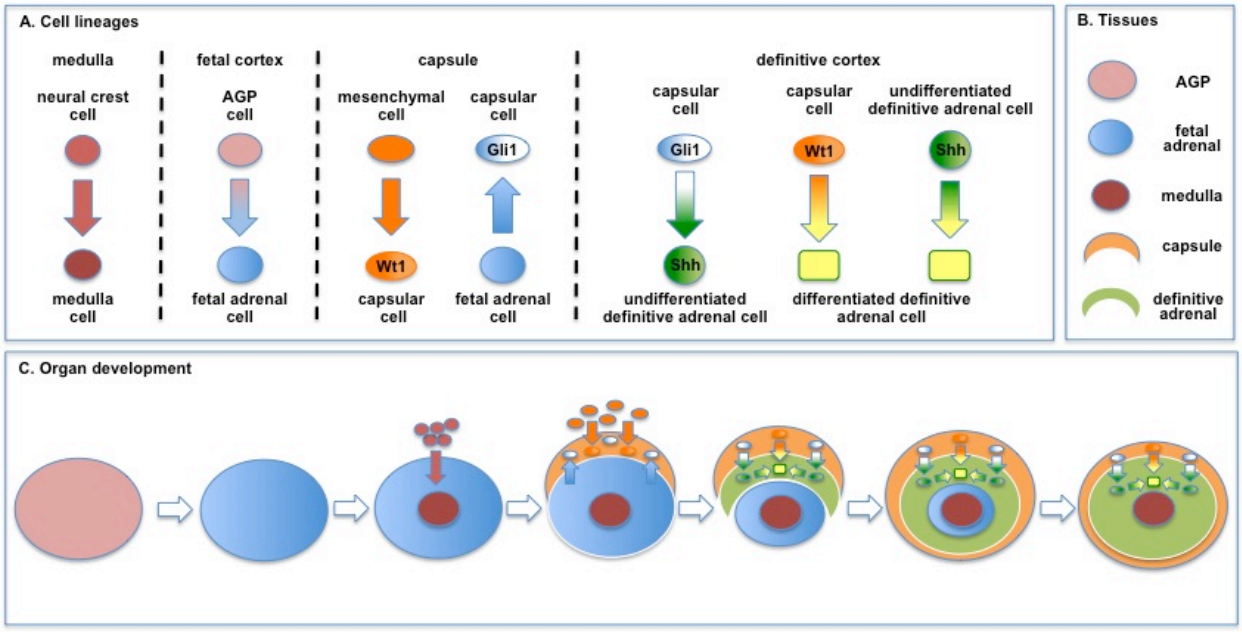

Figure 1.2: Cell lineages in adrenal development and homeostasis.

(A) Multiple cell lineages contribute to the adrenal gland. Neural crest cells (red) are precursors to catecholamine-secreting cells of the adrenal medulla. Wt1-expressing mesenchymal cells contribute to the adrenal capsule (orange). Fetal adrenal cells (blue) give rise to a subpopulation of capsular cells that express Gli1 (blue/white). *Gli1*-expressing capsular cells give rise to undifferentiated Shh-producing cells (green) and differentiated steroidogenic adrenocortical cells (yellow) of the adult gland. Shh-producing cells give rise to and/or differentiate into steroidogenic cells of the definitive cortex. Wt1-expressing cells (orange) give rise to differentiated cells of adult cortex that no longer express Wt1. (B) The tissues represented in C. (C) Adrenal organogenesis begins when adrenogonadal primordia (AGP) form and separate into fetal adrenals and fetal gonads. Cells from the neural crest infiltrate the fetal adrenal gland to form the medulla, while mesenchymal cells participate in encapsulation. Fetal adrenal cells give rise to a portion of adrenal capsule. As the definitive cortex replaces the fetal cortex, *Gli1*-expressing capsular cells serve as precursors to *Shh*-expressing progenitors and steroidogenic adrenocortical cells. Wt1+ cells also contribute to the cortex, albeit infrequently. Upon completion of development, the capsular and subcapsular progenitor cells are retained throughout adulthood and maintain homeostasis of the definitive adrenal cortex. Adapted from [41].

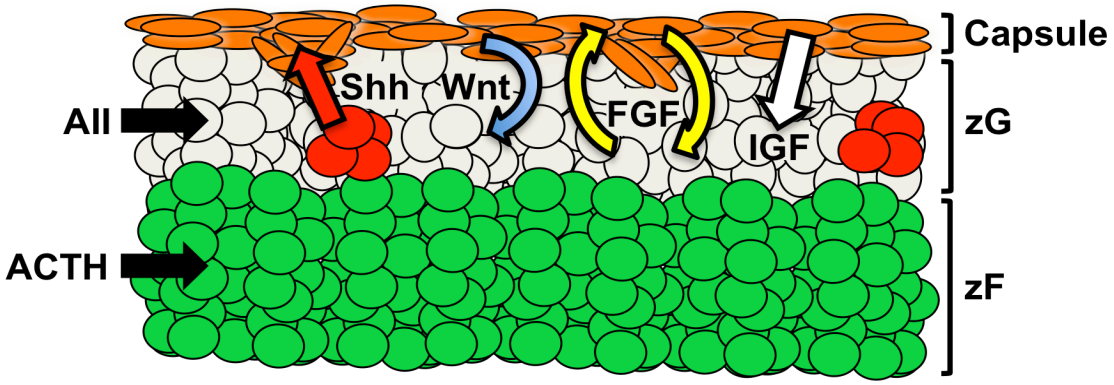

Figure 1.3: Signaling pathways that participate in various aspects of adrenal biology.

AII and ACTH are endocrine signals (black arrows) that stimulate steroidogenesis in differentiated cells of the zG (grey) and zF (green), respectively. Undifferentiated progenitor cells (red) secrete Shh ligands (red arrow) that act on cells within the adrenal capsule (orange). Wnt ligands (blue arrow) are received by cells of the zG, are critical for maintenance of adrenocortical progenitors, and indirectly regulate aldosterone production. FGFs (yellow arrows) are secreted from and received by both the capsule and cortex and may be involved in regulation of proliferation. During adulthood, IGFs are expressed in the capsule, act on the cortex to promote proliferation, and may participate in steroidogenesis.

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CHAPTER II: WNT SIGNALING INHIBITS ADRENOCORTICAL STEROIDOGENESIS BY CELL-AUTONOMOUS AND NON-CELL-AUTONOMOUS MECHANISMS

INTRODUCTION

The adrenal cortex synthesizes and secretes steroid hormones necessary for life. The adrenal is zonated into distinct steroidogenic layers responsible for the production of steroids under the control of differing physiological stimuli. In the outer most subcapsular layer, the zona glomerulosa (zG), mineralocorticoids such as aldosterone are produced under the control of the renin-angiotensin system. The inner zona fasciculata (zF) produces glucocorticoids, such as cortisol in humans and corticosterone in mice that coordinate the mammalian stress response regulated by the hypothalamic-pituitary-adrenal (HPA) axis.

The static adrenocortical zonation is intriguing given the exceptionally dynamic nature of the adrenal cortex; cells proliferate at its periphery, differentiate into steroidogenic cells that are displaced centripetally until they eventually turnover at the adrenocortical-medullary boundary. Cell turnover requires replenishment of adrenocortical cells from a somatic stem/progenitor cell pool for maintenance of adrenal tissue. Historically, it has been debated whether each steroidogenic zone contains its own progenitor cell, or whether a common adrenocortical progenitor within the periphery of the cortex changes its fate as it is centripetally displaced throughout life. In recent years, several studies have provided evidence for the latter. Work by King et al. demonstrated that Shh-producing cells that reside within the subcapsular region serve

as a common progenitor cell for steroidogenic lineages. Using lineage tracing, relatively undifferentiated Shh-producing cells become steroidogenic cells of both adrenocortical zones over time [1]. Additionally, utilizing an Aldosterone Synthase-Cre recombinase in cell lineage tracing experiments of zG cells, Freedman et al. demonstrated that under normal conditions all zF cells are derived from zG cells [2]. Collectively, these data suggest unidirectional differentiation of adrenocortical progenitors first to zG cells that undergo conversion into zF cells as they transit through the cortex. However, how progenitor cells are maintained in the subcapsular region, as well as how unidirectional differentiation is regulated is not well understood.

Canonical Wnt signaling is a paracrine signaling pathway implicated in progenitor cell biology in a variety of organ systems, including the adrenal cortex. In the absence of Wnt ligands, the pathway is inactive, resulting from sequestration of the transcriptional regulator beta catenin (βcat) into cytoplasmic destruction complexes composed of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase 1α (CKIα) and other components. Phosphorylation of βcat by GSK3β and CKIα results in its ubiquitination by β-transductin-repeat-containing protein (β-TrCP) and subsequent degradation by the proteasome. Wnt ligands binding to Frizzled (Fzd) receptors with Lrp5/6 co-receptors results in the inhibition of the destruction complex, leading to βcat stabilization and nuclear translocation. βcat then activates Wnt-responsive genes by binding to TCF/LEF transcription factors at gene enhancers. The Wnt/βcat pathway has been previously implicated in the homeostatic maintenance of the adrenal gland [3]. Partial loss-of-function of βcat in the adrenal cortex leads to depletion of adrenocortical cells upon aging, highlighting a critical role for βcat in maintenance of the progenitor cells in the adrenal cortex [3]. Wnt signaling maintains adrenocortical progenitors in

part through the activation of the βcat target gene, *Nr0b1*, dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1 (Dax1) [4]. Dax1 is an orphan nuclear receptor implicated in maintaining the pluripotency of embryonic stem cells [5]. Dax1 knockout adrenals display enhanced steroidogenesis in early ages with adrenocortical failure at late ages [6], consistent with the precocious differentiation of adrenocortical progenitors in the absence of Dax1 resulting in premature depletion of the progenitor cell pool. Interestingly, Wnt signaling has also recently been implicated in zG cell differentiation. A study by Berthon et al. indicates that βcat indirectly promotes aldosterone production by increasing expression of the Angiotensin receptor (At1r) and Nr4a1/2 genes, which stimulate the expression of Cyp11b2, the terminal enzyme in aldosterone biosynthesis [7]. It remains unclear whether Wnt signaling directly influences zF cell differentiation.

In this study, we report that Wnt-responsive cells colocalize with markers of adrenocortical progenitors and differentiated cells of the zG but not the zF. Stimulation of βcat activity *in vitro* inhibits zF cell differentiation by suppressing steroidogenesis through Sf1-dependent mechanisms as well as through the induction of a new βcat-regulated gene, *Ccdc80*. Our study provides insight into how Wnt signaling maintains the undifferentiated state of adrenal progenitor cells through inhibition of steroidogenesis. Additionally, our data indicate that βcat inhibits the zF cell phenotype, supporting a role for Wnt signaling in the unidirectional differentiation of adrenal progenitors into zG cells.

MATERIALS AND METHODS

Mice

All animal experiments were carried out in accordance with protocols approved by the University Committee on Use and Care of Animals at the University of Michigan. Reporter mice used in these studies have been described elsewhere. TCF/Lef:H2B-GFP mice [8] were obtained from JAX laboratories. Shh^{LacZ} mice [9] were obtained from the laboratory of Andrzej Dlugosz at the University of Michigan. Sf1:eGFP mice [10] were obtained from the laboratory of the late Keith Parker.

Immunofluorescence

Adrenals from 6 week old male Tcf/Lef:H2B-GFP; Shh^{LacZ} mice were fixed for 4 h with 4% paraformaldehyde (PFA), dehydrated in graded ethanol solutions and paraffin-embedded. For BrdU labeling *in vivo*, mice were intraperitoneally injected with BrdU Labeling Reagent (Life Technologies, Carlsbad, CA) 1mL/100g body weight, sacrificed 2 h later and processed as above. For immunofluorescence analyses, adrenal sections (6 μm) were boiled in 10 mM citric acid (pH 2 or pH 6) for 10 min followed by 20 min cooling for antigen retrieval. Slides were washed with phosphate-buffered saline (PBS) and blocked with 2% non-fat dry milk in PBS with 5% goat serum (Thermo Scientific, Wilmington, DE) for 1 h followed by primary antibody incubation at 4°C overnight. Slides were washed with tris-buffered saline with 0.1% Tween (TBS-T), incubated with secondary antibodies for 1 h at room temperature followed by nuclear counterstaining with DAPI and mounted using ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA). Slides were cured overnight and fluorescence microscopy was performed on a Zeiss ApoTome microscope utilizing an AxioCam MRm (Carl Zeiss, Thornwood, NY). Primary antibodies: rabbit α-GFP 1:200 (Life Technologies, Carlsbad, CA),

chicken α-LacZ 1:1000 (Abcam, Cambridge, MA), mouse α-PCNA 1:500 (Santa Cruz Biotechnology, Inc., Dallas, TX), sheep α-BrdU 1:230 (Abcam, Cambridge, MA), mouse α-Cyp11b1 and mouse α-Cyp11b2 1:50 (generous gifts from Dr. C. Gomez-Sanchez) and chicken α-GFP 1:1500 (Abcam, Cambridge, MA). Secondary antibodies: goat α-rabbit Alexa Fluor 488, goat α-mouse Dylight 488, donkey α-chicken Dylight 488**,** goat α-chicken Dylight 549, donkey α-sheep Cy3, goat α-mouse Dylight 649, donkey α-rabbit Dylight 649 - all used at 1:800 (all obtained from Jackson ImmunoResearch, West Grove, PA). For quantification of immunofluorescence data, a total cell count per population (GFP+, LacZ+ or PCNA+) was obtained from 3 separate adrenal sections corresponding to distinct adrenal regions. The number of double-positive GFP+/LacZ+, GFP+/PCNA+ or LacZ+/PCNA+ cells was divided by the total number of GFP+, LacZ+ or PCNA+ cells. Data were averaged from 7 different animals.

Cell culture and Reagents

ATCL7 and 293T cells were grown under standard conditions at 37°C with 5% CO_2. Growth medium for ATCL7 cells was DMEM:F12 containing 2.5% fetal bovine serum (FBS), 2.5% horse serum (HS), 100U/mL pencillin/streptomycin (P/S), 1% Insulin-Transferrin-Selenium X (all obtained from Life Technologies, Carlsbad, CA). For treatment experiments, cells were grown in low serum media (same as normal growth media except with 0.025% FBS and 0.025% HS). 293T cells were grown in DMEM with 10% FBS and 100U/mL P/S. 6-bromoindirubin-3′-oxime (BIO), ACTH (human, rat N-terminal fragment 1-24), and DMSO were obtained from Sigma-Aldrich (St Louis, MO).

Immunocytochemistry

ATCL7 cells were plated at a density of 10^5 cells per well on chamber slides (Lab-Tek). After 24 h, cells were treated with 0.5 μM BIO or DMSO overnight and fixed for 15 min with 4% PFA followed by PBS washes and permeabilization with 0.1% IGEPAL for 3 min at room temperature. Cells were blocked using 2% non-fat dry milk in PBS with 5% normal goat serum (Thermo Scientific, Wilmington, DE) for 1 h, followed by overnight incubation with rabbit α-βcat 1:500 (Santa Cruz Biotechnologies, Dallas, TX) at 4°C. Slides were washed with PBS, incubated with goat α-rabbit Alexa Fluor 488 secondary antibody 1:800 (Jackson ImmunoLabs, West Grove, PA) for 1 h at room temperature, followed by nuclear counterstaining with DAPI and mounted using ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA). Fluorescence microscopy was performed using an Optiphot-2 microscope (Nikon, Melville, NY) with an Olympus DP-70 camera and software (Olympus, Hauppauge, NY).

ATCL7 cell treatments and electroporation

For BIO experiments, cells were plated at a density of $2.5x10^5$ cells per well in 6 well dishes and treated with 0.5 μM BIO for 12 h, followed by 100 nM ACTH stimulation for 6 h. For conditioned media experiments, cells were plated as above and treated with conditioned media for 18 h, followed by 100 nM ACTH stimulation for 6 h. For electroporation of ATCL7 cells, an Amaxa Cell Line Nucleofector R Kit (Lonza, Walkersville, MD) was used following the manufacturer's protocol. Briefly, 10^6 cells per transfection were trypsinized, resuspended in solution R with supplement 1 and mixed with 1 μg pcDNA 3.1 empty vector, 1 μg βcatS33Y and/or 1 μg dnTCF4E expression constructs. Cell mixtures were electroporated using program T-20, plated in 4 wells of a 12 well dish and harvested 24 h later.

Cell fractionation and Immunoblotting

For whole cell lysates, cells were harvested with RIPA buffer (Promega, Madison, WI) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO) and PhosSTOP (Roche, Indianapolis, IN), rocked on ice for 20 min, scraped and sonicated briefly. For immunoblotting, 1/10 of cell lysates (20ug) were subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) 1:1 with PBS. Primary antibodies were diluted in TBS-T with 5% bovine serum albumin (BSA) (Roche, Indianapolis, IN) and incubated at 4°C overnight. Primary antibodies: rabbit α-Sf1 1:1000 (custom), mouse α-Active βcat 1:500 (EMD Millipore, Billerica, MA), rabbit α-βcat 1:2000 (Santa Cruz Biotechnologies Inc., Dallas, TX), mouse α-β-actin 1:5000, mouse α-FlagM2 1:2000 (Sigma-Aldrich, St. Louis), rabbit α-myc 1:1000 (Bethyl Laboratories, Inc., Montgomery, TX), rabbit α-p-Erk1/2 (Thr202/Tyr204), rabbit α-Erk1/2, rabbit α-p-Creb (Ser133) and rabbit α-Creb were all used at 1:1000 (all obtained from Cell Signaling, Danvers, MA). Cell fractionation was achieved following the nuclear extraction protocol utilizing the Nuclear Extract Kit (Active Motif, Carlsbad, CA). For immunoblotting, equivalent proportions of each fraction were analyzed.

RNA extraction and qRT-PCR

Following specified treatments, cells were harvested using the RNeasy Mini Plus Kit (Qiagen, Valencia, CA). 1 ug of RNA was converted to cDNA utilizing the iScript Supermix Reverse Transcriptase Kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Quantitative real-time PCR was carried out using 10 ng of cDNA, 1 μM of specific primers **(see Table 1)** and Power SYBR Green reagent (Applied Biosystems, Foster City, CA) on an ABI

7300 thermocycler (Applied Biosystems, Foster City, CA). Gene expression was normalized to Rplp0 mRNA abundance and calculated using the ΔΔCt method.

Chromatin immunoprecipitation

ATCL7 cells were plated at a density of $6x10^6$ cells per 150 mm dish per treatment for 12 h. Cells were treated with 0.5 μM BIO or DMSO in low serum media for 12 h followed by stimulation with 100 nM ACTH for 6 h. Cells were cross-linked with 1% formaldehyde with rocking at room temperature for 10 min. The reaction was quenched by incubation with 0.125 M glycine with rocking at room temperature for 10 min. Cells were washed three times with ice-cold PBS. Cell membranes were disrupted for 15 min at 4°C in nuclear extraction buffer containing 25 mM HEPES (pH 7.9); 5 mM KCl; 0.5 mM $MgCl_2$; 1 mM DTT; 1% IGEPAL and protease inhibitors (Sigma-Aldrich, St. Louis, MO). Nuclei were isolated from cytoplasmic protein by centrifugation at 1,000xg for 5 min at 4°C. Nuclei were lysed in a buffer containing 1% SDS; 10 mM EDTA; and 50 mM Tris-HCl (pH 8.1) for 10 min on ice**.** Lysates were sonicated at 100% amplitude, with 20 x 30 sec pulses using a Q500 sonicator with a 3" cup horn (Qsonica, Llc., Newtown, CT) to shear genomic DNA between 200 – 400bp. Sonicated lysates were cleared by centrifugation at 14,000xg for 10 min at 4°C and diluted 1:10 in a ChIP dilution buffer containing 0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl (pH 8.1); and 167 mM NaCl. The diluted lysates were pre-cleared for 1 h at 4°C with Protein G Dynabeads (Life Technologies, Carlsbad, CA) prepared in ChIP dilution buffer with 200 μg/mL BSA and 200 μg/mL glycogen (both from Roche, Indianapolis, IN). 30 μg of chromatin per treatment was immunoprecipitated overnight at 4°C using 3 μg of Sf1 antibodies (custom) or rabbit IgG (Santa Cruz Biotechnologies, Inc., Dallas, TX). Immunoprecipitates were recovered for 1 h at 4°C with Protein G Dynabeads as prepared above**.** Precipitates were washed for 5 min

each with the following buffers: low salt buffer [0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl (pH 8.1); and 150 mM NaCl], high-salt buffer [0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl (pH 8.1); and 500 mM NaCl], LiCl buffer [(0.25 M LiCl; 1% IGEPAL; 1% deoxycholate; 1 mM EDTA; and 10 mM Tris-HCl, (pH 8.1)] and Tris-EDTA (pH 8.0) buffer. Immunoprecipitates were eluted from Dynabeads with two 15 min incubations in elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and 1 μg RNase A (Roche, Indianapolis, IN). Protein was digested by incubation for 30 min at 65°C in the presence of 10 mM EDTA, 40 mM Tris-HCl (pH 6.5) and 40 μg Proteinase K (Roche, Indianapolis, IN). DNA fragments were purified using a PCR Purification Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Purified DNA fragments were analyzed by qPCR on an ABI 7300 thermocycler (Applied Biosystems, Foster City, CA) using Power SYBR Green reagent (Applied Biosystems, Foster City, CA). Primer pairs used for ChIP-qPCR are listed in Table 2.

Corticosterone Measurement

Experimental media from treated cells was collected at time of cell harvest and stored at -20°C. The corticosterone content of the media samples was determined using a Mouse and Rat Corticosterone ELISA Kit (ALPCO Diagnostics, Salem, NH). Assays were conducted following the manufacturer's instructions except standard curves were prepared in the cell culture medium. Protein concentration of corresponding cell lysates was assessed using a BCA Assay Kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Corticosterone abundance was normalized to protein concentration and shown as fold change over Vehicle treated samples.

Adrenal Digestion and Cell Sorting

10 adrenals per genotype per sort were minced and digested by incubation in DMEM:F12 containing 0.1% collagenase (Roche, Indianapolis, IN), 0.01% DNaseI (Roche, Indianapolis, IN) for 1 h at 37°C. A single cell suspension was obtained following mechanical dispersion, filtration through a 40 μm nylon cell strainer, centrifugation at 320xg for 5 min followed by re-suspension in sterile PBS containing 10% cosmic calf serum and 10 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO). 10,000 – 50,000 viable GFP positive cells were isolated via FACS using a FACSAria III cell sorter (BD Biosciences, San Jose, CA). Cells were sorted directly into RLT buffer and RNA was harvested using an RNeasy Micro Kit (Qiagen, Valencia, CA).

Microarray analysis

We measured cell transcript abundances in two experiments using Affymetrix Mouse gene ST 1.1 strip arrays with 28944 probe-sets. In both cases RMA algorithms were used to measure log2 transformed transcript abundances. In the *in vivo* experiment, RNA from 4 independent sorts per genotype was used. cDNA were prepared according to the NuGen WT-Pico V2 kit protocol from 5 ng total RNA (NuGEN, San Carlos, CA). Biotinylated single-stranded cDNA were prepared from 3ug of cDNA using the Encore Biotin Module (NuGEN, San Carlos, CA). We used two-sample T-test to compare the two conditions in this data-set. One TCF/Lef:H2B-GFP sample was discarded for quality control reasons. For the *in vitro* experiment, RNA from 4 pairs of DMSO or 0.5 μM BIO treated ATCL7 cell preparations was used. Biotinylated cDNA samples were prepared using an Ambion WT Expression Kit from 250 ng total RNA (Affymetrix, Santa Clara, CA). The data was modeled using paired T-tests for each probe-set. The array data and statistical analysis have been deposited in NCBI's Gene Expression

Omnibus (GEO) [11] and are accessible through GEO Series accession numbers GSE53971 and GSE53981.

Plasmid Constructs and Mutagenesis

For the Ccdc80-luciferase construct, -1957 to +814 surrounding the transcription start site of murine *Ccdc80* was PCR amplified from genomic DNA using Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) and the following primers: fwd, 5'-AAAAGCTAGCAGCTGGTTGTGGGATGGATG-3', adding an Nhe I site; rev, 5'-TGTAAGATCTACTTGGGGACGCAGAGGGGGTATAAT-3', adding a Bgl II site. The PCR product was subcloned into the pGL3-Basic vector (Promega, Madison, WI) using the engineered Nhe I and Bgl II sites. Core elements of TCF binding sites were mutated from AA(A/G)A to CGCT by site-directed mutagenesis using Calbiochem KOD Hot Start Polymerase reagents (EMD Millipore, Billerica, MA) according to the manufacturer's instructions. Primers sets for site directed mutagenesis are listed in Table 3.

Luciferase Assays

293T cells were plated at a density of $2.5x10^4$ cells per well into 24-well plates and incubated for 24 h. For BIO treatment experiments: cells were transiently transfected with 25 ng empty vector pcDNA3.1 construct or 25 ng Sf1 expression construct, 100 ng Star-luciferase reporter construct and 30 ng of Renilla-luciferase reporter construct using Fugene HD (Roche, Indianapolis, IN). 24 h after transfection, cells were treated with 2 μM BIO or DMSO for 24 h and then harvested. For βcatS33Y experiments: cells were transiently transfected with 25 or 125 ng empty vector pcDNA3.1 construct and/or 25ng Sf1 expression construct and/or 100ng βcatS33Y expression construct with 100 ng Star-luciferase reporter construct and 30 ng of Renilla-luciferase reporter construct using Fugene HD (Roche, Indianapolis, IN). Cells were

harvested 48 h post transfection. For Ccdc80-luciferase experiments: cells were transiently transfected with 100 ng specific Ccdc80-luciferase reporter constructs and 30 ng Renilla-luciferase reporter construct using Fugene HD (Roche, Indianapolis, IN). 24 h later cells were treated with 2 μM BIO or DMSO for 24 h and then harvested. Cells were lysed with Passive Lysis Buffer and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer's instructions on a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA).

Preparation of conditioned media

293T cells were plated at a density of $2x10^6$ cells per 100 mm dish and transfected with 19 μg Ccdc80-myc or 19 μg empty vector pcDNA 3.1 construct using Fugene HD (Roche, Indianapolis, IN) in low serum media. 24 h later, conditioned media were collected and centrifuged at 1,000xg for 5 min to pellet floating cells and debris, sterile-filtered through 0.22 μm filters, aliquoted and frozen at -80°C. Thawed conditioned media were mixed 1:1 with fresh ATCL7 low serum growth media just prior to use in experiments.

Statistical analysis

Unless otherwise noted, data in figures is from repeated experiments with 1 sample per condition per experiment, with the means for each condition and the standard deviations for each condition with main experiment effects removed being shown. The analyses are from two-way analysis of variance procedures on log-transformed data with terms for each condition and each experiment, which in the simplest case of two conditions are paired T-tests. Commonly, pairwise contrasts comparing pairs of conditions were tested, but occasional tests of interactions between two treatments required comparing the difference of differences in pairs of conditions. Tests with *p*-values less than 0.05 were considered significant.

RESULTS

Wnt-responsive cells in the adult adrenal cortex are a heterogeneous population *in vivo*

The subcapsular adrenal cortex is the location of both 1) cells that are activated in response to signals from canonical Wnt ligands [3] and 2) cells that produce the ligand Shh [1]. The peripheral cortex is also the primary location where adrenocortical cells proliferate [12]. Because Shh-producing cells primarily serve as relatively undifferentiated progenitor cells for the steroidogenic cortex [1] and Wnt signaling participates in the maintenance of the adrenocortical progenitor pool [3], we sought to delineate any shared identity of Wnt-responsive and Shh-producing cells and determine the mechanisms by which Wnt/βcat-signaling contributes to homeostasis of the adult adrenal cortex. We hypothesized that Wnt-responsive cells would be identical to the Shh-producing progenitors. Additionally, given a role for βcat in the regulation of proliferation during adrenocortical development [3], we speculated that proliferating adrenocortical cells in the adult gland would be Wnt-responsive cells**.** Therefore, to determine whether Wnt-responsive cells colocalize with markers of adrenocortical progenitors and/or proliferating cells, we crossed the Tcf/Lef:H2B-GFP reporter mice with Shh^{LacZ} reporter mice and performed immunofluorescence on adult adrenal sections. We observed that while many GFP+ (Wnt-responsive) cells do not co-express LacZ, most LacZ+ (Shh-producing) progenitor cells costain as GFP+ (Wnt-responsive) cells (Figure 2.1A). Surprisingly, less than 10% of GFP+ (Wnt-responsive cells) colocalize with dividing, PCNA+ cells (Figure 2.1C). This fraction of the GFP+ population constitute less than 30% of the proliferating adrenocortical cells, suggesting that Wnt signaling does not play a primary (cell autonomous) role in regulating adrenocortical proliferation (Figure 2.1A,C). Moreover, less than 1% of LacZ+ (Shh+) cells colocalize with PCNA+ cells, indicating Shh+ progenitor cells are relatively quiescent (Figure 2.1A,C). Similar

results were obtained utilizing BrdU to mark proliferating cells in Tcf/Lef:H2B-GFP; Shh^{LacZ} mice (Figure 2.1B). These data suggest that a subset of Wnt-responsive cells are adrenocortical Shh-producing progenitor cells, however, the proliferation of adrenocortical cells during normal maintenance of the adult gland appears to be independent of a direct effect of Wnt signaling.

In addition to progenitor cells, the peripheral subcapsular region contains differentiated, steroidogenic cells of the zG that express Cyp11b2, the terminal enzyme in aldosterone production. Because a substantial proportion of GFP+ (Wnt-responsive) cells do not co-stain with LacZ (indicative of the undifferentiated Shh+ cells), we asked whether Wnt-responsive cells colocalize with Cyp11b2+ cells using immunofluorescence. We found that most Cyp11b2+ cells colocalized with a subset of GFP+ cells (Figure 2.1D). In contrast, differentiated Cyp11b1+ cells of the zF were not GFP+ cells (Figure 2.1E). Collectively, these data indicate that adrenocortical Wnt-responsive cells constitute a heterogeneous population, containing Shh+ progenitor cells and differentiated Cyp11b2+ cells of the zG.

Adrenocortical cell model of βcat signaling

The mutually exclusive expression of GFP (Wnt-responsive cell) and Cyp11b1 (zF cell) suggest that Wnt signaling may serve to repress the differentiation of progenitor cells into Cyp11b1+ expressing cells of the zF. We therefore examined *in vitro* the effects of Wnt/βcat signaling on steroidogenesis in a Cyp11b1-expressing zF cell line. The Wnt gain-of-function mutations present in most adrenocortical cell lines (i.e. H295A/R, HAC15) necessitate genetic or pharmacologic loss-of-function experiments that are fraught with an inability to achieve adequate suppression of Wnt signaling, making interpretation of results extremely difficult. Moreover, the reduced cell viability induced by even the partial reduction of βcat expression in the H295R cell line is consistent with oncogene addiction and hampers data interpretation [13].

We therefore opted to use the ATCL7 adrenocortical cell line derived from adrenocortical tumors of mice that harbor SV40 large T antigen driven by an Sf1-responsive promoter [14]. Importantly, these cells do not have any gain of function mutations in βcat and hence the Wnt signaling pathway is not basally active but can be stimulated pharmacologically, reflecting more accurately the physiological inducibility of this signaling pathway (Figure 2.2A). Treatment of ATCL7 cells with the GSK3β inhibitor BIO (6-bromoindirubin-3-oxime) induces βcat stabilization and nuclear translocation (Figure 2.2A,B) that results in transcriptional engagement of the canonical Wnt-responsive gene *Axin2* (Figure 2.2C), indicating that ATCL7 cells serve as an excellent model of adrenocortical Wnt/βcat-signaling *in vitro*.

Induction of βcat activity suppresses zF steroidogenesis *in vitro*

Given that Wnt-responsive cells in the adrenal cortex constitute both progenitor cells and differentiated cells of the mineralocorticoid-producing zG that express *Cyp11b2* (and specifically not the differentiated cells of the glucocorticoid-producing zF that express *Cyp11b1)*, we examined whether induction of βcat activity influences the steroidogenic function of ATCL7 adrenocortical cells. ATCL7 cells do not express *Cyp11b2* and therefore do not produce aldosterone. However, they do express the full complement of enzymes involved in production of corticosterone, consistent with a zF cell phenotype. Upon induction of βcat activity with BIO treatment, basal and ACTH-induced corticosterone release was significantly reduced (Figure 2.3A). We therefore assessed if reduction of corticosterone secretion was due to altered expression of steroidogenic enzymes. Treatment of ATCL7 cells with BIO resulted in down-regulation of the basal and ACTH-induced expression of the steroidogenic genes *Cyp11a1*, *Star* and most importantly *Cyp11b1* as measured by qRT-PCR (Figure 2.3C-E). Additional reduction in ACTH receptor (*Mc2r*) gene expression and concomitant downstream signaling through

MAPK/ERK and PKA pathways was also reduced following BIO treatment, as reflected in reduced levels of pCreb and pErk1/2 under basal and ACTH-stimulated conditions (Figure 2.3F,G).

BIO-induced reduction of steroidogenesis is due to effects on Sf1

The above data suggest that the effects of Wnt-activation on steroidogenesis may be mediated by primary effects on *Mc2r* expression and subsequent signaling effects on steroidogenic enzyme expression and corticosterone secretion. However, since *Mc2r* and the majority of steroidogenic enzymes are direct Sf1 target genes, we investigated whether the suppression of steroidogenesis by BIO could be in part mediated by direct effects on Sf1 expression or Sf1 transcriptional activity. Stimulation of βcat in ATCL7 cells with BIO resulted in a marked decrease in Sf1 expression as measured by qRT-PCR and immunoblotting (Figure 2.4A,B). We performed chromatin immunoprecipitation experiments with antibodies directed against Sf1 to examine whether reduction in Sf1 expression results in less Sf1 occupancy at target promoters. Sf1 was markedly reduced at the promoters of *Star*, *Cyp11a1* and *Mc2r* under basal and ACTH-stimulated conditions (Figure 2.4C-E). We next assessed whether BIO treatment decreased Sf1 transcriptional activity independent of changes in Sf1 levels. Stimulation of the heterologous 293T cell line with BIO resulted in a reduction of Star-luciferase activity in the presence of equivalent amounts of Sf1 (Figure 2.4F). These results were recapitulated using βcatS33Y, a mutant βcat that is constitutively active [15] (Figure 2.4G), indicative of a βcat-dependent inhibitory effect of BIO on Sf1-mediated transcription. These results demonstrate that, in addition to modulating Sf1 levels, induction of βcat activity interferes with the ability of Sf1 to transactivate steroidogenic promoters.

Gene expression profiling of Wnt-responsive cells *in vitro* and *in vivo* reveals novel genes regulated by Wnt/βcat signaling in adrenocortical cells

It is plausible that βcat additionally activates (as opposed to inhibits) genes that influence steroidogenesis through Sf1-independent mechanisms. Therefore, in order to determine genes induced by Wnt/βcat signaling *in vitro*, 4 paired samples of BIO or Vehicle treated ATCL7 cells were subjected to gene expression profiling with Affymetrix Mouse gene ST 1.1 strip arrays. We sought to identify genes induced by βcat stimulation *in vitro* that were also expressed in Wnt-responsive adrenocortical cells *in vivo*. To determine the panel of genes more highly expressed in the Wnt-responsive population versus genes expressed throughout the adrenal cortex, we employed two different lines of transgenic reporter mice: Tcf/Lef:H2B-GFP mice express GFP in Wnt-responsive cells, while Sf1:eGFP mice express GFP in all adrenocortical cells. GFP+ adrenocortical cells were obtained from 6 week old male Tcf/Lef:H2B-GFP mice and Sf1:eGFP mice independently via FACS. RNA samples extracted from 4 independent sorts of 10 adrenals per genotype were assessed using Affymetrix Mouse gene ST 1.1 strip arrays. Genes highly expressed in the Tcf/Lef:H2B-GFP population compared to the Sf1:eGFP population were intersected with genes up-regulated in ATCL7 cells by BIO treatment versus Vehicle treatment (Figure 2.5). While known Wnt target genes present in other organ systems were captured at the intersection, such as Tnfrsf19 and Nkd1 [16, 17], we focused our efforts on *Ccdc80,* the gene with the largest fold-increase upon BIO treatment that was also expressed in Wnt-responsive cells *in vivo*.

Ccdc80 is a βcat-regulated gene in adrenocortical cells

Given that *Ccdc80* was enriched in both data sets, we hypothesized that *Ccdc80* was a bona fide direct target gene of Wnt/βcat signaling. Treatment of ATCL7 cells with BIO induced

Ccdc80 expression, consistent with our gene expression profiling (Figure 2.6A). To determine whether the induction of *Ccdc80* was dependent on a transcriptionally competent βcat/TCF complex, we electroporated ATCL7 cells with βcatS33Y alone or in combination with a dominant-negative TCF (dnTCF4E). As transfection of ATCL7 cells is very inefficient, we compared changes in *Ccdc80* expression with induction of *Axin2*. Forced expression of βcatS33Y in ATCL7 cells increased *Ccdc80* expression, commensurate with an increase in *Axin2* levels (Figure 2.6B). This increase was abrogated in the presence of dnTCF4E, suggesting regulation of *Ccdc80* expression by a βcat/TCF complex. Sequence analysis of the promoter region of *Ccdc80* with MatInspector [18] revealed four potential TCF/LEF binding sites within a 2 kb region encompassing the transcription start site (Figure 2.6C). We constructed a series of luciferase reporters containing this region of the *Ccdc80* promoter with each TCF site individually mutated. As with endogenous *Ccdc80* expression, treatment of 293T cells with BIO induced activity of Ccdc80-luciferase (Figure 2.6D). While mutation of putative TCF site 1 did not suppress the induction of Ccdc80-luciferase activity, individual mutation of TCF sites 2, 3 or 4 abolished BIO-induced luciferase activity of these constructs (Figure 2.6D). These data indicate that *Ccdc80* is a direct βcat/TCF-regulated gene and that each of the three putative TCF sites is essential for promoter-proximal Wnt-mediated transcriptional activation of Ccdc80.

Ccdc80 suppresses steroidogenesis *in vitro*

We identified a novel βcat regulated gene in adrenocortical cells, *Ccdc80*. While little is known about the function of Ccdc80, it has been recently shown to be a secreted protein implicated in pre-adipocyte differentiation and extracellular matrix biology, including the potentiation of growth factor / growth factor receptor interaction [19, 20]. Because βcat signaling activates *Ccdc80* expression and suppresses Sf1-mediated zF cell steroidogenesis, we sought to

determine whether Ccdc80 also contributed to Wnt-mediated steroidogenic inhibition in adrenocortical cells. We first generated Ccdc80-conditioned media (CM) to confirm its identity as a secreted protein and to allow for an evaluation of its role as an extracellular regulator of adrenocortical steroidogenesis. Immunoblot analysis revealed that Ccdc80-myc expressed in 293T cells is present in both cell lysates and collected media. Ccdc80-myc migrates on SDS-PAGE at ~110 kDa, with multiple higher molecular weight bands likely due to its glycosylation (Figure 2.7A). Treatment of ATCL7 cells with Ccdc80-containing CM resulted in a reduction in basal and ACTH-induced corticosterone secretion in comparison to treatment of cells with CM from 293T cells transfected with an empty vector (Figure 2.7B). As in our previous experiments with βcat stimulation, treatment with Ccdc80 CM resulted in a significant decrease in expression of *Star*, *Cyp11a1* and *Cyp11b1* (Figure 2.7C-E). In contrast, treatment of ATCL7 cells with Ccdc80 CM did not affect expression of *Mc2r* or *Sf1* (Figure 2.7F,G,I). Given that treatment of ATCL7 cells with Ccdc80 CM partially phenocopies the induction of βcat in these cells, we assessed βcat levels and transcriptional activity. Treatment with Ccdc80 CM does not induce βcat stability or *Axin2* expression (Figure 2.7H,I). Together with the findings that pErk and pCreb levels are unchanged in the presence of Ccdc80 CM (Figure 2.7I), these data indicate that Ccdc80 is likely suppressing steroidogenesis through mechanisms distinct from signaling events initiated through Wnt or Mc2r signaling.

DISCUSSION

Wnt signaling plays diverse, tissue-specific roles that include progenitor cell maintenance, self-renewal, proliferation, cell survival, cell fate commitment and differentiation in multiple organ systems. The mechanisms by which Wnt signaling participates in such a wide variety of processes is complicated by the multiplicity of ligand/receptor pairings and the presence of a host of secreted repressive coregulators. The mammalian Wnt pathway has 19 different ligands and 10 different Fzd receptors; each Wnt can bind every receptor (with varying affinity) and has the capacity, depending on the cellular context, to elicit canonical (through βcat) or noncanonical Wnt pathway activation [21]. Concentration gradients of competing secreted Wnt ligands and Wnt inhibitors can activate and potentiate distinct genetic programs dependent upon the relative abundance of stabilized βcat [22]. The rules that dictate how these various components are temporally and spatially integrated with other intersecting signaling pathways (FGFs, IGF) to regulate physiologic Wnt responses in the mammalian adrenal cortex are relatively unknown.

The current study aimed to characterize the cells in the mouse adrenal cortex that participate in Wnt-mediated organ homeostasis. In this report, we demonstrate that the Wnt-responsive cells in the peripheral cortex of the adrenal gland are indeed a heterogeneous population containing both undifferentiated Shh-producing progenitor cells and differentiated mineralocorticoid-producing cells of the zG. Surprisingly, the Wnt-responsive cells constitute only a small fraction of the proliferating adrenocortical cells in the adult mouse under normal conditions. These results were unanticipated given the increased adrenocortical cell proliferation observed in the adrenals of mice with genetic gain of Wnt pathway function, versus the decreased proliferation present in mice with loss of Wnt pathway activation [3, 23-25]. It has

remained unclear whether the Wnt-responsive cells or perhaps their descendants are the bona fide proliferating cells in these mutant adrenal cortices. It is plausible that during simple homeostatic organ maintenance, Wnt signaling contributes indirectly to adrenocortical proliferation through up-regulation of paracrine factors that promote cell proliferation in a non-cell-autonomous fashion, and/or, that during organ re-growth following injury repair (as opposed to organ maintenance), Wnt-responsive cells contribute more directly to the proliferating pool of adult adrenocortical cells.

Previous studies have highlighted the role of both βcat and the βcat target genes Dax1 and inhibin-α (Inhα) in the maintenance of the undifferentiated state of adrenocortical progenitors (Dax1) and the fidelity of an adrenal versus a gonadal fate of these progenitors (Inhα) [3, 6, 26]. Additionally, adrenals from mice with adrenocortical-specific knockout of APC, a component of the βcat destruction complex, exhibit cell accumulation at the cortical-medullary boundary that express Sf1, stabilized βcat and Dax1, but not markers of differentiated zG or zF cells [23]. Collectively, these findings demonstrate that βcat maintains the undifferentiated state of adrenocortical progenitors through suppression of steroidogenesis and restriction of differentiation to an adrenal fate. However, the interpretation of these data are challenging in light of a recent study supporting a stimulatory role of adrenocortical Wnt signaling in zG differentiation through indirect regulation of aldosterone synthesis [7]. DeltaCat mice engineered to express a genetically modified, constitutively active βcat, partially phenocopy mice with adrenocortical-specific knockout of APC, with aberrant cell accumulation at the cortical-medullary boundary. These cells express Sf1 and stabilized βcat, however a subset displays ectopic expression of Cyp11b2 resulting in overt hyperaldosteronism [24]. Supporting such a role of Wnt signaling in aldosterone production in the zG is the loss of secreted Wnt

inhibitors recently observed in aldosterone-producing adenomas (APA) [7]. In our current study, we now report that stimulation of βcat reduces fasciculata steroidogenic enzyme expression and corticosterone secretion. Our molecular data indicates that βcat signaling may actively repress zF cell differentiation. This is supported by our observation that Wnt-responsive cells do not overlap with Cyp11b1+ cells *in vivo*. Additionally, deltaCat adrenals that have stabilized βcat throughout the adrenal cortex have fewer zF cells and lower *Cyp11b1* expression compared to wildtype animals [24]. The combined data support an emerging model whereby Wnt signaling in the adrenal cortex serves to inhibit a fasciculata fate and facilitate a glomerulosa fate.

An interesting aspect of our study is that the repressive effect of βcat signaling on fasciculata steroidogenesis is partially mediated through effects on Sf1 expression and activity. Here we find that stimulation of βcat activity results in down-regulation of Sf1 expression and decreased occupancy of Sf1 from steroidogenic promoters. While transcriptional regulation of Sf1 expression in the adult adrenal is not well characterized, it is plausible that βcat can directly repress the Sf1 gene through conserved Tcf/Lef binding sites upstream of its transcription start site (Zubair and Hammer, unpublished observation), or that βcat can activate genes that negatively regulate Sf1 expression. Nonetheless, independent of changes in Sf1 expression, we find that in a heterologous cell line, βcat reduces the ability of Sf1 to transactivate steroidogenic promoters. Additionally, in a fasciculata cell line, stimulation of βcat decreases expression of a number of steroidogenic enzymes including Cyp11b1 that is responsible for glucocorticoid production and defines the differentiated state of these cells. Because Sf1 has been shown to actually repress the expression of Cyp11b2 that is responsible for mineralocorticoid production [27], it is plausible that the observed Wnt-mediated facilitation of zG fate [7, 24] involves in part the release of Sf1-mediated repression of Cyp11b2. Similarly, in the developing gonad, the roles

of Wnt signaling as an activator versus repressor of Sf1 activity are temporally and spatially distinct. In the ovary, βcat blocks Sf1-mediated testis differentiation by removing Sf1 from the *Tesco* enhancer of *Sox9* [28]. Given that Sf1 and βcat synergistically co-activate a subset of genes involved in progenitor cell biology, including Dax1 and Inhα, a model emerges whereby Wnt pathway activation potentially titrates Sf1 away from the zF promoters of pro-differentiation/steroidogenic genes to co-regulate genes involved in progenitor cell biology (Dax1, Inhα) and subsequent activation of a zG fate.

In this report we also unveil a unique Sf1-independent mechanism of Wnt-mediated suppression of zF steroidogenesis. In search of Wnt-activated targeted genes, *Ccdc80* emerged as a novel βcat-regulated gene that encodes a secreted protein in the peripheral adrenal cortex. *Ccdc80* was originally cloned as *Urb*, a gene up-regulated in brown adipose tissue in bombesin-receptor-3 knockout mice [29] and subsequently shown to be down-regulated in adipose tissue in obese mouse models versus wild-type mice [30]. The exact role for Ccdc80 in adipose differentiation is unclear as both overexpression and knockdown of Ccdc80 resulted in suppression of pre-adipocyte differentiation [19]. In human cancer cell lines Ccdc80, also known as Dro1 (Down-regulated-by-oncogenes-1), was demonstrated to have reduced expression compared to primary tissues and its expression was down-regulated by various oncogenic insults in immortalized cells [31]. Re-expression of Ccdc80/Dro1 reduced anchorage-independent cell growth and sensitized several cancer cell lines to induced apoptosis in numerous studies [31-33]; however, how Ccdc80 elicits these effects is unclear. Several studies on the chicken ortholog of Ccdc80, Equarin, have revealed that it is critical for lens differentiation through two functional outputs of heparan sulfate proteoglycan binding: promoting cell adhesion and facilitating FGF signaling [20, 34]. Little is known about the role of the extracellular matrix and cell adhesion in

steroidogenesis or the adrenal progenitor niche. A role for FGF signaling in the adrenal cortex has only recently been appreciated. Guasti et al. found expression of FGF ligands and receptors in both the adrenal capsule and the adrenal cortex. Global Fgfr2IIIb knockout adrenals exhibit adrenal hypoplasia by e15.5 in part due to reduced proliferation in both the adrenal capsule and cortex, reflecting a role for FGF signaling in adrenal development [35]. Any potential roles of the extracellular matrix and signaling pathways in Ccdc80 regulation of adrenal homeostatic maintenance will be an important area of future research, as will potential roles of Ccdc80 on zG function.

In summary, we propose a model in which the fate of adrenocortical progenitor cells (maintenance vs. differentiation) is regulated by temporal and spatial integration of paracrine and endocrine stimuli (Figure 2.8). Our data, in combination with other studies, suggests that Wnt signaling is primarily maintaining the undifferentiated state of adrenal progenitors, yet 'priming' them to become zG cells upon endocrine differentiation signals (AngII/ACTH). In this model, paracrine signals (Wnt) would keep progenitor cells undifferentiated (through expression of Dax1, [6]) yet primed to differentiate directly to a zG cell (through expression of the Angiotensin receptor (At1r) and Nr4a1/2 genes, [7]), while zF differentiation is actively repressed (by effects on Sf1 and through Ccdc80, as shown in this study). In times of greater physiological need for increased steroid production, endocrine signals could override paracrine inputs and promote differentiation and steroidogenesis. Evidence for such phenomena are supported in this study, where ACTH stimulation reduces the induction of βcat signaling, reflected in a decrease of *Axin2* levels induced by BIO (Figure 2.3), and in other studies, where AII stimulation of H295R cells decreases the expression of βcat target genes *Axin2* and *Lef1* [7]. How paracrine and endocrine signals are integrated or override one another to dictate the fate of adrenal progenitors

remains unclear, but likely involves thresholds and temporal regulation. Freedman et al. demonstrated that, under normal conditions, all fasciculata cells are derived from glomerulosa cells [2]. Stem and progenitor cells in multiple organ systems engage differently depending on pathological perturbation or injury repair vs. normal homeostatic maintenance [36, 37]. Indeed, ACTH clears transcriptional activators from the Dax1 promoter, thereby shutting off this gene [38]. It remains possible that under great physiological need to replenish zF cells, such as regrowth following dexamethasone-induced atrophy of the zF, a portion of progenitor cells could differentiate directly into zF cells without a zG intermediate. Future studies of dynamic models of injury repair will be necessary to further define the diverse cellular populations engaged in the complex process of adrenal homeostasis.

ACKNOWLEDGEMENTS

Author Contributions: Isabella Finco performed BrdU experiments depicted in Figure 2.1B. Natacha Bohin performed experiments and analysis depicted in Figure 2.4F-G. Rork Kuick analyzed the microarray data and created heatmaps presented in Figure 2.5. Elisabeth Walczak performed all other experiments and analysis. The data presented in this chapter resulted in a first author manuscript submitted for publication in *Molecular Endocrinology*.

FIGURES

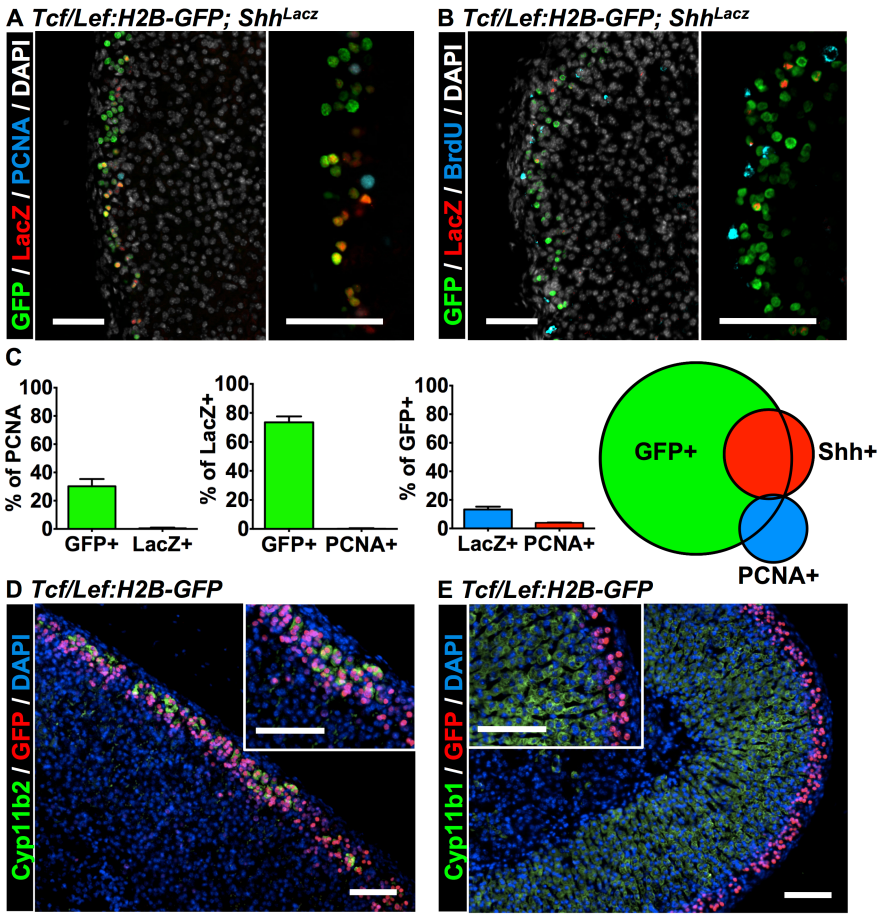

Figure 2.1: The Wnt-responsive population is heterogeneous *in vivo.*

Adrenal glands from 6 week old male Tcf/Lef:H2B-GFP, Shh^{LacZ} mice were evaluated on paraffin sections. (A) Representative coimmunofluorescence image for LacZ, GFP, PCNA and nuclear counterstain DAPI. Inset at the right is an overlay without DAPI. (B) Coimmunofluorescence images for BrdU, GFP, LacZ staining. Mice were harvested 2 h after one single BrdU injection. Inset at the bottom is an overlay without DAPI. (C) Quantification of data from (A) from 3 sections each of n=7 animals, error bars represent SEM. Diagram at the right represents the relative size and overlap of each population graphically. (D) Coimmunofluorescence images of Cy11b2 and GFP. (E) Coimmunofluorescence images of Cyp11b1 and GFP. Scale bars: 100 μm.

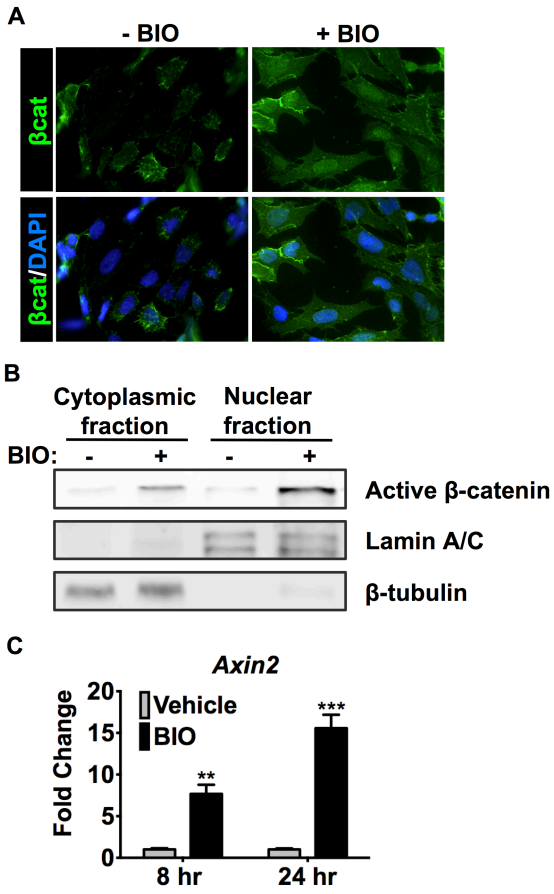

Figure 2.2: Adrenocortical cell model of βcat activity.

ATCL7 cells were treated with 0.5 μM BIO or Vehicle (DMSO) in low serum media for 24 h. (A) Subcellular localization of βcat was assessed using immunocytochemistry with βcat antibodies. (B) ATCL7 cells were separated into nuclear and cytoplasmic fractions. Fractionation of βcat was determined by SDS-PAGE and immunoblotting. Nuclear Lamin A/C and cytoplasmic β-tubulin serve as loading and fractionation controls. (C) qRT-PCR assessing *Axin2* expression 8 and 24 h post treatment. Values represent the mean±SD, where Vehicle treated cells were normalized to 1 (n = 4, ** $p < 0.005$) *** p <0.0005).

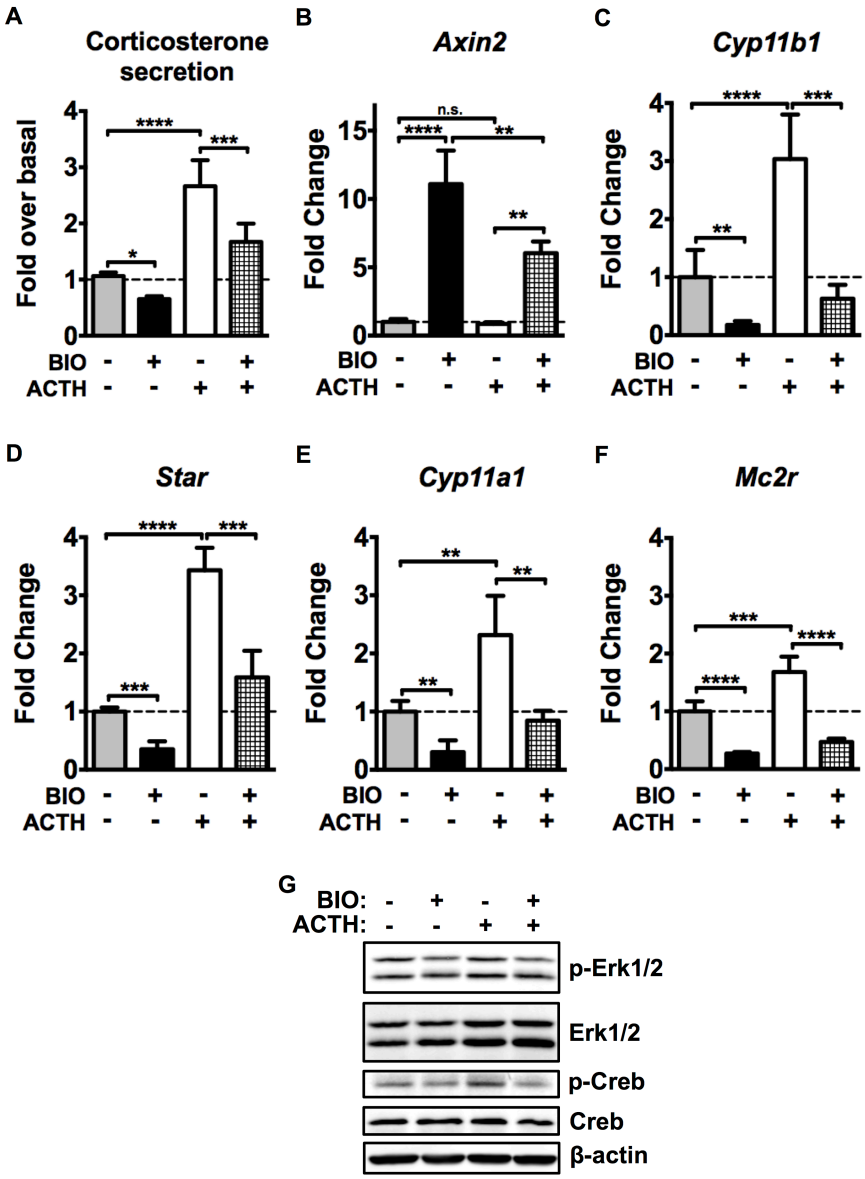

Figure 2.3: Stimulation of βcat activity inhibits basal and ACTH-induced zF steroidogenesis *in vitro.*

ATCL7 cells were pretreated with 0.5 μM BIO or Vehicle for 12 h in low serum media followed by 100 nM ACTH stimulation for 6 h and harvested. (A) Harvested media were subjected to ELISA to measure corticosterone release (n = 4). Experimental values were calculated as ng corticosterone/mg of protein and normalized to double Vehicle treated cells. (B-F) qRT-PCR assessment of changes in expression of *Axin2* (B), *Cyp11b1* (C), *Star* (D), *Cyp11a1* (E) and *Mc2r* (F). Values represent the mean±SD, where Vehicle treated cells were normalized to 1 (n = 4, **** $p<0.00005$, *** $p<0.0005$, ** $p<0.005$, *$p<0.05$, n.s. = not significant). (G) Protein level changes were assessed by immunoblotting for p-Erk1/2 (Thr202/Tyr204), total Erk1/2, p-Creb (Ser133), total Creb and β-actin (n = 3, representative data shown).

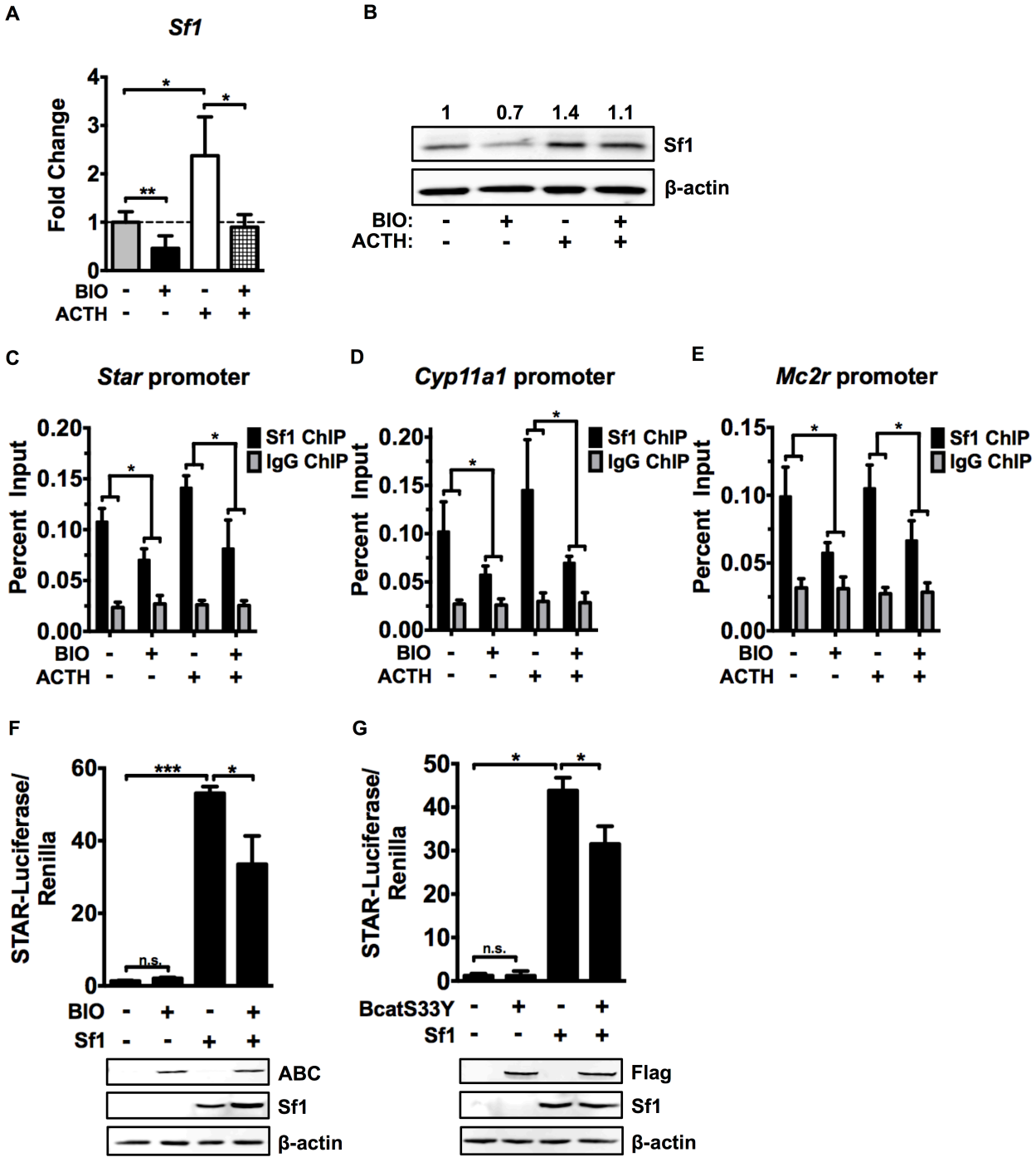
A
Sf1
Fold Change
BIO
ACTH
B
1
0.7
1.4
1.1
Sf1
β-actin
BIO:
ACTH:
C
Star promoter
Percent Input
Sf1 ChIP
IgG ChIP
D
Cyp11a1 promoter
E
Mc2r promoter
F
STAR-Luciferase/ Renilla
n.s.
ABC
G
BcatS33Y
Flag

Figure 2.4: βcat activity affects Sf1 levels, promoter occupancy and transcriptional activity

ATCL7 cells were pretreated with 0.5 μM BIO or Vehicle for 12 h in low serum media followed by 100 nM ACTH stimulation for 6 h and harvested. (A) Changes in Sf1 mRNA levels were assessed by qRT-PCR. Values represent the mean±SD, where Vehicle treated cells were normalized to 1 (n = 4, ** $p<0.005$, * $p<0.05$). (B) Sf1 protein levels were assessed by immunoblot (n = 4, representative data shown). Quantification of changes in Sf1 protein level is normalized to β-actin levels. (C-E) Changes in Sf1 DNA occupancy was measured by ChIP assays using Sf1 antisera. Immunoprecipitates were analyzed by qPCR using primers for the proximal promoters of *Star* (C), *Cyp11a1* (D) and *Mc2r* (E). Pre-immune IgG antisera were used as a negative control. Experimental values are normalized to 2% input (n = 4, * $p<0.05$). (F) 293T cells were transfected with Star-luciferase reporter construct, Renilla-luciferase internal control construct and empty vector or Sf1 expression construct for 24 h. Transfected cells were treated with 2 μM BIO or Vehicle for 24 h before passive lysis. Luciferase activity was measured and normalized to Renilla luciferase for transfection efficiency. Values represent the mean±SD (n = 4, *** $p<0.0005$, ** $p<0.005$, *$p<0.05$, n.s. = not significant). Protein levels of Active-βcat (ABC), Sf1 and β-actin were measured by immunoblotting (lower panels, n = 4, representative data shown). (G) 293T cells were transfected with Star-luciferase reporter construct, Renilla-luciferase internal control construct and empty vector, Sf1 or flag-tagged βcatS33Y alone or in combination for 48 h before passive lysis. Luciferase activity was measured and normalized to Renilla luciferase for transfection efficiency. Values represent the mean±SD (n = 4, *** $p<0.0005$, ** $p<0.005$, *$p<0.05$). Protein levels of Flag-βcatS33Y, Sf1 and β-actin were measured by immunoblotting (lower panels, n = 4, representative data shown).

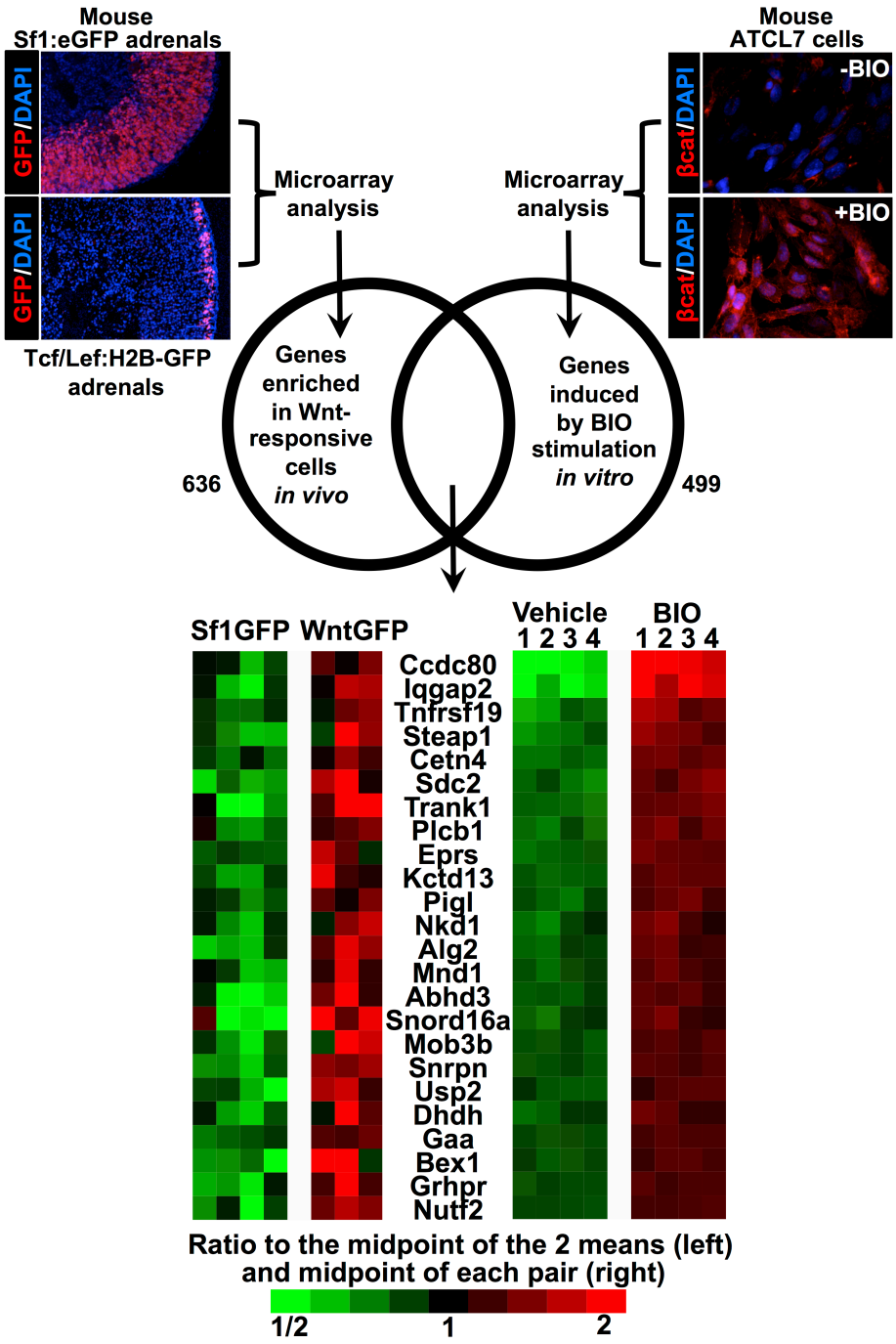
Mouse
Sf1:eGFP adrenals
GFP/DAPI
GFP/DAPI
Tcf/Lef:H2B-GFP
adrenals
Microarray
analysis
Mouse
ATCL7 cells
βcat/DAPI
-BIO
βcat/DAPI
+BIO
Microarray
analysis
Genes
enriched
in Wnt-
responsive
cells
in vivo
636
Genes
induced
by BIO
stimulation
in vitro
499
Sf1GFP WntGFP
Vehicle
1 2 3 4
BIO
1 2 3 4
Ccdc80
Iqgap2
Tnfrsf19
Steap1
Cetn4
Sdc2
Trank1
Plcb1
Eprs
Kctd13
Pigl
Nkd1
Alg2
Mnd1
Abhd3
Snord16a
Mob3b
Snrpn
Usp2
Dhdh
Gaa
Bex1
Grhpr
Nutf2
Ratio to the midpoint of the 2 means (left)
and midpoint of each pair (right)
1/2
1
2

Figure 2.5: Assessment of the gene expression profile of Wnt-responsive cells *in vitro* and *in vivo.*

Schematic representation of microarray workflow. (Left, Top) GFP+ adrenocortical cells were obtained from 6 week old male TCF/Lef:H2B-GFP mice and Sf1:eGFP mice independently via FACS. RNA was extracted from 4 independent sorts per genotype. Using two-sample T-tests we asked that probe-sets give $p<.05$ and average fold-changes of at least 1.5, which selected 636 increased and 907 decreased probe-sets in Tcf/Lef:H2B-GFP+ cells, 15% of which were expected to be false positives bases on analysis of data-sets where the sample labels were randomly permuted (Right, Top) ATCL7 cells were treated for 24 h with 0.5 μM BIO or Vehicle in low serum media prior to harvesting and RNA extraction. 4 pairs of treated cells were used for expression profiling. We asked that paired T-tests give $p<.05$ and average fold-changes or at least 1.5, which selected 499 up and 746 down probe-sets in BIO treated cells, of which we expect 0.1% to be false positives based on analysis of data-sets where sample labels were permuted within the pairs of samples. The intersection of up probe-sets in the two experiments was 25 probe-sets, representing the 24 distinct genes shown in the heatmap at bottom

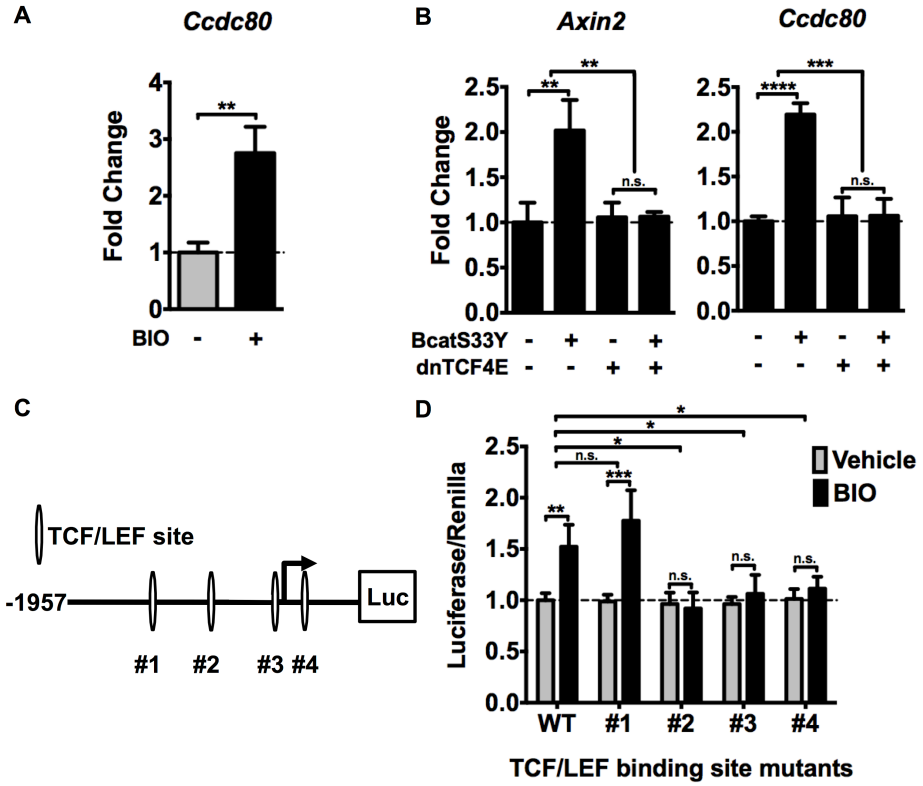

Figure 2.6: Ccdc80 is a novel βcat regulated gene in adrenocortical cells

ATCL7 cells were treated with 0.5 μM BIO or Vehicle for 18 h in low serum media and harvested. (A) Changes in *Ccdc80* expression were assessed by qRT-PCR. Values represent the mean±SD, where Vehicle treated cells were normalized to 1 (n = 4, ** p<0.005). (B) ATCL7 cells were electroporated with empty vector, βcatS33Y or dnTCF4E alone or in combination. 48 h post transfection, cells were harvested and changes in *Axin2* expression (left) and *Ccdc80* expression (right) were measured by qRT-PCR. Values represent the mean±SD, where Vehicle treated cells were normalized to 1 (n = 4, **** p<0.00005, *** p<0.0005, ** p<0.005, n.s. = not significant). (C) Diagram of Ccdc80-luciferase reporter construct. -1957 to +814 upstream of the transcription start site of *Ccdc80* was cloned into pGL3b luciferase reporter plasmid. Four putative Tcf/Lef binding sites identified using Genomatix MatInspector are labeled #1-4. (D) 293T cells were transfected with wildtype Ccdc80-luciferase construct or constructs with each individual Tcf binding site mutated (1-4) and Renilla luciferase internal control construct for 24 h. Cells were treated with 2 μM BIO or Vehicle for 24 hr before passive lysis. Luciferase activity was measured and normalized to Renilla luciferase for transfection efficiency. Values represent the mean±SD, where WT, Vehicle treated cells were normalized to 1 (n = 4, *** p<0.0005, ** p<0.005, *p<0.05, n.s. = not significant).

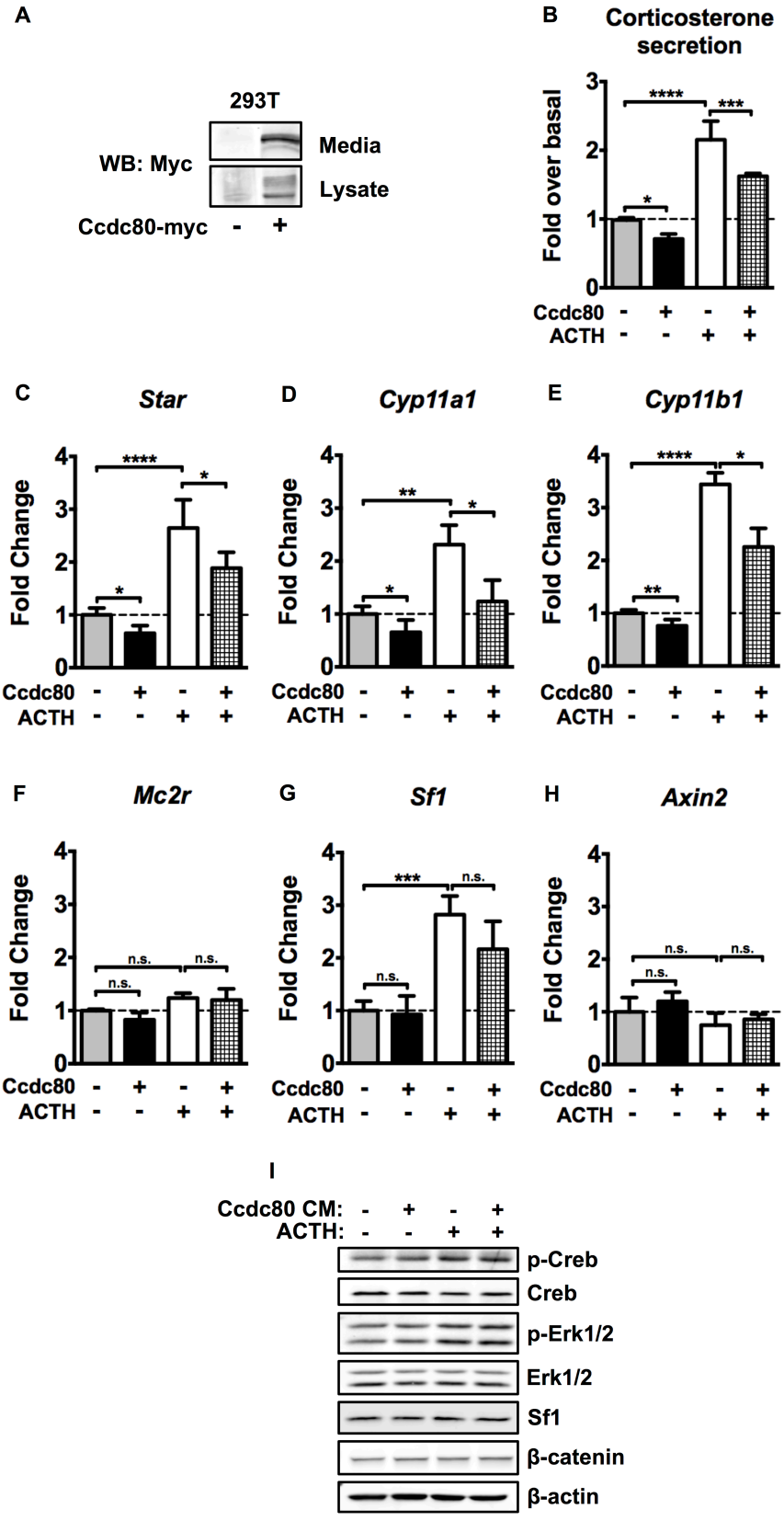
A
293T
WB: Myc
Media
Lysate
Ccdc80-myc - +
B
Corticosterone secretion
Fold over basal
****
***
*
Ccdc80 - + - +
ACTH - - + +
C
Star
D
Cyp11a1
E
Cyp11b1
Fold Change
F
Mc2r
G
Sf1
H
Axin2
n.s.
I
Ccdc80 CM: - + - +
ACTH: - - + +
p-Creb
Creb
p-Erk1/2
Erk1/2
Sf1
β-catenin
β-actin

Figure 2.7: Ccdc80 suppresses basal and ACTH-induced zF steroidogenesis *in vitro*

(A) 293T cells were transfected with Ccdc80-myc or empty vector for 24 h and harvested. Media was collected at time of harvest. Concentrated conditioned media and cell lysates were subjected to immunoblotting for Myc. (B) ATCL7 cells were treated with Mock or Ccdc80-conditioned media derived as in (A) for 18 h followed by 100 nM ACTH stimulation for 6 h and harvested. Media collected at harvest were subjected to ELISA to measure corticosterone release (n = 4). Experimental values were calculated as ng corticosterone/mg of protein and normalized to untreated cells. (C-H) qRT-PCR assessment of changes in expression of *Star* (C), *Cyp11a1* (D), *Cyp11b1* (E), *Mc2r* (F), *Sf1* (G) and *Axin2* (H) (n = 4, **** $p<0.00005$, *** $p<0.0005$, ** $p<0.005$, * $p<0.05$, n.s. = not significant). (I) Protein level changes were measured by immunoblotting for p-Erk1/2 (Thr202, Tyr204), total Erk1/2, p-Creb (Ser133), total Creb, Sf1, βcat and β-actin (n = 3, representative data shown).

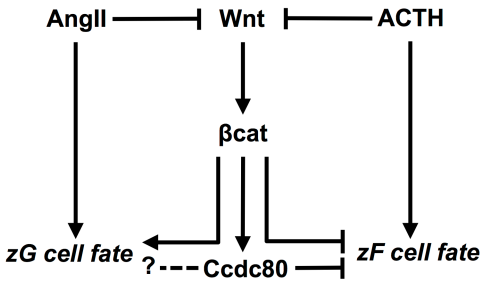

Figure 2.8: Integration of Wnt and endocrine signals dictate adrenocortical cell fate.

AngII and ACTH promote steroidogenesis in the zG and zF respectively, defining the differentiated state of the two adrenocortical cell types. Wnt signals stimulate βcat that inhibits the transcription of steroidogenic genes and transcriptionally activates Ccdc80, a secreted protein that in turn also inhibits steroidogenic gene expression. The rules that govern the balance the paracrine (Wnt/Ccdc80) and endocrine (ACTH/AngII) signals in this model are predicted to involve organotypic temporal and spatial cues. Coupled with the known role of βcat in the regulation of pro-zG genes, these data collectively suggest that Wnt signaling facilitates the unidirectional centripetal differentiation of adrenocortical progenitor cells. Specifically, the model predicts that Wnt-mediated inhibition of zF-associated steroidogenesis, maintenance of the progenitor cell pool and priming of zG cell fate, serves to assure that adrenocortical progenitor cells differentiate into zG cells before becoming zF cells as supported by recent work. See discussion for details.

TABLES

Table 2.1: Primer sets for qRT-PCR		
Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
Rplp0	GAAACTGCTGCCTCACATCCG	GCTGGCACAGTGACCTCACACG
Axin2	GCAGGAGCCTCACCCTTC	TGCCAGTTTCTTTGGCTCTT
Cyp11b1	GCCATCCAGGCTAACTCAAT	CATTACCAAGGGGGTTGATG
Star	AAGGCTGGAAGAAGGAAAGC	CCACATCTGGCACCATCTTA
Cyp11a1	AAGTATGGCCCCATTTACAGG	TGGGGTCCACGATGTAAACT
Mc2r	GTAAGTCAACGGCAAACACC	GTGTCATTGGTGTGTTCATACG
Sf1	CGCTGTCCCTTCTGCGGCTT	AGCACGCACAGCTTCCAGGC
Ccdc80	AGGCATGCAATTTTGGTCTGC	ACATCTTCCCGCTCAACGAT

Table 2.2: Primer sets for ChIP-qPCR		
Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
Star	AGAGGGTCAAGGATGGAATGATT	CAGTCTGCTCCCTCCCACC
Cyp11a1	GGGAGGTCACCGCTCCATCAGC	GCCAGCATACTGTCCCCACGACT
Mc2r	AACCAACCAGACATCCCCTGCC	CAAGGACCTCTCCTCCCACAAAGC

Table 2.3: Primers for site directed mutagenesis of Ccdc80-luciferase construct	
Gene	**Primer (5'-3')**
Tcf site 1 Forward	GGCTCTCATACTGCGAGAAGCCGCTACTTTACCAGCTGAGG
Tcf site 1 Reverse	CCTCAGCTGGTAAAGTAGCGGCTTCTCGCAGTATGAGAGCC
Tcf site 2 Forward	CCATGGGCTTTTCCAACCCAGAACTCCGCTCATAAAGCAGGCAG
Tcf site 2 Reverse	CTGCCTGCTTTATGAGCGGAGTTCTGGGTTGGAAAAGCCCATGG
Tcf site 3 Forward	CGCTCGGAAACCGCTGGACTCGGCCAGACTGCAGG
Tcf site 3 Reverse	CCTGCAGTCTGGCCGAGTCCAGCGGTTTCCGAGCG
Tcf site 4 Forward	CCTCATGCCTGCCGCCTGTAAATCCGCTCTGGATTTCCTGC
Tcf site 4 Reverse	GCAGGAAATCCAGAGCGGATTTACAGGCGGCAGGCATGAGG
The underlined letters represent the mutations introduced to abrogate transcription factor binding.	

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CHAPTER III: SUMMARY AND FUTURE DIRECTIONS

Previous studies established Wnt signaling as integral for maintenance of adrenal homeostasis. The data presented in this thesis provides mechanistic insight into how βcat contributes to this process and provides a deeper characterization of the Wnt-responsive adrenocortical progenitor cell population.

In chapter two, phenotypic characterization of Wnt-responsive adrenocortical cells revealed a previously unappreciated heterogeneity of this population. Given that Wnt signaling is critical for proper adrenal development and homeostasis, we hypothesized that Wnt-responsive cells constituted a transit-amplifying progenitor cell pool [1]. Therefore, it was predicted that Wnt-responsive cells would have a shared identity with Shh-producing cells that serve as undifferentiated progenitors to steroidogenic adrenocortical cells [2]. While a portion of the Wnt-responsive population is indeed *Shh*-expressing, a subset of the Wnt-responsive cells unexpectedly colocalize with differentiated cells of the zG. One caveat to this study is that our observations are based on transgenic reporter mice, which, depending on the half-life of the reporter protein could have expression artifacts or mark cells that have recently but no longer are responding to Wnt ligands. However, while our studies were in progress, data emerged supporting a role for Wnt signaling in zG biology [3], and thus reinforced our observations made with Wnt reporter mice.

Given that Wnt signaling can be mitogenic and promotes tumorigenesis in many tissues including the adrenal cortex [4], it is additionally unexpected that Wnt-responsive cells only infrequently colocalize with proliferating cells. Even more surprising is the observation that Shh-producing progenitor cells proliferate even less than Wnt-responsive cells. These results raise many important questions for future study. If *Shh*-expressing progenitors are not 'transit-amplifying', is there an undetermined population of transit-amplifying progenitors? Also, what are the major mitogenic stimuli of adrenocortical cells under normal homeostatic conditions, if not Wnt ligands? It seems reasonable that IGF and/or FGF signaling could be responsible for maintaining proliferation of adult adrenocortical cells. *IGF2* is the most highly expressed gene in adrenocortical carcinomas [5], and IGFs are potent adrenal mitogens *in vitro* and *in vivo* [6-9]. Igf1r is enriched in the subcapsular cortex [10], positioning the IGF-receiving cells in proximity to where the bulk of proliferation occurs. As with IGFs, FGF2 stimulates proliferation in primary cultures and adrenocortical cell lines [11, 12]. FGF ligands and receptors are situated in the capsular and subcapsular region of the adrenal gland [13]. Mice deficient for either FGFR2 isoforms or specifically isoform IIIb exhibit hypoplastic adrenals with disorganized capsules [13, 14]. The embryonic lethality of these mice precludes investigation into the role of FGF signaling in postnatal adrenal homeostasis and growth. Conditional ablation of FGFR2 IIIb and further characterization of the FGF-responsive adrenal cells will be an important area for future research.

It remains plausible that Shh+ 'progenitor' cells are slowly cycling and/or relatively quiescent and more 'stem'-like. Lineage tracing of *Shh*-expressing cells utilizing *Shh*-CreERT2 mice crossed with $R26R^{mT/mG}$ reporter animals revealed continuous patches of clonal GFP+ cells extending from the outer cortex to the cortical-medullary boundary [2]. These data suggest that

either *Shh*-expressing cells divide, or *Shh*-expressing cells proliferate after they extinguish the genetic program that includes *Shh* expression. While the vast majority of adrenocortical cells are undergoing continuous centripetal displacement, studies have uncovered a subpopulation of long-term retained cells within the capsular/subcapsular niche. Under normal physiological conditions, a newly divided, BrdU-labeled cell in the peripheral region transits the length of the cortex in 12-14 weeks [15]. 5-6 months following 2 weeks of BrdU infusion *in vivo*, a small population of label-retaining cells remained in the subcapsular region as well as the capsule [16]. Data emerging from the Laufer laboratory suggests that Shh+ cells are indeed a long-term retained population in the adrenal cortex. In lineage tracing experiments, Shh+ cells labeled shortly after birth persist in the outer cortex up to a year post-labeling (Dr. Edward Laufer, oral communication, 2012 Conference of the Adrenal Cortex). It will be of great interest to assess if the slowly cycling, long-term retained cells observed by Chang et al. are *Shh*-expressing cells. If true, these data would suggest that Shh+ cells are the adult stem cells of the adrenal cortex. A hierarchy of which adrenal cells are the 'stem' or 'progenitor' has not been clearly delineated. It is established that, in addition to Shh+ cells, several capsular populations (Gli1+, Wt1+) can give rise to adult adrenocortical cells [2, 17, 18]. However, the relative frequency with which these different cell types contribute to the adrenal cortex under normal homeostasis vs. regeneration following injury is unknown. Lineage tracing experiments from Gli1+, Wt1+ or Shh+ cells under regeneration paradigms, such as enucleation or dexamethasone-induced adrenal atrophy will help address this issue. Additionally, it will be of interest to investigate the gene expression profiles of the 'stem' cells taking a similar approach as in chapter two. GFP+ cells from Shh^{gfpcre} or Gli1:GFP adrenals could isolated via FACS and subjected to gene expression analysis via Affymetrix microarrays or RNA-Sequencing.

It will also be essential to determine whether long-term retained cells in the cortex are Wnt-responsive and if Wnt signaling is in part responsible for their maintenance. βcat activates transcription of Dax1 and Dax1 deficient adrenals partially phenocopy adrenal failure observed in βcat knockout adrenals [1, 19, 20]. These data together suggest that βcat-mediated Dax1 expression maintains the quiescent and/or undifferentiated state of Shh+ cells, but this has not been formally demonstrated. It will be important to re-examine the phenotypes of βcat and Dax1 knockout animals crossed with Shh and Gli1 transgenic reporter animals. Are there less Shh+ cells and/or long-term retained cells without βcat or Dax1? Long-term BrdU pulse-chase studies utilizing Shh^{LacZ};*Sf1/Cre*^{low}*:Ctnnb1*^{tm2kem} or Shh^{LacZ}; $Dax^{-/Y}$ will address this question. If the Shh+ population does not decrease in these animals, then this would indicate βcat and Dax1 adrenal knockout phenotypes could be due to non-cell-autonomous signaling defects on the capsular stem/progenitor cell niche. Lineage tracing of Shh+ and Gli1+ cells in the context of βcat or Dax1 deficiency would provide insight into this hypothesis. Additionally, it is unclear whether Wnt signaling somehow contributes to the genetic program that enacts *Shh* expression. While unpublished data from our laboratory suggest that the Shh pathway positively influences Wnt pathway activation, no data exists suggesting a reciprocal relationship in the adrenal gland (Finco and Hammer, unpublished observations). We observe that Shh-producing cells are Wnt-responsive cells, but it is unclear if Wnt pathway activation is critical for inducing *Shh* expression. Therefore, it will be important to identify Wnt target genes in the Shh+ population. βcat ChIP-Seq performed on GFP+ cells isolated from Shh^{gfpcre} adrenals would help resolve this question.

The characterization of Wnt-responsive cells in chapter two demonstrates that the majority of proliferating adrenocortical cells are not Wnt-responsive cells. It is plausible that Wnt signaling contributes to proliferation in a non-cell-autonomous fashion. Gene expression profiling presented herein used strict criteria to prioritize genes for further study; only genes that were enriched in the Wnt-responsive population *in vivo* and stimulated by βcat activity *in vitro* were considered for further analysis. Genes intersecting these data sets did not include any growth factors or cell-cycled related genes. It is possible that mitogenic paracrine factors are indeed under the control of Wnt signaling *in vivo*, but were not stimulated by induction of βcat activity in the fasciculata-like ATCL7 cells used for our *in vitro* studies. Future investigation could analyze only the genes enriched in the Wnt-responsive population alone. Our phenotypic analysis revealed that the Wnt-responsive population is heterogeneous and contains most, if not all of the differentiated cells of the zG. It is likely that subset of genes enriched in the Wnt-responsive population may simply be zG genes that are not directly regulated by Wnt/βcat signaling. Accordingly, it may be fruitful to compare the genes enriched in our *in vivo* dataset with other adrenal gene expression data sets characterized by high Wnt signaling. For example, one could overlay genes from the Wnt-responsive population to those upregulated in the adrenals of APC knockout mice versus wildtype mice. Another comparison could include genes downregulated in βcat deficient adrenals versus wildtype. These experiments would keep the gene expression analysis within the same species and would consider both Wnt pathway gain- and loss-of-function *in vivo*. Target genes emerging from these studies are predicted to shed light on additional pro-growth genes regulated by Wnt signaling, and could additionally provide insight into how Wnt signaling promotes tumorigenesis in the adrenal cortex.

Data presented in this thesis demonstrates that one contribution of Wnt signaling to adrenocortical homeostasis is the inhibition of zF cell fate by the suppression of zF cell steroidogenesis. Combined with recent data reflecting a role for Wnt signaling in zG biology [3, 21], our results suggest that Wnt signaling ensures unidirectional differentiation of adrenal progenitors into cells of the zG and not the zF, therefore aiding in the establishment of adrenocortical zonation. To test this notion further, it would be advantageous to investigate the distribution of differentiated cells in each steroid-producing layer in βcat knockout adrenals. It is predicted that, prior to progenitor cell loss and ultimate adrenal failure, βcat deficient adrenals may have less *Cyp11b2*-expressing zG cells and a higher proportion of *Cyp11b1*-expressing zF cells.

Our studies indicate that Wnt signaling inhibits zF steroidogenesis through suppression of *Mc2r* gene expression and subsequent downstream signaling from Mc2r, as well as through direct effects on Sf1-meditated transcription of other steroidogenic genes. Very recently, researchers determined that canonical Wnt signaling augments zG steroidogenesis through indirect regulation of aldosterone production [3]. While it appears contradictory that Wnt signaling could both positively and negatively influence steroidogenesis within the same cell, several explanations exist to address this contradiction. Steroidogenesis in the zG is under control of the RAAS, intracellularly involving PLC, PKC and calcium signaling, whereas the HPA axis and cAMP/PKA signaling regulates the steroidogenesis in zF cells. Our laboratory and others have only considered the effects of canonical Wnt/βcat pathway on steroidogenesis and have not investigated noncanonical Wnt signaling. It is interesting to note that calcium signaling is a branch of noncanonical Wnt pathway [22]. It has been demonstrated that the same Wnt can promote both canonical and noncanonical Wnt signaling dependent on cellular context [23], and

that canonical/noncanonical signaling within a single cell is not mutually exclusive [24]. It will be important to determine which Wnt ligands are expressed in the adrenal, in addition to which branch of Wnt signaling they stimulate. Wnt4 is expressed in subcapsular cells and has been shown to engage both the canonical and noncanonical pathway in different tissues [25, 26]. As Wnt4 is a target of the canonical pathway itself [27, 28], it is unclear whether Wnt4 promotes feed-forward activation of the canonical pathway, and/or if it can elicit calcium signaling to promote zG fate and steroidogenesis or both. Mice globally deficient for *Wnt4* exhibit less *Cyp11b2* expression at birth suggesting that Wnt4 does impact zG steroidogenesis [29]. It will important to determine if Wnt4 is eliciting these effects through a canonical or non-canonical mechanism with *in vitro* studies utilizing adrenal cell lines that do not contain gain-of-function mutations in the Wnt pathway yet have the capacity to express *Cyp11b2* and synthesize aldosterone. *Wnt4* global knockout mice die perinatally due to kidney malformation [30]. Accordingly, it would be advantageous to employ a conditional allele of *Wnt4* in the steroidogenic cell lineage to investigate the potential role of Wnt4 on adrenal zonation, homeostasis and aldosterone production during adulthood.

The molecular data in chapter two indicates that βcat inhibits zF cell steroidogenesis by suppressing Sf1-mediated transactivation of steroidogenic genes. In the ATCL7 zF-like cell line, induction of βcat activity reduced Sf1 expression at the mRNA and protein level. It is unclear whether βcat affects Sf1 protein stability. Pulse-chase experiments utilizing ^{35}S-methionine would clarify this issue. Our data demonstrates that when Sf1 protein levels are equivalent, βcat suppresses Sf1 activation of steroidogenic promoters in reporter assays. These data are likely a reflection of reduced Sf1 occupancy or reduction in recruitment of coactivators, or both. These

data are interesting in light of studies demonstrating that βcat and Sf1 synergistically activate transcription of several genes, including Dax1 and Inhα, which are critical for adrenal progenitor biology. It is possible that βcat titrates Sf1 away from steroidogenic promoters and recruits Sf1 to the promoters of Dax1 and Inhα for expression of genes critical for maintenance of progenitors cells. It is also likely that the suppression of Sf1-mediated expression of steroidogenic promoters could be due to effects on co-regulator recruitment. Our data indicates that reduced Mc2r expression is correlated with a reduction in PKA signaling, resulting in less phosphorylation of Creb, a known coactivator of Sf1 for steroidogenic gene expression [31]. It would be worthwhile to assess if loss of occupancy of pCreb or other factors is correlated with and contributes to loss of Sf1 on steroidogenic promoters.

Gene expression profiling in chapter two unveiled *Ccdc80* as a novel Wnt-responsive gene encoding a secreted factor that participates in the suppression of zF steroidogenesis. It is unclear how Ccdc80 blunts basal and ACTH-induced steroidogenesis, as Mc2r-initiated cAMP/PKA signaling and Sf1 levels are unaffected in the presence of Ccdc80 conditioned media. The function of Ccdc80, as well as its potential signaling mechanism, is poorly understood. In the chick eye lens, reports suggest that Ccdc80 binds heparan sulfate proteoglycans and modulates FGF signaling [32]. Aside from potential mitogenic effects of FGFs (as discussed above), it is unknown if FGF signaling influences adrenal steroidogenesis. FGF stimulates the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway in a context-dependent manner [33]. A recent report implicated JAK2 in prolactin-mediated adrenocortical steroidogenesis [34]. Therefore, it is intriguing to speculate that the inhibitory effects of Ccdc80 on corticosterone production could be due to modulation of an intra-

adrenal FGF-JAK/STAT signaling axis. To test this hypothesis, it will be essential to determine whether adrenal-expressed FGFs induce JAK/STAT activation and whether manipulation of these factors impact steroidogenesis. One intriguing aspect of Ccdc80 is its subcellular localization. While predominantly secreted in most cell lines examined [35-38], Ccdc80 is present in the cytoplasm in some reports [38, 39] and in the nucleus of thyroid cells [40]. While its intracellular actions are not well characterized, O'Leary et al. demonstrated that Ccdc80 is a binding partner of JAK2. While Ccdc80-binding does not increase the level of phosphorylation of JAK2, this binding event somehow facilitates JAK2-mediated phosphorylation of STAT proteins. The experiments performed in chapter two utilizing Ccdc80 conditioned media only investigated the extracellular effects of Ccdc80 on steroidogenesis. It will be critical to determine whether Ccdc80 is retained intracellularly and if Ccdc80 influences JAK/STAT signaling through extracellular or intracellular mechanisms.

It is likely that the dual role for Wnt signaling in progenitor maintenance and zG differentiation is dictated by the integration of Wnts with endocrine signals and paracrine factors such as Ccdc80. Data presented in this thesis demonstrates that Ccdc80 inhibits zF-associated steroidogenesis, however it remains unknown whether Ccdc80 impacts zG biology. If Ccdc80 stimulates basal or AII-stimulated aldosterone production, this would suggest that Ccdc80 contributes to the unidirectional differentiation of adrenal progenitors and thus adrenal zonation. However, it is equally possible that Ccdc80 suppresses aldosterone synthesis. This would implicate Ccdc80 in maintaining the undifferentiated state of adrenocortical progenitors, akin to the actions of Dax1. An evaluation is needed on the potential role for Ccdc80 on aldosterone synthesis *in vitro* and adrenal zonation *in vivo.* It is of great interest to determine whether Ccdc80

deficient adrenals will phenocopy βcat and Dax1 knockout adrenals and implicate Ccdc80 in adrenocortical homeostasis.

In summary, the data presented in this thesis demonstrate that the heterogeneous Wnt-responsive population contains progenitors and differentiated cells of the zG, but not the zF. Wnt signaling likely promotes the unidirectional differentiation of adrenal progenitors into zG cells by inhibiting zF cell fate. This is achieved in part through the novel inhibitory actions of βcat on Sf1-mediated zF-associated steroidogenesis. βcat also induces expression of Ccdc80, a paracrine factor, with an unknown mode of signal transduction, which additionally participates in the inhibition of zF steroidogenesis through yet determined mechanisms. Collectively, these data highlight the complexity with which Wnt signaling contributes to adrenocortical homeostasis.

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